THE DISTRIBUITON OF Bf PHENOTYPES IN HUMAN SERA IN BEIJING AREA AND DETECTION OF Bf PHENOTYPES IN BLOODSTAINS

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Properd in factor B (Bf) was first discovered in 1972 by Alper et al[1], Besides the two common alleles BfF and Bfs, and the less common alleles BfF1 and Bfs1(Bfs07), More than 20 rare variants and Bf° genes have been discovered. subtypes of BfF were studied using PAGIEF in 1982 by Geserick. The subtypes of Bfs was reported in 1983 by David et al[2].

In our investigation, the distribution of Bf phenotypes from 234 Chinese subects was studied using IEF. Bf typing time limit of 22 bloodstains with Bf types stored at room temperature was determinated.

Materials and methods

EDTA plasma samples from 234 healthy and unrelated donors in Beijing area were collected . The sera was stored at -20℃.

Bloodstains samples from 22 donors with Bf phenotypes was absorbed on filter paper and dried at room temperature.

The samples were examined after different time intervals. Before electrophoresis the stains cut in 5×5 mm pieces and treated with 20 μl 10% glycine.

1-2 μl or 5-7 μl extract of bloodstains was submitted to IEF on thin layer PAG in the PH range 3.0-9.5, 4-6, 5-7(voltage 2000v power 20 time 2.5h). A specific goat anti- human Bf (Shanghai) was layered on the gel .After incubating for 30 min at 37℃ in a moist condition. the proteins other than Bf were washed

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Table 2 : Relations between the degree of longitude/HP*, degree of latitude/HP* and the degree of longitude-latitude/HP* for 37 hitherto HP subtyped European population samples by means of Spearman's correlation analysis (a), simple linear regression analysis (b) and multiple linear regression analysis (c)

	degree of longitude				degree of latitude				degree of longitude-latitude	
HP*	(a) R	mit p<	(b) F	mit p<	(a) R	mit p<	(b) F	mit p<	(c) F	mit p<
HP*1S	-0,239	0,284	0,097	0,759	0,133	0,556	0,053	0,820	0,089	0,915
HP*1F	-0,180	0,422	7,849	0,015	0,092	0,683	1,184	0,290	5,846	0,011
HP*2FS	0,231	0,301	0,135	0,717	-0,212	0,344	0,154	0,699	0,174	0,842
HP*2FF	-0,357	0,102	9,675	0,006	-0,278	0,210	2,887	0,105	5,970	0,010
HP*2SS	0,203	0,365	4,344	0,050	0,214	0,340	0,050	0,826	2,339	0,124
HP*VAR	-0,327	0,138	3,139	0,091	-0,121	0,594	0,377	0,546	1,541	0,240
HP*1	-0,540	0,010	6,726	0,017	0,277	0,211	1,371	0,255	5,896	0,010
HP*2	0,541	0,009	6,679	0,018	-0,278	0,211	1,405	0,250	5,912	0,010

subtype allele. A significant increase is only observed concerning the rare HP*2FF. A mistyping of HP2FF (cathodic drift effect) in some included investigations is not impossible (Zischler,1988). Therefore a check of up-to-date surveys seems advisable in a few years time.

It is of desicive importance that the conventional as well as subtyped HP-system shows a clear geographic distribution pattern in Europe. The observed distribution gradients reveal some paralleles with other known polymorphic blood group systems of man e.g. AB0 and RHESUS. Statements as to what extent physiological factors, evolutionary mechanisms or the history of the European populations contributed to the geographic HP distribution pattern in Europe can be only hypothetic at present.

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Table 1: HP phenotypes and allele frequencies in three European population samples (Hannover, Thuringia, Tyrol)

	Hannover		Thuringia		Tyrol	
Phenotypes	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
HP 1S	16	21,06	13	13,07	19	16,25
HP 1S-1F	26	22,78	13	14,61	18	16,62
HP 1F	7	6,16	4	4,08	2	4,25
HP 2FS	73	74,84	60	60,81	97	93,97
HP 2FS-1S	87	79,40	58	56,39	69	78,15
HP 2FS-1F	38	42,94	32	31,51	44	39,97
HP 2SS	--	0,14	--	0,45	--	0,11
HP 2SS-1S	2	3,42	5	4,87	5	2,73
HP 2SS-1F	2	1,85	4	2,72	--	1,39
HP 2FS-2SS	8	6,44	10	10,50	6	6,55
HP 2FF	--	0,00	--	--	--	0,02
HP 2FF-1S	1	0,28	--	--	1	1,00
HP 2FF-1F	--	0,15	--	--	1	0,51
HP 2FS-2FF	--	0,53	--	--	2	2,39
HP 2SS-2FF	--	0,02	--	--	--	0,08
Total	260	260,01	199	199,01	264	263,99
	$X^2_{(10)} = 6,797$ $p > 0,70$		$X^2_{(6)} = 1,323$ $p > 0,95$		$X^2_{(10)} = 7,415$ $p > 0,50$	

Alleles	Hannover	Thuringia	Tyrol
HP*1S	0,2846	0,2563	0,2481
HP*1F	0,1539	0,1432	0,1269
HP*2FS	0,5365	0,5528	0,5966
HP*2SS	0,0231	0,0477	0,0208
HP*2FF	0,0019	----	0,0076
	1,0000	1,0000	1,0000

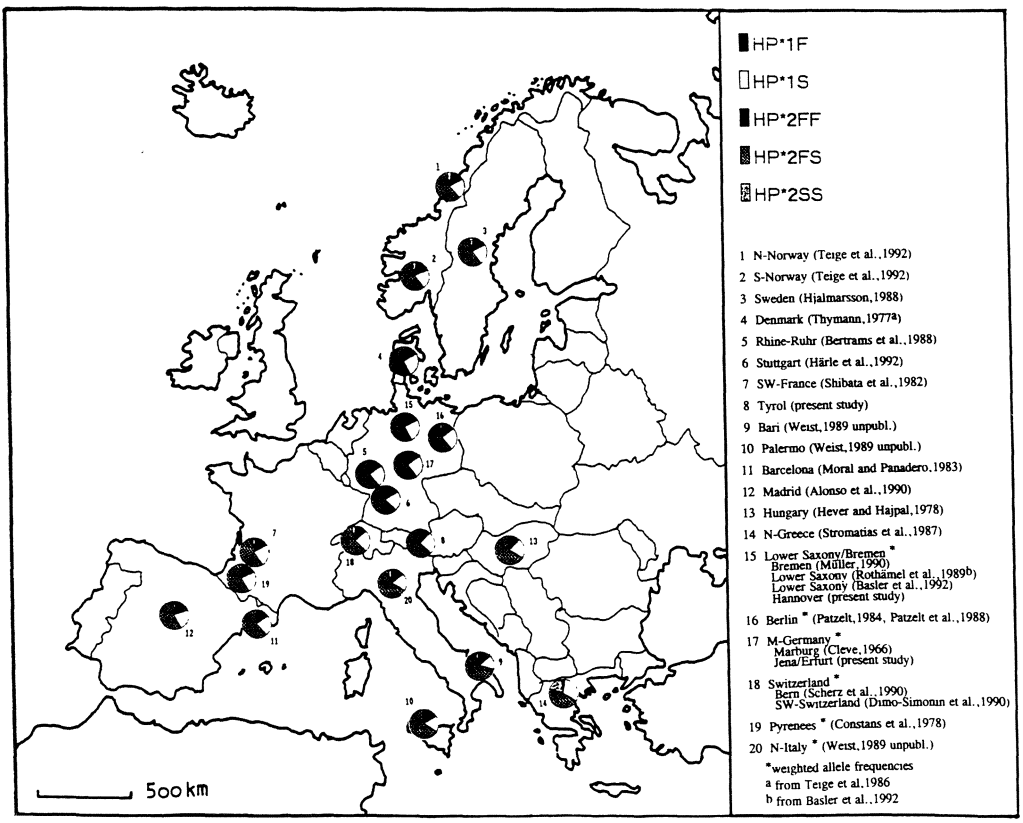

Fig.1: Distribution of HP subtype allele frequencies in European populations

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Investigations on the distribution pattern of haptoglobin subtype allele frequencies in European populations
- Preliminary report -

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Introduction

Human haptoglobin (HP) is a polymorphic serum glycoprotein with various biological activities (Lange,1992). It consists of two types of polypeptide chains, α and ß, covalently linked through disulfide bridges. The genetic polymorphism is essentialy based on variations in the aminoacid sequence of the α-chains. Today the most common codominant HPα-alleles are HP*1S and HP*1F (subtype alleles of HP*1) and HP*2FS, HP*2SS, HP*2FF (subtype alleles of HP*2), controlling 15 different phenotypes, respectively genotypes. In addition, several rare α- and ß-variants have been observed. The distribution of HP alleles indicates a high diversity in the different populations of the world (Roychoudhury and Nei,1988). Within European populations the HP*1 allele frequencies show a marked geographical distribution pattern of decline from Northwest to Southeast Europe (De Stefano et al.,1987).

In this paper some new HP subtype data for European populations are given. Furthermore the distribution pattern of HP subtype allele frequencies within Europe will be analyzed statistically.

Material and Methods

EDTA serum of unrelated individuals (blood donors) from three European population samples (Hannover, n=260, Thuringia, n=199, Tyrol, n=264) has been subtyped.
They were collected in 1991 in coopération with Prof.Dr.W.Stangel (Hannover), Dr.J. Schimmel (Jena), Dr.U.Jaeger (Jena), Prof.Dr.R.Uhlig (Erfurt) and Prof.Dr.H.Reissigl (Innsbruck,Austria). HP subtyping was carried out according to the method proposed by Patzelt (1984) and Bertrams et al.(1988). The gel was prepared with a modified ampholyte mixture (LKB): 0,28ml pH 3,5-10, 0,46ml pH 5-7, 0,18ml pH 6-8 in 18,5ml total gel volume. If samples turned out to be only weakly stained by the classical Coomassie staining, destaining and drying, this process was followed by a rapid silver staining excepted stop-reagent according to Willoughby and Lambert (Westermeier,1990). For the statistical analysis the relations between the degree of longitude/HP*, the degree of latitude/ HP* and the degree of longitude-latitude/HP* were examined for 37 hitherto HP subtyped European population samples by means of Spearman's correlation analysis as well as by simple and multiple linear regression analysis (Sachs,1984).

Results and Discussion

After PAGIEF, each HPα-polypeptide chain is visible as a single major band corresponding with a characteristic minor band. By the modified ampholyte composition the discrimination of the HP 2FS and HP 1F allele products could be improved. Formerly we frequently had to control the results adding an HP typing by means of vertical PAGE. The addition of silver staining optimizes the visualisation of weak bands which can be observed by this subtyping method in some instances (low HP serum concentration, low HPα output, intense destaining etc.).
The distribution of HP phenotype and allele frequencies in the three population samples under study is shown in Table 1. Genetic equilibrium can be assumed for all of them. All possible 5 HP subtype allele products including HP 2FF were observed. The investigated allele frequencies are in good accordance with those obtained from other European population studies. In general there is a relatively high interregional HP subtype variability within European populations (Fig.1). In Europe the most frequently occuring HP*1 subtype allele is HP*1S (0,1573-0,3110) and concerning the HP*2 subtype allele HP*2FS (0,4630-0,6653).

The correlation and regression analysis verify the results of De Stefano et al.(1987) with respect to the HP subtype data. R-, F- and p-values (Table 2) indicate that the HP system shows a clear Northwest to Southeast distribution gradient in Europe with the most prominent gradient from West to East. This pertains to the conventional haptoglobin polymorphism as well as to the subtyped one. The decrease frequency of the HP*1 mainly reflects a decrease in HP*1F. The parallele increase frequency of HP*2 cannot be attributed to a specific HP*2

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water, dried at room temperatue.

11. Observe the result: If a visible blue brown color appears on the papera, the Ag-Ab reaction has occurred, if no color changes, no Ag-Ab reaction has occurred.

Selection of Extraction reagents

We select a extraction reagent that can rapidly dissolve ABH substances from red cell membrane without changing the activity of these antigens.

Selection of enzyme-labeled antibodies

Antibody that is IgG class can be used in determination of ABO blood group in bloodstains by Dot-ELISA. Antibody that is IgM class can not be used in determination of ABO blood group in bloodstains by Dot-ELISA.

Blind study and sensitivity test

200 samples were tested by Dot-ELISA. Their results is the same as known blood group.

Sensitivity test: 1 ul extract of bloodstain diluted 3200 fold could still be correctly typed by Dot-ELISA. This amount corresponds to 150 ng bloodstain cottonthread. The color of the reaction becomes weaker and weaker as bloodstain extract concentration decreases.

Discussion

In 1987, J. Michael Cecka etc described determintion of ABO blood group in bloodstains by Dot-ELISA. In 1992, Nu Ruiguang etc directly labeled the enzyme onto the anti-A, anti-B, and anti-H monoclonal antibody for ABO blood gtoup by Dot-ELISA. The latter, sample treatment is time-cosuming and require special equipment. Extraction reagent we prepared can rapidly dissolve ABH substances form red cell membrane without changing the activity of these antigens. Sample treatment using the extraction reagent requires no special equipment. In the time being, this method for determination of ABO blood group in bloodstains is the most sensitive, and take only about 30 min. The extract reagent treatment takes time longer for the bloodstain aged up to 1.5 years.

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6. Blocking: Soak the papers in 1-2% BSA for 5 to 10 min.
7. Incubation: put the papers into the appropriate diluted (with blocking solution) enzyme-labeled monoclonal antibodies solution (A. B. H) for 5 to 60 min, respectively.
8. Washing: Repeat the washing (see step 3)
9. Development: soak the papers in developing solution for about 1 min.
10. Cease reaction: Development reaction was terminated by washing the paper in water, dried at room temperatue.
11. Observe the result: If a visible blue brown color appears on the papers, the Ag-Ab reaction has occurred, if no color changes, no Ag-Ab reaction has occurred.

Selection of extract reagent

These reagents such as XT-100, NH_4OH, N_AOH, PBS Tris-HCI etc. were made up into more than 10 extract reagents. Bloodstain samples were treated with these reagents respectively for determination of ABO blood group.

Selection of enzyme-labeled antibodies

Monocloral anti-A antibodies from 3 cell lines, monoclonal anti-B astibodies from 3 cell lines. and monoclonal anti-H antibodies from 2 cell lines, were purified respectively and labeled with horseradish peroxidase (HRP) for detemination of ABO blood group.

Blind study and sensitivity Test

200 samples were tested by Dot-ELISA. Their results were compared with known ABO blood group in bloodstains.

Sensitive test: bloodstains are determined by the Dot-ELISA at different dilutions.

Result

Dot-ELISA method for ABO blood group in bloodstains.

1. samples treatment: Bloodstain samples soak in extraction reagent.
2. Dot samples: About 1 ul extract was applied on three pieces of nitrocellulose paper, dried at room temperature.
3. Washing: put the papers in PBS-T and wash it two times for about two min/time.
4. H_2O_2 treatment: Soak the papers in 3% H_2O_2 for 3 min.
5. Washing: Repeat the washing (see step 3).
6. Blocking: Soak the papers in 1-2% BSA for 5 to 10 min.
7. Incubation: put the papers into the appropriate diluted (with blocking solution) enzyme-labeled monoclonal antibodies soiution (A. B. H) for 10 to 20 min, respectively.
8. Washing: Repeat the washing (see step 3).
9. Development: Soak the papers in developing solution for about 1 min.
10. Cease reaction: Develpment reaction was terminated by washing the paper in

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Study of Rapid Determination of ABO Blood Group in Bloodstains by the Improved Dot-ELISA with Monoclonal Antibodies

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In this paper, a determination of ABH blood group substances in bloodstains by the improved Dot-ELISA with horseradish peroxdase coupled monoclonal antibodies is described. The enrire test can be performed in about 30 munites by this method. This method is more simple, sensitive, correct and inexpensive than conventional methods and needs no complicated equipments. 1ul dilution of bloodstains as low as 3200 fold(or 150 ng bloodstained cottonthread) is sufficient for detection of ABO blood groups by this method. ABH blood group substances from the bloodstains aged up to2 years can be detected. The key of this method is a extraction reagent we prepared, which can rapidly dissolve ABH substances from red cell membranewithout changing the activity of these antigens.

Key Words: ABO blood grouping, Bloodstains, Dot-ELISA, Monoclonal antibody, Extraction reagent

Bloodstain samples is one of the most important forensic biologic evidence. Determination of ABO blood group substances in bloodstains is a standard laboratory procedure. For a long time, methods of determination of ABO blood group were mainly the absorption and elution-absorption testes. These conventional methods are either time-consuming or requiring special equipment and are also cumbersome. It is difficult that these methods are used in the scence of crime.

Based on the method, we established for determination of ABO blood group in body fluids, in 1992. Now again we have establish a method for determination of ABO blood group in bloodstains. Determination of ABH blood group substances by this method has been used in practical cases. This method is rapid, simple, sensi-tive and accurate. It produces clear results, and requires no equipment.

Materials and Methods

Materials

1. ABH enzyme-labeled monoclonal antibodies: produced by this institute
2. Extraction reagent: XT-100, NH_4OH, N_AOH, PBS, Tris-HCI etc.
3. Substrate solution: 4-chloro-1-naphthol, 3. 3′-diaminobenzidine(DAB), 30% hydrogen peroxide
4. Nitrocellulose Paper: purchased from Beijing chemical-works School
5. Blocking solution: bovine serum albumin (BSA)
6. Washing buffer: 0. 2M PH7. 4 phosphate buffered saline (PBS)
7. Bloodstain samples aged from 1 month up to 5 years

Dot-ELISA for determination of ABO blood group in bloodstains

1. Sample treatment: Bloodstain samples are soaked in a variety of extraction reagents, respectively.
2. Dot samples: About 1 ul extract were applied on three pieces of nitrocellulose paper, dried at room temperature.
3. Washing: put the papers in PBS-T and wash it two times for about two min/time.
4. H_2O_2 treatment: Soak the papers in 3% H_2O_2 for 1-10 min.
5. Washing: Repeat the washing (see step 3).

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RESULTS AND DISCUSSION

Figure 1 shows the bond patterns of three different types analyzed by SDS-PAGE under native and desialylated conditions using polyclonal and monoclonal antibodies. The OR35 and OR48 antibodies recognized ORM1 and ORM2 bands, respectively. On the other hand, the OR40 antibody reacted to both ORM1 and ORM2 bands and provided essentially similar patterns obtained with the polyclonal antibodies. The three monoclonal antibodies reacted to native and desialylated ORM.

ORM phenotyping was performed on 658 individuals from nine subpopulations of Aboriginal Taiwanese by IEF and immunoprinting using polyclonal antibody. Some samples were difficult to determine the phenotype, as shown in lanes 2–4 of figure 2A. The monoclonal antibodies were used to find the correct ORM phenotyps. The OR35 monoclonal antibody reacted only to ORM1 alloproteins and OR48 reacted only to ORM2 bands. On the other hand, OR40 reacted strongly to ORM1 bands and faintly to ORM2 bands (figure 2B–D). The results revealed the bands corresponding to ORM2 M to originate only from ORM2 M band, and the samples in the lanes 2–4 of figure 2 were identified as ORM1 F1–Q0/ORM2 M, Q0/M and S–Q0/M, respectively. The distributions of ORM phenotypes and allele frequencies are given in Table 1. In the present study, five ORM1 alleles (*ORM1*F1*, *ORM1*S*, *ORM1*dF1S*, *ORM1*B9* and *ORM1*Q0*) and three ORM2 alleles (*ORM2*M*, *ORM2*H19* and *ORM2*H2*) were recongnized by IEF method in combination with immunodetection using monoclonal and polyclonal antibodies.

In conclusion, we have found the technique using monoclonal and polyclonal antibodies to be of great value in ORM phenotyping of *ORM1*Q0* carrier. Futher study will be necessary to confirmation by genetical analysis using nucleic acids.

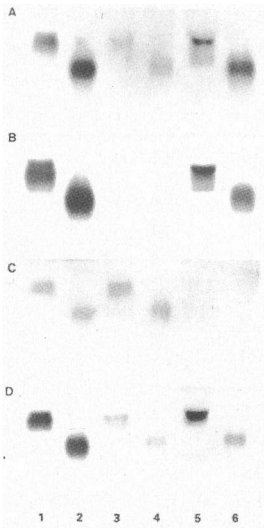

Fig.1. SDS-PAGE and immunoblot analysis of ORM before (1,3,5) and after (2,4,6) treatment with sialidase using polyclonal (A;DAKO) and monoclonal (B;OR35, C;OR48, D;OR40) antibodies. 1,2: ORM1 F1/ORM2 M, 3,4: Q0/M, 5,6: S/Q0.

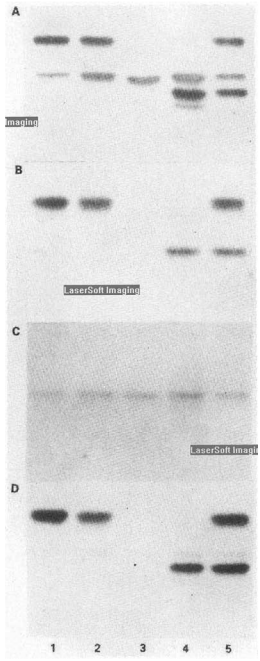

Fig.2. Immunoprint (A) using polyclonal antibody to ORM (Dako) and immunoblot using monoclonal antibodies to ORM with: (B) OR35, (C) OR48 and (D) OR40. 1: ORM1 F1/ORM2 M, 2: F1–Q0/M, 3: Q0/M, 4: S–Q0/M, 5: F1–S/M.

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Table 1. Distribution of ORM phenotypes and allele frequencies in nine subpopulations of Aboriginal Taiwanese.

Phenotypes		Atayal	Saisiat	Bunun	Tsou	Ami	Puyuma	Rukai	Paiwan	Yami
ORM1	F1	49	49	37	56	48	38	47	42	75
	F1-S	23	11	34	19	13	18	4	14	3
	S	0	0	6	3	0	1	0	1	0
	F1-dF1S	0	2	4	0	0	2	0	1	0
	F1-B9	0	0	0	0	3	0	0	0	0
	S-B9	0	0	0	0	0	1	0	0	0
	F1-Q0	25	1	4	2	8	3	3	2	0
	S-Q0	2	1	1	0	0	0	0	0	0
	Q0	1	0	1	0	0	0	0	0	0
Total		100	64	87	80	72	63	54	60	78
Allele frequencies										
*ORM1*F1*		0.730	0.875	0.667	0.831	0.833	0.786	0.935	0.842	0.981
*ORM1*S*		0.125	0.094	0.270	0.156	0.090	0.167	0.037	0.133	0.019
*ORM1*dF1S*		-	0.016	0.023	-	-	0.016	-	0.008	-
*ORM1*B9*		-	-	-	-	0.021	0.008	-	-	-
*ORM1*Q0*		0.145	0.016	0.040	0.013	0.056	0.024	0.028	0.017	-
ORM2	M	99	64	84	79	62	56	53	54	78
	M-H19	1	0	2	1	10	7	1	6	0
	M-H2	0	0	1	0	0	0	0	0	0
Total		100	64	87	80	72	63	54	60	78
Allele frequencies										
*ORM2*M*		0.995	1.000	0.983	0.994	0.931	0.944	0.991	0.950	1.000
*ORM2*H19*		0.005	-	0.012	0.006	0.069	0.056	0.009	0.050	-
*ORM2*H2*		-	-	0.006	-	-	-	-	-	-

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OROSOMUCOID PHENOTYPING USING MONOCLONAL ANTIBODIES

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INTORODUCTION

Orosomucoid(ORM) is a major glycoprotein in human serum and shows extensive variation. About 30 alleles heve been described at ORM1 and ORM2 locus, respectively (Yuasa et al. 1993). The ORM1 and the ORM2 alloproteins are encoded by two closely linked genes on the long arm of the chromosome 9 (Dente et al. 1987) and are distinguishable in band intensity from each other (Yuasa et al. 1986). In the course of ORM phenotyping, we have occasionally encountered some bands which are difficult to determine whether they belong to ORM1 or ORM2 product. In this study to overcome such confusions we attempted to prepare locus-specific monoclonal antibodies.

MATERIALS AND METHODS

Monoclonal antibodies – Female BALB/c mouse was immunized twice at 2-weeks interval by intraperitoneal injection of the purified desialylated ORM (about 100 μg protein, Cosmo Bio, Tokyo, Japan) emulsified in 100 μl Hunter's TiterMax adjuvant (CytRx, Norcross, GA). Three monoclonal antibodies, OR35 (IgG1), OR40 (IgM) and OR48 (IgG1), were selected. The ascitic fluid was used for immunological detection of ORM bands without further purification.

SDS-PAGE and immunoblot analysis – Sera reduced with 2-mercaptoethanol were separated on SDS polyacrylamide gel (8-16%), and blotted onto an Immobilon-P membrane (Millipore, Bedford, USA) by electroblotting. Then the filter was reacted to monoclonal or polyclonal antibodies for 40 min. Immunostaining was performed using Alp-labeled goat anti-mouse Ig (Dako, Glostrup, Denmark) or Alp-labeled porcine anti-rabbit Ig (Dako).

Subjects tested – Plasma samples from 658 Aboriginal Taiwanese were collected from the following a subpopulations: 100 Atayal, 64 Saisiat, 87 Bunun, 80 Tsou, 72 Ami, 63 Puyuma, 54 Rukai, 60 Paiwan and 78 Yami. Preliminary genetic studies on them were reported by Umetsu et al. (in press).

ORM phenotyping – Polyacrylamide gel IEF was carried out as described elsewhere (Yuasa et al. 1987), except that the concentration of Triton X-100 was decreased from 0.2% to 0.05%. The ORM patterns were developed by immunoprinting with polyclonal antibody, and also the ORM phenotyping was performed by immunoblotting with monoclonal antibodies. The symbols of these alleles were based on the nomenclature proposed by Yuasa et al. (1993).

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Although anti-H did not stain the mucous cells in the posterior lingual glands and tracheal glands from A1 donors, UEA showed moderate to weak reactivity in these cells. This result indicates that UEA reacts with at least two different types of terminal fucose residue in the mucous cells; one is the UEA specific residue which is unreactive with anti-H, and the other is the terminal residue of H antigen recognized by the anti-H used in this study.

The results obtained in this study showed that the expression of A and H anti-gens in selected tissues is different between A1 and A2 individuals.

Table 1. Staining with monoclonal antibodies and lectins in various human tissues from blood group A1 and A2 secretor donors.

	Hassalls corpuscles		Sublingual gl.		Tracheal gl.		Posterior lingual		Ebner's gl.		Endothelial cells	
Blood group	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
Reagents												
MoAB-A	++	++	++	++	++	++	++	++	++	++	++	++
MoAB-A1	−	−	−	−	−	−	−	−	−	−	−	−
MoAB-H	W	++	M	++	−	+	−	M	M	+	−	+
HPA	++	++	++	++	++	++	++	M	++	++	++	++
DBA	+	−	+	M	+	+	+	M	M	W	−	−
UEA	W	++	+	++	W	+	+	+	+	+	W	+

MoAB;monoclonal antibody, ++;intense reactivity, +;positive reactivity, -;negative reactivity, W;weak reactivity, M;mosaic pattern,

Summary

The expression of blood group antigens in selected tissues from blood group A1and A2 donors was examined using monoclonal anti-A,-H antibodies and blood group A or H specific lectins. Monoclonal anti-A antibody and HPA showed good reactivity with secretory cells of salivary glands and tracheal glands from both A1 and A2 donors. Endothelial cells and Hassall's corpuscles were also stained by these reagents. DBA showed good reactivity with these cells from A1donors and with mucous cells of tracheal and posterior lingual glands from A2 donors. However, it reacted feebly with the serous cells of von Ebner's glandsand exhibited no reactivity with Hassall's corpuscles from A2 donors. Endothlial cells from both A1 and A2 donors did not stain with DBA. Monoclonal anti-H antibody showed better reactivity with endothelial cells from A2 donors than those from A1.

Ackowlegements

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no reactivity with the reagents used in this study.

Lingual gland

The mucous cells in the posterior lingual glands from A1 individuals showed homogeneous staining with anti-A, HPA, DBA and UEA. Anti-H antibody exhibited feeble or no staining with these cells. The serous cells in von Ebner's glands showed strong but mosaic reactivity with these reagents including anti-H antibody. The mucous cells from A2 individuals exhibited a mosaic reactivity with anti-A, HPA and DBA and homogeneous reactivity with anti H and UEA. The serous cells in von Ebner's glands from A2 individuals exhibited essentially the same reactivity with these reagents except that DBA showed feeble reactivity.

Endothelial cells of blood vessels

Endothelial cells from A1 individuals were strongly stained with anti-A and HPA, feebly with UEA and not with anti-H, whereas cells from A2 individuals were clearly stained with anti-H and UEA as well as anti-A and HPA. DBA exhibited no reactivity with these cells from both A1 and A2 individuals.

The results obtained in this study are summarized in Table 1.

Discussion

The differences in the chemical nature of A1 and A2 erythrocytes has been defined. In the A1 phenotype, the determinant structure of A antigen is found on type 1, 2, 3 and 4 core structures. However, the corresponding structure in the A2 erythrocytes is found only on type 1 and 2, and is less abundant[5].

The results obtained in this study indicate that the pattern of expression of A and H antigen varies between the organs and is complicated. Since the A type3 or 4 determinants recognized by anti-A1 have been found on glycolipids[5], these antigens were presumed to be lost during the embedding process. In fact, the anti-A1 antibody used in this study exhibited no reactivity with the secretory cells, endothelial cells and erythrocytes but reacted with Hassall's corpuscles from A1 donors. The anti-H used in this study, which recognizes type 1 and/or 2 H antigen, exhibited little or weak reactivity with the mucous cells of tracheal glands, sublingual glands, and posterior lingual glands from A1 donors, and showed good reactivity with those from A2 donors. These results indicate that nearly all the H antigens were converted into A antigens in the mucous cells of A1 phenotypes while substantial amounts of H antigen remained unchanged in A2 phenotypes. The serous cells in von Ebner's glands from both A1 and A2 individuals were stained with anti-A and -H, showing a mosaic-like pattern. This result may indicates the presence of a mechanism for inhibiting the conversion of H antigen into A antigen in von Ebner's glands as observed in the serous cells of the submandibular glands. Here only the H antigen was expressed irrespective of the ABO group but dependent on the secretor status of the individual[4].

The reactivity with HPA or DBA varied among the tissues and organs, as seen in Hassall's corpuscles, secretory mucous cells, von Ebner's glands, endothelial cells. DBA did not react with endothelial cells from both A1 and A2 donors, and showed good reactivity with secretory cells from A1 donors, weak to moderate reactivity with secretory mucous cells from A2 donors and feeble reactivity with the serous cells of von Ebner's glands and Hassall's corpuscles from A2 donors. On the other hand, HPA and anti-A showed intense reactivity with these cells from both A1 and A2 individuals. These differences in staining intensity may be explained by differences in binding specificity of these lectins and structurural differences of the A antigens at each tissue site. Torres et al.[6] and Baker et al.[7] reported that HPA has six carbohydrate binding sites and exhibits much broader specificity for terminal GalNac than DBA which has only two carbohydrate binding sites. Since it seems that nearly all the H antigens are converted into A antigens in the secretory cells from A1 donors but remained unchanged in A2 donors, more A antigens are present in tissues from A1 donors than those from A2 individuals. Thus, the present results may be alternatively explained simply by a difference in the amount of A antigens at each tissue sites.

The results of the present study also showed that nearly all the H antigens in the endothelial cells from A1 donors may be converted into A antigen while substantial amount of H antigens remained unchanged in A2 individuals.

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COMPARATIVE LECTIN AND IMMUNO-HISTOCHEMISTRY ON ANTIGEN EXPRESSION IN BLOOD GROUP A1 AND A2 INDIVIDUALS

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Blood group A has been divided into the major subgroups, A1 and A2, since it was first described by von Dungern and Hirszfeld in 1911[1].

Although the serology and chemical structures, and cDNA encoding transferases of A1 and A2 have been well established[2], the different of antigen expressionin tissue sites between these subgroups has remained an unresolved problem.

The aim of the present work was to elucidate possible differences in the antigen expression and the reactivity with lectins within several organs from A1 and A2 secretor individuals using lectin- and immunohistochemical methods.

Materials and Methods

Specimens of the sublingual gland, trachea, and tongue were obtained at autop-sies. Blood goup A subtypes and Lewis phenotypes of the donors were determined by the routine hemagglutination test. Twenty A1 secretor and 8 A2 secretor individuals were used in this study. Specimens of the thymus from 5 A1 and 3 A2 donors were also examined. The specimens were fixed in 10% formalin, embedded in paraffin and serially sectioned at 5μm. The experiments were performedas previously described using monoclonal anti-A or -H antibodies, and blood groupA or H specific lectins conjugated with horseradish peroxidase(HRP)[3,4]. Monoclonal anti-A and -A1(type 3 and 4 specific) antibodies were purchased from Biotest, Germany. Anti-H(type 1 and 2 chain specific) antibody was obtained from Chembiomed, Canada. HRP-labelled Helix pomatia agglutinin(HPA), Dolichos biflorus agglutinin(DBA) and Ulex europaeus agglutinin I(UEA) were purchased from E.Y.Laboratories, USA. Following incubation with antibodies and lectins, tissue sites reactive with the reagents were visualized using a Strept-Avidin Biotin immunostaining kit(Nichirei,Japan) and/or diaminobenzidine-H_2O_2 medium.

Results

Hassall's corpuscles

The Hassall's corpuscles from A1 secretor individuals showed good reactivity with anti-A, HPA and DBA, and relatively weak with anti-A1 and UEA. Weak or no staining was observed with Anti-H. The Hassall's corpuscles from A2 secretors were clearly stained with anti-A and -H antibodies. They also reacted with HPA and UEA but not with anti A1 and DBA.

Sublingual gland

Nearly all the mucous cells from A1 individuals were stained with anti-A, HPA,DBA and UEA. Anti-H antibody exhibited good reactivity with a small number of the mucous cells and no reactivity with the remaining cells showing a mosaic distribution pattern of H antigen. Although the mucous cells from A2 individuals were clearly stained with anti-A,-H, HPA and UEA, DBA exhibited feeble or no reactivity with these cells. Anti A1 antibody showed no reactivity with the mucous cells in the sublingual gland. No positive reactions were recognized with these reagents in the serous cells of the glands.

Tracheal gland

In the mucous cells of the tracheal glands, the reactivity with monoclonal antibodies and lectins was essentially the same as that observed in the sublingual glands except that DBA showed good reactivity with the mucous cells from both A1 and A2 groups. The serous cells of the tracheal glands exhibited

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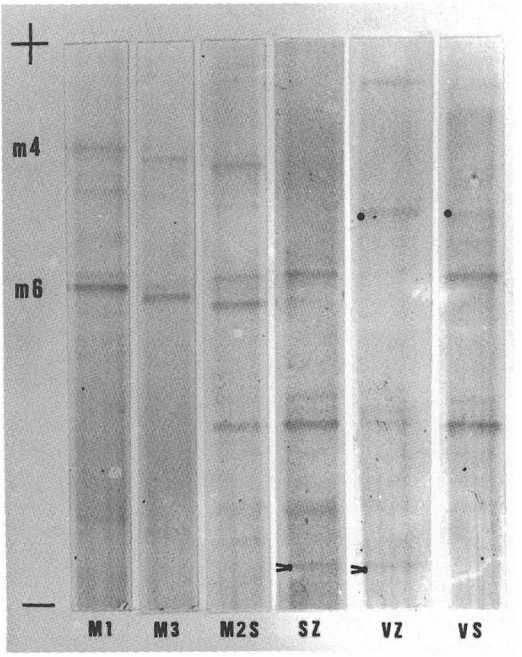

Figure 1.
PI subtypes obtained by HIEF in a 4.3-4.8 pH gradient. The main bands corresponding to PI V and PI Z are marked with · and > respectively.

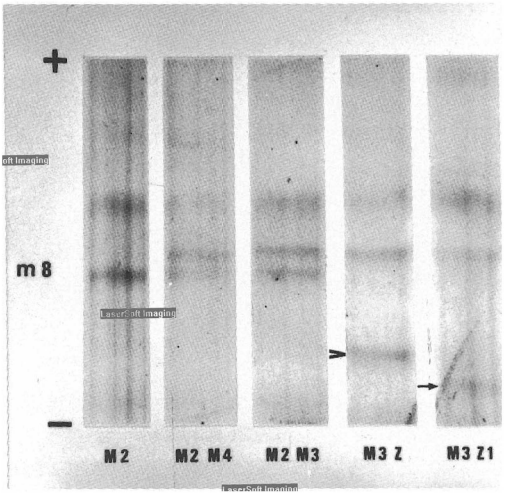

Figure 2.
PI subtypes obtained by HIEF in a 4.52-4.72 pH gradient. The main bands corresponding to PI Z and PI Z1 are marked with > and -> respectively.

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Portuguese sample is characterized by increased PI*M2 and decreased PI*M3 frequencies.

Formal Genetics

Segregation analysis was performed in a sample with 132 families with 301 offspring. No mother-child exclusions were found. Due to the small numbers per mating type inherent to the high degree of polymorphism of this system, the segregation of each gene product against all others was studied sequentially. With this analysis, a significant deficiency of M1 offspring from M1- x M1- mating type was found (Table 2).

TABLE 1. PI phenotype distribution in NW Portugal

Phenotype	observed	expected
M1	86	79.13
M1M2	54	58.55
M1M3	17	21.28
M1M4	5	2.76
M1S	28	30.93
M2	14	10.83
M2M3	5	8.07
M2S	12	11.44
M2V	1	0.41
M2Z	2	0.82
M3	5	1.50
M3S	6	4.26
S	3	3.02
SZ	1	0.43
SZ1	1	0.11
VZ	1	0.02
others	0	7.44
total	241	

PI*M1= 0.573 ± 0.023
PI*M2= 0.212 ± 0.019
PI*M3= 0.079 ± 0.012
PI*M4= 0.010 ± 0.005
PI*S = 0.112 ± 0.014
PI*V = 0.004 ± 0.003
PI*Z = 0.008 ± 0.004
PI*Z1= 0.002 ± 0.002

chi-square = 5.68
2 df
.10 > P > .05

TABLE 2. Segregation analysis of M1- x M1- mating type

nr. of		offspring phenotypes					
families	offspring	M1	M1-	-	chi^2	df	P
24	50	5 (12.50)	31 (25.00)	14 (12.50)	6.12	2	.050-.025

"-" stands for any allele except M1

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PI (α-1-ANTITRYPSIN) SUBTYPES IN NW PORTUGAL

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INTRODUCTION

The study of α-1-antitrypsin (PI) locus at protein level by isoelectric focusing has revealed a high degree of genetic variation both within and between most human populations.

Among European populations, Iberia (including the Basque Country) shows a peculiar distribution, with the highest PI*S frequencies reported so far.

In this work we present formal and population genetics data on PI subtypes in NW Portugal.

MATERIAL AND METHODS

Blood samples were obtained by venipuncture and EDTA plasmas were stored at -20°C.

PI subtyping was done by hybrid isoelectric focusing (HIEF) at pH ranges 4.3-4.8 and 4.52-4.72 in polyacrylamide gels (T=5%; C=3%) at 8°C. Immobilized pH gradients were generated according to LKB Application Note 324. The dried gels were rehydrated for 2 hours with 20% (w/v) sucrose and 2% (v/v) carrier ampholytes pH 4.2-4.9 (Pharmacia). Electric settings were: 4000V, 4mA, 12W for 6 hours and 5000V, 25mA, 8W for 8 hours for the pH ranges 4.3-4.8 and 4.52-4.72, respectively. Anolyte was glutamic acid 10mM and catholyte was NaOH 10mM. Samples were directly applied using Whatman nr.1 filter paper (0.5x0.5cm). Staining was done with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Fig.1 presents PI phenotypes as revealed by HIEF with the 4.3-4.8 pH range. Using this interval PI M3 and PI M4 gene products were not discriminated. A rare variant (V) with intermediate mobility between the so-called m4 and m6 zones was detected, showing a decreased band intensity when compared to both M and S.

Fig.2 shows the separation achieved in the m8 zone with the pH range 4.52 - 4.72. PI Z1 is a gene product with a pI higher than PI Z and similar band intensity. These properties are coincident with PI Zaug gene product characterized by FABER et al.(1990) suggesting that the Z substitution (Glu^{342}-> Lys^{342}) occurred in a M subtype different from M1.

Population Genetics

Tab.1 shows the distribution of PI subtypes in a random sample of 241 individuals from NW Portugal.

The high frequency of PI*S of our sample (in association with a reduction of PI*M1 frequency) is consistent with other reports on Spanish populations (Alonso et al., 1991); however, the

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DISCUSSION

The results of this study show that 24% of the patients are positive for Ag allo-antibodies in comparison to 27% of 178 patients tested in 1978/1981. Ag(x) and Ag(al) (7.6%) are the most common specificities found. Except for one case, Ag antibodies were not detected in the intermedia and sickle/thalassaemia patients who had received very low numbers of blood transfusions. Sex, age and splenectomy did not affect significantly the frequency of the Ag antibodies, whereas there was a correlation with the number of transfusions per year.

History of severe allergic reactions associated with blood transfusions was statistically significantly higher in the anti-Ag positive patients in comparison with the negative ones.

The results of this study confirm previous findings of the high frequency of allo-immunization against low density lipoproteins in multiply-transfused patients. However, there is a declining incidence of these antibodies over a period of 10 years either because of desensitization of the patients as a result of repeated contact with more common antigens(5) or because of improvements in the quality of transfused blood(6).

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Table 3: Frequency of Ag-antibodies by age and number of transfusions

	Ag (+)		Ag (-)	
	n	%	n	%
Total	35	24.1	110	75.9
Age				
<15	3	37.5	5	62.5
15-19	7	24.1	22	75.9
20-24	11	23.4	36	76.6
25-29	10	33.3	20	66.7
30-34	2	11.1	16	88.9
35+	2	15.4	11	84.6

Table 4: Specificities of Ag-antibodies in total sample and in the positives

	Total sample		Ag positives	
	n	%	n	%
Total	145	100.0	35	100.0
a1	11	7.6	11	31.4
c	7	4.8	7	20.0
d	2	1.4	2	5.7
g	2	1.4	2	5.7
h	7	4.8	7	20.0
x	26	17.9	26	74.3
y	3	2.1	3	8.6

Table 5: Comparative studies

1978-1981
84.6% of the patients remained positive
15.4% lost their antibody (z specificity)

1978-1991
69.2% remained positive
30.8% lost their antibody

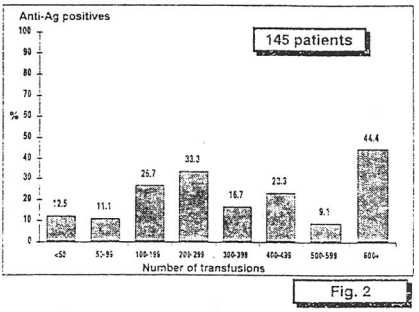

Fig. 2

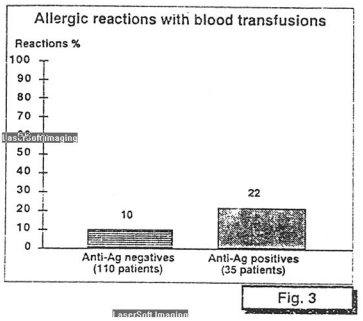

Fig. 3

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IMMUNIZATION OF THALASSAEMIC PATIENTS AGAINST THE Ag SYSTEM

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INTRODUCTION

Patients with homozygous b-thalassaemia may develop a variety of allo-antibodies against blood cell antigens and serum protein antigens as a result of multiple transfusions. Previous studies in our thalassaemia unit and elsewhere have shown that the incidence of red cells alloantibodies is lower than expected, whereas the frequency of allo-antibodies against low density lipoproteins in high (1-3).
The aim of this study is to investigate the frequency and specificities of allo-antibodies against IgA and low density lipoprotein and to compare these results with the corresponding data ten years ago.

MATERIALS AND METHODS

Sera from 145 multiply transfused Greek thalassaemic patients were tested for the presence of allo-antibodies against IgA (anti-IgA) and low density lipoprotein (anti-Ag) using a haemagglustination assay (HA) and immunodiffusion tests. The patients had all been receiving red cell concentrates (RCC) which, after 1985, were leukocyte poor with washing and filtration procedures (Table 1). The prevalence of the detected allo-antibodies are analyzed in relation to age, splenectomy, number of blood transfusions and allergic reactions associated with tranfusion.
Data were analyzed by logistic regression.

RESULTS

Table 1

	Sample description	
	n	%
Total patients	145	100.0
Females	76	52.4
Males	69	47.6
	Diagnosis	
Thalassaemia major	127	87.6
Intermedia thalassaemia	9	6.2
Sickle/Thalassaemia	9	6.2
	Splenectomy	
Yes	85	58.6
No	60	41.4
Age (years) Median	Min	Max
23	3	50
Transfusions 353	2	980

Table 2: Frequency of Ag-antibodies (by sex, diagnosis and splenectomy)

	Anti-Ag +		Anti-Ag -		
	n	%	n	%	
Total	35	24.1	110	75.9	
Sex					
Females	18	23.7	58	76.3	$x^2_1=0.00$
Males	17	24.6	52	75.4	p=1.00
Diagnosis					
Major thal.	34	26.8	93	73.2	$x^2_1=3.88$
Intermedia thal.	1	11.1	8	88.9	p=0.049
Sickle/b-thal.	0	.0	9	100.0	
Splenectomy					
Yes	17	20.0	68	80.0	$x^2_1=1.41$
No	18	30.0	42	70.0	p=0.23

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Table 1. The expression of ABH antigens and lectin reactivities of taste buds and secretory cells in tongue from some mammalian species.

Rat	TB	EG	LG	RBC	End	Epi	Rabbit	TB	EG	LG	RBC	End	Epi
Reagents							Reagents						
A	+	+	W+	-	-	+	A	+	+	-	-	-	+
B	+	+	-	-	-	-	B	W+	-	-	+	-	-
H	-	+	W+	-	-	-	H	W+	W+	-	-	-	+
Le x	-	-	-	-	-	-	Le x	W+	-	-	-	-	-
Le y	W+	+	-	-	-	-	Le y	+	+	-	-	-	-
Le a	-	+	-	-	-	-	Le a	W+	-	-	-	-	-
Le b	-	+	-	-	-	-	Le b	W+	-	-	-	-	-
HPA	+	+	+	-	-	+	HPA	+	+	W+	-	-	+
DBA	-	+	+	-	-	+	DBA	W+	M+	-	-	-	+
UEA-I	+	W+	+	-	-	-	UEA-I	-	-	-	-	-	-
PNA	-	+	-	-	-	-	PNA	-	W+	+	-	-	-
ECA	-	+	-	-	-	-	ECA	-	+	+	-	-	-
WGA	W+	+	+	+	+	+	WGA	+	+	+	-	-	-

Dog	TB	EG	LG	RBC	End	Epi	Monkey	TB	EG	LG	RBC	End	Epi
Reagents							Reagents						
A	+	+	+	-	-	+	A	-	-	-	-	-	-
B	-	-	-	-	-	-	B	+	M+	M+	-	+	+
H	W+	+	-	-	-	+	H	W+	+	+	-	-	+
Le x	-	+	-	-	-	-	Le x	-	W+	-	-	-	-
Le y	+	+	M+	-	-	+	Le y	-	M+	+	-	-	-
Le a	+	+	-	-	-	-	Le a	+	+	-	-	-	-
Le b	+	+	M+	-	-	-	Le b	W+	+	+	-	-	-
HPA	+	+	+	-	-	+	HPA	-	-	-	-	-	-
DBA	-	-	+	-	-	+	DBA	-	-	-	-	-	-
UEA-I	-	-	M+	-	-	+	UEA-I	+	+	+	-	-	-
PNA	-	-	-	-	-	-	PNA	-	-	-	-	-	-
ECA	-	-	+	-	-	-	ECA	-	+	+	-	-	-
WGA	+	-	+	-	-	+	WGA	+	+	+	-	-	-

Human*	TB	EG	LG	RBC	End	Epi	Human**	TB	EG	LG	RBC	End	Epi
Reagents							Reagents						
A	+	+	+	+	+	+	A	+	+	+	+	+	+
B	-	-	-	-	-	-	B	-	-	-	-	-	-
H	+	+	-	-	-	+	H	-	+	-	-	-	-
Le x	W+	+	W+	-	-	-	Le x	W+	+	W+	-	-	-
Le y	W+	+	W+	-	-	+	Le y	-	+	-	-	-	-
Le a	+	+	+	-	-	-	Le a	+	+	+	-	-	-
Le b	+	+	+	-	-	-	Le b	-	+	-	-	-	-
HPA	+	+	+	+	+	+	HPA	+	+	+	+	+	+
DBA	+	+	+	-	-	-	DBA	+	-	-	-	-	-
UEA-I	+	+	+	-	-	+	UEA-I	-	-	-	-	+	+
PNA	-	-	-	-	-	-	PNA	-	-	W+	-	-	-
ECA	-	-	-	-	-	-	ECA	-	-	W+	-	-	-
WGA	+	+	+	+	+	+	WGA	-	+	+	+	+	+

EB;taste buds, EG;von Ebner's glands, LG;posterior lingual glands,
RBC;red blood cells, Ebd;Endothelial cells, Epi;Papillary epithelia.
+;positive reaction, w+;weak reaction, M+;mosaic distiribution,
-;negative reaction.
*;blood group A secretor, **;blood group A non-secretor.

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Numerous nuclei in the serous cells of von Ebner's glands from nearly all the individuals of japanese monkeys were unexpected stained by anti Le^a. This antibody showed clear reactivity the nuclear cell membrane and chromatin of von Ebner's glands and no reactivity with the nucleus in other tissue sites and organs even from the same donor.

ABH antigens in tissues from non-secretor individuals were not recognized in this study except some human donors.

The results obtained in this study are summarized in Table 1. The results described in Table 1 were obtained from the following blood group donors; Rat: blood goup AB, Rabbit: blood group A+, Dog: blood group A, Japanese monkey: blood group B, Human: blood group A (secretor and non-secretor).

Discussion

Taste buds consist of neuroepithelial cells with secretory and/or taste receptor functions. The significance of salivary secretion for taste perception has been pointed out by Gurkan and Bradley(3).

Mucous substances, which are secreted by the taste cells themselves and by vonEbner's glands or by salivary glands of the oral cavity, have been implicated in the taste stimulation process. Gurkan and Bradley(3) described that secretions from von Ebner's glands may influence the response of the taste receptors by controlling access of the taste stimuli to the taste buds, by changing the transepithelial resistance as a consequence of secretory mechanisms, and by changing the ionic or macromolecular composition of the micro-environment in which the initial events of taste transduction take place. Schmale et al.(4) found a glycoprotein in the mucous of von Ebner's glands, which was chemically related to the order binding protein that may play a key role in olfactory reception. The finding of ABH related antigen in the taste buds and von Ebner's glands may provide an important clue to the role of these antigens and carbohydrates in the chemoreceptor systems, since the taste buds are tissues that possesses a limited function as the taste receptor.

Summary

The localization of the ABH and related antigens in the taste buds and the lingual glands was examined by means of immmuno- and lectin-histochemical methods. The ABO blood type of each mammalian individual was determined by the reactivity of anti A, B, and H antibodies with secretory cells of lingual glands. The serous cells of von Ebner's glands of all the rats examined reacted with anti A and B antibodies. The rabbits were grouped into A(+) or A(-) individuals. The dogs were typed into blood group A or O, and the japanese monkeys were typed into blood group B or O. Although the reactivity of MoAbs against ABH related antigens and blood group specific lectins with the tissue site of tongue, such as taste buds, von Ebner's glands, posterior lingual glands, epithelial cells, and endothelial cells of blood vessels were different and varied among the animal species, the individuals belonging to the same species and the same blood group showed similar reactivity with each reagent apart from the staining intensity.

The finding of ABH and related antigens in the taste buds and von Ebner's glands may provide an important clue to elucidating the role of these antigens in the taste receptor systems.

Acknowledgement

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LECTIN- AND IMMUNO-HISTOCHEMISTRY ON MUCOUS SUBSTANCES OF THE TASTE BUDS AND LINGUAL GLANDS IN SOME MAMMALS

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The ABO blood group antigens were discovered at the beginning of this century. Although the biochemical and molecular-biochemical nature of the ABH and related antigens is now quite well characterized and it is presumed that they are concerned with oncodevelopmental and/or cell recognition phenomena(1), no clear explanation of the biological significance of these antigens has been proposed. Characterization of cells with regard to their surface and secretory glycoconjugates is important in order to understand the basis of their physiological and interactive behaviour. The taste buds of vertebrates are covered by mucous substances and it has been suggested that the carbohydrate residues of the mucous substances are essential for chemoreceptor processes in the function of taste buds. Therefore, in the present study, we examined the distribution of different types of carbohydrate antigens in the taste buds cells and lingual glands using monoclonal antibodies(MoAb) against blood group antigens and lectins conjugated with horseradish peroxidase(HRP).

Materials and Methods

Tissue specimens of tongue from mammals including man ,japanese monkey, rat, rabbit and dog were used in this study. Tissues from these animals were obtained from the Institute for Experimental Animals of Shiga University of Medical Science. Human tissue specimens were obtained at autopsy. Tissues were fixed in 10% formalin, embedded in paraffin and serially sectioned at 5μm. The experiments were performed as previously described using MoAb, A, B, H, Le^a, Le^b, Le^x and Le^y, and a battery of lectins conjugated with HRP(2). MoAbs against A,B, Le^a and Le^b antigens were purchased from Biotest,Germany, anti-H(type 1 and 2 specific) from Chembiomed, Canada, anti Le^x and Le^y from Signet, England. Helix pomatia agglutinin(HPA), Dolichos biflorus agglutinin (DBA), Ulex europaeus agglutinin I(UEA), Arachis hypogaea agglutininn(PNA), Triticum vulgaris agglutinin (WGA), Glycine max agglutinin(SBA), and Erythrina cristagalli agglutinin(ECA) were purchased from E.Y. Laboratories, USA.

Results

The ABO blood type of individuals examined in this study could be determined by the reactivity of anti A, B, and H MoAb with secretory cells of lingual glands. The serous cells of von Ebner's glands from all the rats examined reacted with anti A and B MoAb. The rabbits were grouped into A(+) and A(-) individuals. Secretory cells from A(-) individuals showed no reactivity with anti A, B and H antibodies. The dogs were typed into blood group A or O. The japanese monkeys were typed into blood group B or O. Although the reactivity of each reagent with the tissue sites of the tongue, such as taste buds, von Ebner's glands, posterior lingual glands, epithelial cells, and vascular endothelial cells were different and varied, the tissues from individuals belonging to the same species and blood groups showed similar reactivity with these reagents apart from the staining intensity. The reactivity of blood group specific lectins with the taste buds from each mammal was dependent on the ABO blood group of the donor. The Le^y antibody showed clear reactivity with the taste buds from all mammals, except the non-secretor human individuals. The anti-Le^x showed weak reactivity with the taste buds from the rabbits and humans, and moderate reactivity with serous cells of von Ebner's gland, as well as the mucous cells of posterior lingual glands from non-secretor human donors.

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In the present method, the polymorphism on ITI has been detected with the use of a commercially available anti-ITI antiserum that reacts with all of the ITI subunits. In subsequent experiments, we found that there was an ability to detect ITI variation in anti-H chains, but not in anti-L chain fractionated from the antiserum by affinity chromatography (data not shown). Recently, the cDNA sequences of H1, H2 (Salier et al. 1987) and that of L coding genes (Bourguignon et al. 1985) were determined and moreover DNA polymorphisms in the ITI H2 gene were demonstrated (Léveillard et al. 1990a, 1990b). Relationship of ITI polymorphisms between protein and cDNA is still unknown, however, it might be solved in further studies.

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RESULTS AND DISCUSSION

Fig. 1 shows ITI phenotypes by means of PAGIEF and immunoblotting. In comparison with common types, the mother and her child were phenotyped as ITI 2 and ITI 1, respectively (Fig. 1, lane 3 and 4), thus resulting in the denial of maternity. However, no additional incompatibility between mother and child was observed in other genetic marker systems including AHSG demonstrating the transmission of a rare allele (AHSG*15) from mother to child (Fig. 2, lane 2 and 3). Although we could not observe apparent and considerable decrease of ITIs in both sera judging from the intensity of ITI bands (Fig. 1) and a quantitative rocket immunoelectrophoresis (data not shown), we considered that the inconsistency might be elucidated by the presence of a silent allele (ITI*Q0).

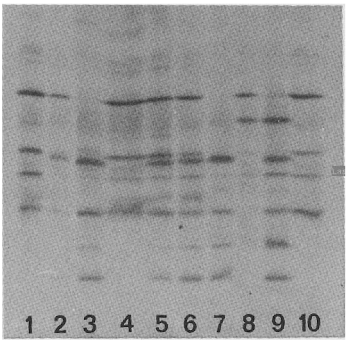

Fig. 1. Band patterns of ITI phenotypes of desialized sera analyzed by IEF in 2.5 M urea and the range of pH 5.5-7.7 followed by immunoblotting. Anode is at the top.

Lane 1 and 10	:	ITI 1
2 and 6	:	2-1
3(mother)	:	2
4(child)	:	1
5(father)	:	2-1
7	:	2
8	:	3-1
9	:	3-2

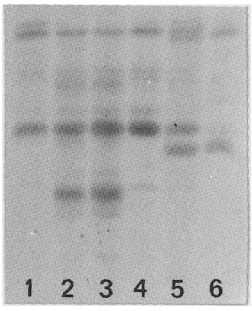

Fig. 2. AHSG phenotypes of desialized sera analyzed by IEF in 2.5 M urea and the range of pH 5-6 followed by immunoblotting. Anode is at the top.

Lane 1	:	AHSG 1
2(mother)	:	15-1
3(child)	:	15-1
4(father)	:	1
5	:	2-1
6	:	2

Vogt et al. (1991b) reported one paternity case excluded fatherhood in ITI system, and suggested that the ITI phenotype in younger children below 18 months having genotype ITI*1-2 might be typed as homozygous phenotype of ITI 1 or ITI 2 by reason of incomplete expression of ITI. This assumption still remains in the present case because the child was 9 months old when she was tested.

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HUMAN INTER-ALPHA-TRYPSIN INHIBITOR (ITI) SILENT ALLELE FOUND IN A CASE OF DISPUTED PATERNITY

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INTRODUCTION

Inter-alpha-trypsin inhibitor (ITI) is a 180-kDa glycoprotein having the role of a serine protease inhibitor. It has been revealed that ITI is not a single polypeptide chain structure, but a complex of three kinds of subunits, two heavy (H1 and H2) chains and a light (L) chain (Bourguignon et al. 1983) with chondroitin sulfate cross-links (Jessen et al. 1988). In situ hybridization showed that the H1, H2 and L chains were encoded by separate genes located on chromosomes 3, 10 and 9, respectively (Diarra-Mehrpour et al. 1989). Genetic variation of ITI on an isoelectric point was reported by Vogt and Cleve (1990) being controlled by mainly three alleles (ITI*1, ITI*2 and ITI*3) with autosomal codominant inheritance. The utility of ITI system has been supported from the results of several population studies (Luckenbach et al. 1991; Vogt et al. 1991a, 1991b; Yuasa et al. 1991; Vogt et al. 1992). However, Vogt et al. (1991b) indicated the cautious application of ITI for paternity testing due to an incomplete expression of ITI phenotypes among infants.

We present here a case of disputed paternity with ITI silent allele (ITI*Q0) in a mother-child pair, supposing from the fact that no inconsistent result of maternity in 26 other genetic marker systems containing the rare variant allele in alpha-2-HS-glycoprotein system (AHSG*15) was observed.

MATERIALS AND METHODS

Serum samples obtained from individuals in paternity testing were treated with neuraminidase and subjected to isoelectric focusing (IEF) on polyacrylamide gel (PAG) containing 2.5 M urea and 2.5% carrier ampholyte of either pH 3.5-9.5 or pH 5.5-7.7 (pH 6-8 : pH 7-9 = 3 : 1). After IEF, ITI bands were visualized by immunoblotting using anti-ITI antiserum (Dako-patts) mostly according to the previous report (Nakayashiki and Katsura 1989). In this case, following genetic markers were phenotyped; 7 red cell antigens (ABO, MNSs, Rh, Duffy, Kidd, Lewis, P), 10 serum proteins (HP, GC, TF, PI, AHSG, GM, KM, HF, ZAG, ITI), 5 red cell enzymes (ACP1, PGM1, PGD, GPT, ESD) and 3 HLA antigens (HLA-A, -B, -C). Additionally, 2 DNA polymorphisms (VNTRs: ApoB and YNZ22) by PCR technique were tested.

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the five populations of Sri Lanka constitute different subpopulations, maintained by cultural religious and ethnic barriers. To understand the effect of these genetic differences will have on paternity and forensic testing , we calculated the Power of exclusion (PE), Probability of Match (PM), Discriminant Probability (DP) for each system (Table 1). There is a considerable spread of PE values in Sri Lankan populations. It ranges from 0% in Kell to 45% in MNSs. The MNSs system is the most informative system followed by PGM subtypes and GC subtypes in all the five populations. Using only the blood groups, one can exclude around 83% of all falsely accused fathers in three populations (Sinhalese, Tamils and Moors), while this exclusion power of the blood groups is slightly better in Burghers (86%) and Moors (87%). Using the battery of red cell enzymes, cumulative power of exclusion (CPE) range between 73-78%, the highest found in the Moors. For serum proteins, CPE shows a narrow range 80-83%. Overall using these conventional systems more than 99% of wrongly alleged fathers could be excluded from each of these populations. Sixteen systems have been found to be very informative in all the five populations (ABO, Rhesus, MNSs, FY, JK, ACP, ESD, GLO, GPT, BF, ORM1 and subtypes of PGM, GC, TF, PI and HP) excluding 99% of alleged fathers in Burghers and Moors and 98% in Sinhalese, Tamils and Malays.

A further analysis was attempted to calculate the probability of match (PM) and discriminant probability (DP) of these twenty seven conventional systems. A trend similar to that observed in PE values was observed in PM and DP estimates. In all populations MNSs shows lowest PM values, and therefore has the highest probability of being able to discriminate between blood samples. Other systems with low PM and high DP are PGM and GC subtypes. For blood groups, there is a very similar range of PM estimates in all the five populations with the lowest in the Moors (1.14×10^{-3}) and highest in the Malays (2.26×10^{-3}). A similar trend was observed for red cell enzymes and serum proteins. Moors and Burghers showed the lowest PM values for both red cell enzymes and serum proteins. The combined probability that two blood samples match on all twenty seven genetic markers varies from 1.09×10^{-8} (Sinhalese) to 2.64×10^{-9} (Moors). Using the battery of sixteen systems, PM values varied from from 1.11×10^{-7} (Sinhalese) to 2.64×10^{-8} (Moors).

In conclusion, it seems that in spite of the wide variation in allele frequencies among the five populations, the CPE values for each group are very similar, thus the substructure of the population may have very little effect in paternity testing. Due to considerable variation in each genetic system in different populations, appropriate genetic database for traditional markers is still required. The cumulative probability of match is quite different in the investigated groups therefore, for forensic testing individual subpopulation analysis is essential and warranted.

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Table 1. PE, PM and DP estimates in five endogamous populations of Sri Lanka

System	Sinhalese			Tamils			Burghers			Moors			Malays		
	PE	PM	DP	PE	PM	DP	PE	PM	DP	PE	PM	DP	PE	PM	DP
ABO	0.253	0.346	0.654	0.296	0.295	0.705	0.330	0.377	0.623	0.307	0.285	0.715	0.330	0.310	0.690
RHESUS	0.260	0.338	0.662	0.229	0.340	0.660	0.345	0.252	0.748	0.325	0.299	0.701	0.183	0.454	0.546
MNSs	0.455	0.183	0.817	0.410	0.203	0.797	0.411	0.186	0.814	0.453	0.179	0.821	0.413	0.193	0.807
KELL	0.000	1.000	0.000	0.010	1.000	0.000	0.027	0.886	0.114	0.000	1.000	0.000	0.000	1.000	0.000
DUFFY	0.183	0.367	0.633	0.172	0.394	0.606	0.178	0.401	0.599	0.169	0.406	0.594	0.165	0.418	0.582
JK	0.184	0.374	0.626	0.172	0.392	0.608	0.187	0.336	0.664	0.187	0.339	0.661	0.181	0.364	0.636
COB	0.005	0.981	0.019	0.000	1.000	0.000	0.032	0.873	0.127	0.000	1.000	0.000	0.005	0.981	0.019
P1	0.187	0.611	0.389	0.187	0.597	0.403	0.187	0.632	0.368	0.183	0.543	0.457	0.183	0.558	0.442
ACP	0.143	0.507	0.493	0.169	0.426	0.574	0.188	0.428	0.572	0.297	0.261	0.739	0.177	0.410	0.590
ADA	0.106	0.604	0.396	0.094	0.662	0.338	0.077	0.699	0.301	0.096	0.644	0.356	0.063	0.775	0.225
ESD	0.140	0.541	0.459	0.074	0.752	0.248	0.134	0.585	0.415	0.137	0.511	0.489	0.091	0.683	0.317
GLO	0.146	0.480	0.520	0.140	0.496	0.504	0.173	0.391	0.609	0.147	0.479	0.522	0.155	0.457	0.543
GPT	0.182	0.377	0.623	0.191	0.397	0.603	0.180	0.407	0.593	0.180	0.373	0.627	0.187	0.361	0.639
AK	0.061	0.759	0.241	0.084	0.692	0.308	0.067	0.761	0.239	0.057	0.792	0.208	0.085	0.687	0.313
PGI	0.010	0.959	0.041	0.010	0.961	0.039	0.000	1.000	0.000	0.008	0.967	0.033	0.005	0.981	0.019
PGP	0.045	0.820	0.180	0.030	0.885	0.115	0.051	0.805	0.195	0.014	0.938	0.062	0.005	0.943	0.057
PGMS	0.353	0.211	0.789	0.376	0.199	0.801	0.340	0.235	0.765	0.361	0.199	0.801	0.367	0.200	0.800
PGD	0.010	0.961	0.039	0.000	1.000	0.000	0.000	1.000	0.000	0.007	0.974	0.026	0.014	0.943	0.057
BF	0.175	0.459	0.541	0.222	0.402	0.598	0.170	0.429	0.571	0.183	0.397	0.603	0.179	0.389	0.611
C3	0.037	0.862	0.138	0.079	0.712	0.288	0.105	0.599	0.401	0.107	0.592	0.408	0.091	0.697	0.304
GC-S	0.305	0.239	0.761	0.247	0.335	0.665	0.260	0.307	0.693	0.283	0.282	0.718	0.336	0.226	0.774
PLG	0.110	0.629	0.371	0.061	0.757	0.243	0.118	0.606	0.394	0.109	0.591	0.409	0.101	0.624	0.376
PI-S	0.227	0.366	0.634	0.276	0.273	0.727	0.249	0.328	0.672	0.247	0.341	0.659	0.192	0.445	0.555
TF-S	0.160	0.531	0.469	0.188	0.461	0.540	0.202	0.471	0.529	0.189	0.438	0.562	0.158	0.520	0.480
HP-S	0.217	0.396	0.604	0.190	0.452	0.548	0.287	0.286	0.714	0.231	0.364	0.636	0.227	0.351	0.649
ORM1	0.174	0.428	0.572	0.168	0.439	0.561	0.176	0.436	0.564	0.167	0.437	0.563	0.155	0.461	0.539
ORM2	0.035	0.868	0.132	0.039	0.849	0.151	0.014	0.943	0.057	0.010	0.958	0.042	0.039	0.849	0.151
Combined Estimates															
Blood groups	**0.838**	**1.76E-03**	**9.98E-01**	**0.823**	**1.87E-03**	**9.98E-01**	**0.868**	**1.16E-03**	**9.99E-01**	**0.859**	**1.14E-03**	**9.99E-01**	**0.821**	**2.26E-03**	**9.98E-01**
Enzymes	**0.738**	**3.63E-03**	**9.96E-01**	**0.734**	**4.88E-03**	**9.95E-01**	**0.743**	**3.99E-03**	**9.96E-01**	**0.776**	**2.13E-03**	**9.98E-01**	**0.727**	**4.30E-03**	**9.96E-01**
Serum Proteins	**0.801**	**1.69E-03**	**9.98E-01**	**0.807**	**1.54E-03**	**9.98E-01**	**0.832**	**8.69E-04**	**9.99E-01**	**0.820**	**8.91E-04**	**9.99E-01**	**0.810**	**1.21E-03**	**9.99E-01**
16 systems	**0.984**	**1.11E-07**	**1.00E+00**	**0.983**	**1.32E-07**	**1.00E+00**	**0.988**	**5.64E-08**	**1.00E+00**	**0.989**	**2.64E-08**	**1.00E+00**	**0.983**	**1.27E-07**	**1.00E+00**
All systems	**0.992**	**1.09E-08**	**1.00E+00**	**0.991**	**1.41E-08**	**1.00E+00**	**0.994**	**4.05E-09**	**1.00E+00**	**0.994**	**2.17E-09**	**1.00E+00**	**0.991**	**1.18E-08**	**1.00E+00**

Genetic Diversity in Sri Lanka: Some implications in Paternity and Forensic testing.

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INTRODUCTION:

In the Indian Subcontinent a vast number of subpopulations exist, originating from different racial stocks and their diversity is dynamically maintained by linguistic, cultural, religious and geographical barriers. The genetic markers that are polymorphic in the populations of this region, show extensive magnitude of variation in allele frequency among subpopulations in different regions (Papiha et al 1982, Mastana and Papiha 1994). In the countries of the Indian subcontinent, pooling of genetic information from different populations for medico-legal problems will lead to spurious results. The necessity to study the genetic composition of various subpopulations for paternity and forensic testing has recently been advocated by Lewontin and Hartl (1991) and others. We have studied five main endogamous groups of Sri Lanka and have seen a considerable variation in their individual allele frequencies for several genetic systems. The aim of this paper is to examine how these allele frequency differences influence parameters like PE (Power of Exclusion), PM (Probability of Match), and DP (Discriminant Probability) in each of the five endogamous subpopulations.

MATERIAL & METHODS:

Populations:

Sinhalese: The Sinhalese are the largest community on the Island and occupy most of the south, west and east of Sri Lanka. Religiously the Sinhalese follow Buddhism.

Tamils: The Tamils (Sri Lankan and Indian) are the largest *Hindu* ethnic minority in Sri Lanka. They are primarily concentrated in the North and North-eastern regions.

Burghers: The Burghers are the descendants of intermarriages between the Sinhalese and the Portuguese and Dutch traders. They practise *Christianity* and are concentrated in the Western side of the Island.

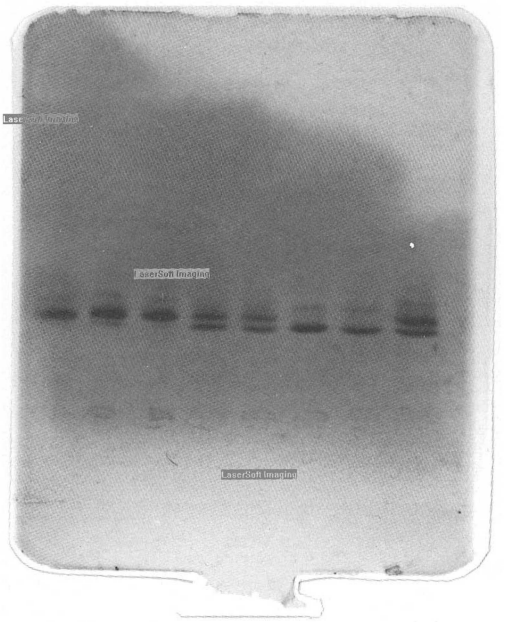
Moors: The Moors (*Muslims*) originated through the intermarriages of local people with Arab traders. Moors are also concentrated on the West Coast.

Malays: The Malays in Sri Lanka are descendants of intermarriages between the Malays (Malaysia) and local women. The Sri Lankan Malays embrace *Islam* and live practically in every part of the island.

Blood samples from Sinhalese (100), Tamils (102), Moors (100) Burghers (103) and Malays (102) were collected and transported by air to the Department of Human Genetics, Newcastle University for processing and analysis. Standard serological methods were used for analysis of blood groups (ABO, MNSs, Rhesus, Kell, FY, P, JK and Colton), while red cell enzymes (AK, ACP, ADA, ESD, GLO, GPT, PGP, PGI, 6PGD, G6PD, LDH, MDH and PGM1 subtypes) and serum proteins (C3, BF, PLG, ORM1, ORM2 and subtypes of HP, GC, TF and PI) were analysed using starch gel and iso-electric focusing electrophoresis. Power of exclusion, Probability of Match and Discriminant Probability was calculated using the methods of Garber and Morris (1983) and Jones (1972) respectively.

RESULTS AND DISCUSSION:

The allele frequency variation in all the five populations investigated was compared by Chi-sqaure analysis and overall heterogeneity was observed for 30% of the systems (Rhesus, KIDD, ACP, ESD, HP, C3, TF, GC systems and PGM1*2+, PLG*1 alleles). Sinhalese differed significantly from other populations in about 12% of the systems (11 out of 92 comparisons), and a higher number of significant differences were found in Tamils (12), Burghers (13), Moors (17) and Malays (14). This diversity among the number of genetic systems indicates that



Alpha-2-glycoprotein phenotypes in dental pulp.
From left to right: 1-1/1-1/1-1/2-1/2-1/2-2/2-2/2-1.

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Isoelectric focusing conditions for the PhastSystem:

ORM and α-2-HS

Sep 1.1	2000 v	10.0 mA	2.0 w	15°C	75 vh
Sep 1.2	2000 v	5.0 mA	1.5 w	15°C	20 vh
Sep 1.3	2000 v	25.0 mA	4.0 w	15°C	600 vh
Transferrin					
Sep 2.1	2000 v	2.0 mA	3.5 w	15°C	75 vh
Sep 2.2	200 v	2.0 mA	3.5 w	15°C	20 vh
Sep 2.3	2000 v	2.0 mA	3.5 w	15°C	700 vh

RESULTS AND DISCUSSION

We obtained clear reading of phenotypes when using recent pulps, but when teeth were very old or contaminated we could not determine phenotypes for Pi or Gc. Tf, α-2-HS and ORM showed more resistant and in most cases could be phenotyped using a minimum amount of sample (2 microlitres of eluate) even in the oldest specimens. Neuraminidase was the best extracting solution for these three serum proteins. A simple Coomassie Blue staining was used to determine phenotypes for Tf and α-2-HS. A silver staining showed more suitable for ORM in old dental pulps.

We were able to phenotype Tf, ORM and α-2-HS using the PhastSystem (Pharmacia) reducing considerable the time for assay.

We can therefore determine serum proteins such as the three mentioned above from very old dental pulp. This is especially useful when we have a very small amount of biological material because we use the same extracting solution (neuraminidase) and a minimum quantity of eluate.

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SERUM PROTEIN DETECTION IN OLD DENTAL PULP

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INTRODUCTION

Dental pulp has proven to be a very good source of biological evidence for forensic casework. Its location in a especially protected cavity makes dental pulp very valuable. When environmental conditions are unfavourable we can still find well preserved proteins in this location.

We investigated some genetic polymorphisms associated with serum proteins in dental pulp from teeth stored at room temperature for as long as five years. We tried to find out which proteins were more resistant to unfavourable envinronmental conditions.

MATERIAL AND METHOD

We used isoelectric focusing methods to obtain phenotypes for the following proteins: Gc protein (Gc), alpha-1-antitrypsin (Pi), transferrin (Tf), alpha-2-glycoprotein (α–2 -HS) and orosomucoid (ORM).

We tried different solutions for extraction (urea, neuraminidase, distilled water) and incubation time varied from 12 to 48 hours at 4°C. Isoelectric focusing was followed by immunofixation and Coomassie Blue staining or silver staining. For Tf, α-2-HS and ORM we used the PhastSystem (Pharmacia) separation and development unit but for Gc and Pi we used conventional isoelectric focusing methods.

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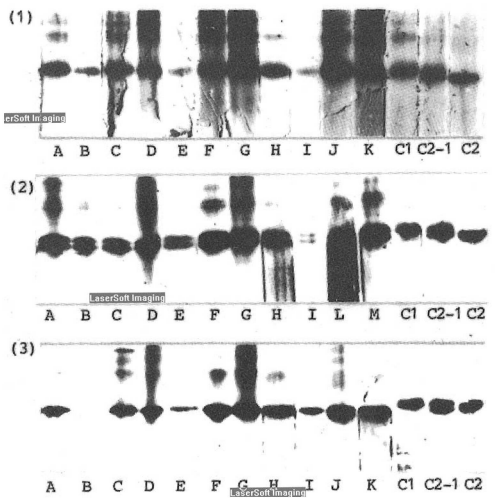

Fig. 2. Isoelectric focusing patterns of TF subtypes in fresh human tissues. (1) male, TF C1; (2) female, TF C2-1; (3) male, TF C2. A: spleen; B: pancreas; C: heart; D: liver; E: muscle; F: lung; G: kidney; H: skin; I: brain; J: prostate; K: testis; L: ovary; M: uterus; C1: serum sample for TF C1; C2-1: serum sample for TF C2-1; C2: serum sample for TF C2. The anode is at the top.

storage. No significant difference in loss of intensity was observed among the allelic products. Storage for 2 weeks yielded much poorer results and it was difficult to identify the TF bands in most organ tissues.

In mass disaster such as automobile accidents, aircraft crashes or explosions, human organs are often submitted for personal identification. The TF subtype system can be a useful means for medicolegal identification of human organs in such cases.

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tissue homogenates was estimated by rocket immunoelectrophoresis. The mean value (μg/ml) was: spleen 342±87, pancreas 143±20, heart 251±11, liver 132±40, muscle 111±8, lung 568±113, kidney 259±81, skin 162±28, brain 69±4, prostate 311±22, testis 346±68, ovary 631±167 and uterus 601±40. These values are equivalent to 1/40 - 1/4 of the amount of TF in human serum. A typical example of rocket immunoelectrophoresis of cardiac tissues is shown in Fig. 1.

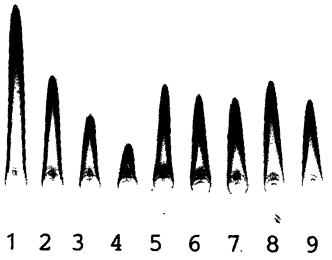

Fig. 1. Rocket immunoelectrophoresis of cardiac tissues in 5 cases. 1-4: serially diluted TF references (1: 134 μg/ml, 2: 67 μg/ml, 3: 33 μg/ml, 4: 17 μg/ml), 5-9: 5-fold diluted cardiac supernatants (5: 60 μg/ml, 6: 53 μg/ml, 7: 51 μg/ml, 8: 63 μg/ml, 9: 49 μg/ml). The anode is at the top.

Table 1 summarizes the results for the determination of TF subtypes in fresh and stored tissues. In fresh tissues of

Table 1. Positive results for the determination of TF subtypes from various human organ tissues

Phenotype	No. tested	Period of storage	Tissue												
			Spleen	Pancreas	Heart	Liver	Muscle	Lung	Kidney	Skin	Brain	Prostate	Testis	Ovary	Uterus
C1	8	48 hours	8	4	8	8	8	8	8	6	8	7	7	1	1
	(M: 7, F: 1)	1 week	6	3	8	5	8	8	8	4	8	7	7	1	1
		2 weeks	2	0	6	3	5	6	5	1	2	3	1	1	1
		3 weeks	2		3	0	3	4	3	1	2	3	1	1	1
		4 weeks	0		2		1	3	3	1	0	0	1	0	1
C2-1	4	48 hours	4	2	4	4	4	4	4	3	4	3	3	1	1
	(M: 3, F: 1)	1 week	2	0	4	2	4	4	4	0	4	3	3	1	1
		2 weeks	2		1	0	2	2	3		2	2	2	0	0
		3 weeks	2		1		2	2	2		2	1	2		
		4 weeks	0		0		1	1	2		1	1	1		
C2	1	48 hours	1	0	1	1	1	1	1	1	1	1	1		
	(M: 1)	1 week	1		1	1	1	1	1	0	1	1	1		
		2 weeks	1		1	1	1	1	1		1	1	1		
		3 weeks	0		1	0	1	1	1		0	1	1		
		4 weeks			1		0	1	1			0	1		

M: male, F: female

almost all the organs examined clear TF subtype patterns were obtained except 7 samples of the pancreas and 3 samples of the skin. The pattern in organ tissues agreed completely with that in the corresponding serum samples (Fig. 2). When stored for 1 week, 3 common TF subtypes were correctly determined from tissues of heart, muscle, lung, kidney, brain, prostate, testis, ovary and uterus. The bands became fainter and more indistinct with increasing period of

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Transferrin Subtyping in Human Organ Tissues

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INTRODUCTION

Serum transferrin (TF) exhibits genetic polymorphism with 2 codominant alleles, TF*C1 and TF*C2 (Kühnl and Spielmann 1978, Thymann 1978). By means of sensitive immunoblotting techniques, the TF subtypes have been demonstrated also in bloodstains (Yamaba *et al.* 1991), semen (Yamaba *et al.* 1991), urine (Kishi *et al.* 1990) and dental pulps (Kido *et al.* 1993). In this study we report the results of TF subtyping in human organ tissues using immunoblotting after isoelectric focusing.

MATERIALS AND METHODS

Following tissue materials were obtained from 13 cadavers (11 males, 2 females) who were medicolegally autopsied within 48 h after death: spleen, pancreas, heart (cardiac muscle), liver, muscle (m. rectus abdominis), lung, kidney, skin including adipose tissue, brain, prostate, testis, ovary and uterus. 5 g of the tissue sample was stored in a glass bottle at room temperature and examined at 1-week intervals. The tissue weighing 0.5 g was homogenized in 0.5 ml of distilled water using an Ultra-Turrax homogenizer (Janke & Kunkel KG, Staufen in Breisgau, FRG). The homogenate was centrifuged at 10,000 rpm for 60 min and the supernatant was retained for analysis. Blood samples were also taken from the same subjects. Quantitation of TF was carried out by the method of rocket immunoelectrophoresis reported previously (Kido *et al.* 1993).

8 µl of the supernatant or serum was treated with 2 µl of 1 M potassium phosphate buffer (pH 7.0) containing 50 U/ml neuraminidase from *Clostridium perfringens* (type V, Sigma, USA) for 18 h at room temperature. The desialated supernatant was diluted 1:20 and the desialated serum 1:100 with distilled water. They were applied to the gel using 5 x 6 mm filter paper strips (Whatman No. 3, UK). Isoelectric focusing and immunoblotting were described in our previous report (Kido *et al.* 1993).

RESULTS AND DISCUSSION

The amount of TF in supernatants of various human organ

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Results and Discussion

1. The result of ABO blood grouping from old bloodstains spread on the filter papers were shown in Table 1.
These were weaker reaction than Table 2. The gelatin particles suspension of Lot. No.082 and 088 were not reacted. These are caused by not difference of the particle diameter but the electric ratio, because it is same condition, that was treated with tanned acid and sensitized with antibody concentrations. Lot.No.091 and already used particles suspension were well specific reaction.
2. The result of A, B and O(H) antigens were transferred from old bloodstains to wet gauze by rubbing were shown in Table 2.
This method is most suitable for the practice of forensic medicine. Accordingly, either fresh or old detained bloodstains were simply collected and identified by this method at the scene.
The authors were identified the ABO blood groups from old bloodstains, that were collected five years ago. It is used very simple and useful method, that is developed and invented by one of the authors. The method is ultra micro reverse passive agglutination. In generally, the method of ABO groups identity from old bloodstains is used by heat elution technique. However, this technique is simple but need some hours. Use the method of this investigation, that was successfully performed in more short time and could be detected correctly and simply.

Table 1 ABO Blood Grouping from Old Bloodstains Spread on the Filter Papers

	A Group	B Group	AB Group	O Group
082-Anti A	−	−	−	−
082-Anti B	−	−	−	−
088-Anti A	−	−	−	−
088-Anti B	−	−	−	−
091-Anti A	+	−	+	−
091-Anti B	−	+	+	−
042-Anti A	+	−	+	−
042-Anti B	−	+	+	−

Table 2 Old Bioodstains were Transferred from Filter Paper to Wet Gauze by Rubbing

	A Group	B Group	AB Group	O Group
082-Anti A	−	−	−	−
082-Anti B	−	−	−	−
088-Anti A	−	−	−	−
088-Anti B	−	−	−	−
091-Anti A	++	−	++	−
091-Anti B	−	++	++	−
042-Anti A	++	−	++	−
042-Anti B	−	++	++	−

Conclusion

The results obtained in this study show that the method of ultra micro reverse passive agglutination was successfully performed in a short time and could be detected correctly and simply in comparison with others.

Reference

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1) Treatment of tanned acid to gelatin particles.
The medium is 0.15 mol, pH.6.5 Phosphate Buffered Saline include 30 ppm tanned acid, this is optimum condition for treatment of tanned acid to the particles at this investigation.
The gelatin particles is suspended with pH.6.5, P.B.S. to 1.25 v/v%, and added equal volume of the above tanned acid solution. Stored at 37 ℃, 30 min. in water bath. Then, wash with cold saline at 1 time, and after, this tanned acid treated particles is resuspended with the same P.B.S. to 1.25v/v%.
2) Sensitization of monoclonal anti-A,-B antibodies.
To begin with divide in three of the above tanned acid treated gelatin particles suspension.
The first divided suspension is sensitized with monoclonal anti-A antibody.
The second divided suspension is sensitized with monoclonal anti-B antibody.
The third divided suspension is not sensitized for negative controls.
The first and second suspension, severally suspension is mix with equal volume Anti-A or Anti-B antibody. Each mixed suspension is stored 37 ℃, 1 hour in water bath, sometimes shake to mixture. And after that, the each sensitized gelatin particle suspension is wash with cold saline at 2 times to eliminate of surplus antibody. Then, each sensitized suspension is resuspended with reaction medium, that is composed of 0.15 mol, pH.7.2, Phosphate Buffered Saline include 1.0 % Bovine Serum Albumin, 0.01 % Tween 80 and 0.01 % NaN_3 as an antiseptic.
3) Prepare of sample
In this paper, ABO blood groups of old bloodstains on filter paper was performed by the two methods.
In one method, each sample was cut to a suitable size,and fixed with methanol on a slide glass. Then, the fixed sample is scratched by a needle like a pin, and got a some fine fibrils of old bloodstains.
In other method, A, B and O(H) antigens were transferred from old bloodstains on the filter papers to wet gauze by rubbing. The gauze was fixed with methanol and unbround' by a needle. So that got a some fine fibrils.
4) Reaction
1. Fill the each holes of Terasaki's microplate with the correct amount of liquid paraffin. We shows the Lot. No. of gelatin particles, and was sensitized with monoclonal anti-A antibody at the upper row and anti-B at under row in the Table 1 and 2. Then, each sample put into the liquid paraffin of the each holes to vertical line as the Tables.
2. Each 3 μl of the sensitized gelatin particle suspension add into the hole, already include the sample, to horizontal line as the Tables, by Terasaki's dispenser.
These are mixed during about 10 minutes by micromixer and reacted at room temperature, and after, the agglutination read under a substance microscopy.

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ABO Blood Grouping of Old Blood Stains by Ultra Micro Reverse Agglutination with Monoclonal Anti-A, -B Antibodies

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Introduction

In the practice of forensic medicine, especially in the case of identification, it is very important that the personal identification include the collection of bloodstains and blood grouping. Blood grouping from old bloodstains as well as hemolytic blood is a very complicated process and is difficult to identify. The authors have already introduced the use of a simple, practical methods by ultra micro passive agglutination with monoclonal anti-A,-B,-H antibodies for the ABO blood grouping of hemolytic blood. The old bloodstains were collected through the months of October and November of 1987, during the medico-legal autopsy. The blood type was identified, and then, the blood was spread out on to filter paper. These were stored for five years at room temperature. Recently, the gelatin particles were presented to us by Fuji Rebio Inc. Three types of gelatin particles were investigated to the comparison of specificities under the same condition with one known-identity.

Materials and Methods

1.Materials.

1) Gelatin particles; Three types of gelatin particles of previously unknown-identity were used, along with one known type*, totaling four types, these are mentioned below.

	Electrophoresic ratio	Particle diameter
Lot.082	0.763μm/cm/sec/v/cm	3.09 μm
Lot.088	0.751μm/cm/sec/v/cm	3.04 μm
Lot.091	0.739μm/cm/sec/v/cm	3.05 μm
Lot.042*	0.730μm/cm/sec/v/cm	3.75 μm

These are artificial particle carrier, that construct from soluble polysaccharide include gelatin and metaphosphoric acid, are treated to bridging and unsolvability.

2) Monoclonal antibodies:

Anti-A antibody ; agglutination titer is x256 or more(saline)
Seraclone Anti-A (mouse) Lot.790581 Biotest AG

Anti-B antibody ; agglutination titer is x256 or more(saline)
Seraclone Anti-B (mouse) Lot.790591 Biotest AG

3)Samples:Bloodstains that were collected during the medico-legal autopsy, a through of October and November of 1987 were spread on the filter paper, dried at room temperature, then stored in individual envelopes marked with autopsy number and blood types.

2.Methods.

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to be 1F-1F1A2, which resulted the child should be GC 1F1A2. A position effect which was already pointed out in the original observation[1] may be the most plausible explanation for the weak 1F factors in the homozygote.

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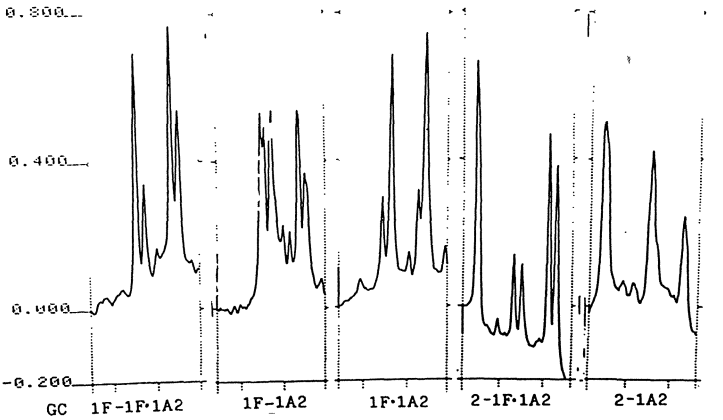

Fig. 2. Densitometric traces of the GC variants.

beam electroscanner(CS-930).

The GC patterns are shown in Fig. 1. In case 1, mother and the proven father had GC phenotype 2-1S and 1F-1A2, re - spectively, while their baby revealed a unique 5-band type, sharing only one band(GC 2) with the mother. The remaining four bands were obviously inherited from the father. The pattern is very similar to the socalled duplicated[1)] or recom- bined 3-allele[4)] variant in the previous reports. Therefore, the genotypes of the child and father were determined to be 2-1F1A2 and 1F-1F1A2,respectively.

In case 2, the mother and alleged father showed both GC 1F-1A2 phenotype, and their child had four GC bands in a position identical to those of 1F and 1A2, with the former bands very weak in intensity. Since the type is clearly different from that of GC 1A2 homozygote, we assumed it also arose from the same variant allele, and tried to examine it by scanning approach.

The densitometric traces in the samples with GC variants are presented in Fig. 2. It is evident that the scanning profile for GC 1F-1F1A2 is different from that of GC 1F-1A2. From the area calculation the ratio of 1F:1A2 in GC 1F-1F1A2 (father of case 1) is about 67:33, whereas the average ratio in GC 1F-1A2 is calculated to be 55:45. Using these figures the genotypes of the parents in case 2 were identified both

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Two Cases of Duplication of the 1F and 1A2 Genes in the Vitamin D-Binding Protein (GC) System

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GC polymorphism in Japan is a bit unique in distribution of the phenotypes, because a variant allele(1A2) also retains in a polymorphic frequency (around 0.02) in addition to the three common alleles(GC 1F,1S & 2). Recently Yasuda et al. (1989)[1] found a new GC variant, GC 2-1F1A2, which is characterized by having double double-banded components with a common GC 2 band by PAGIF, and presumed it had arisen from duplication of the GC 1F and 1A2 genes. Later the same variant type was observed by others[2)-4)] in Japanese populations, and it is estimated such duplication is occurring with a frequency of 0.001 [2]. However, the variant has sofar been identified only in the samples with GC 2.

The samples described here were obtained from two cases of paternity test and their accused men were proven their fatherhood. GC types were determined by PAGIF(pH4.5-5.4, gel 0.2mm thick) followed by immunofixation. Densitometry was performed on stained membranes employing a Shimadsu double

1. Mother GC 2-1S
2. Child GC 2-1F1A2
3. Father GC 1F-1F1A2
4. Mother GC 1F-1F1A2
5. Child GC 1F1A2
6. Father GC 1F-1F1A2

Fig. 1. Photograph showing the GC variants.

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RESULTS AND DISCUSSION

Results of the transformation in genetic markers before and after BMT in recipients with different phenotypes from the donor follows. The red cell antigens transformed as follows; type O to type A, type O to type B, type AB to type A and type A to type O (the pre-BMT recipient type to the post-BMT donor type), MN/ss to M/ss, MN/ss to N/ss, NSs to Nss, CcDEe to CCDee, CcDee to CCDee, CCDee to CcDEe, CcDEe to ccDEE, P1(+) to P1(-), P1(-) to P1(+), Jk(a+b+) to Jk(a+b-), Jk(a+b+) to Jk(a-b+), Jk(a-b+) to Jk(a+b+), Jk(a+b-) to Jk(a+b+), Fy(a+b+) to Fy(a+b-), Xg(a+) to Xg(a-), Xg(a-) to Xg(a+). The red cell enzyme types transformed as follows; EsD2-1 to EsD1-1, EsD1-2 to EsD1-1, PGM2-1 to PGM1-1, PGM1(1+1-) to PGM 2-1(2+1+), PGM1-1(2-1+) to PGM1-1(1-1-), ACP A to ACP AB. Some genetic markers which did not transform when the donor phenotypes differed from the recipient phenotypes include the Lewis, Hp, Tf, FXIII-B, Se, Pa, Pr, Db, and PmF markers. Other genetic markers, including the Lutheran, Kell-Cellano, Xg, HLA, Gc, Gm, Km, Bf, PLG, GLO, 6PGD, Pb, PIF, Amy_1, and various complementing systems could not be evaluated because the donor and recipient phenotypes were identical.

Many transformations in genetic markers which accompany BMT illustrate the relationship between gene products and the bone marrow. Various antigens from red cell membrane substances of the glycolipid and glycophorin systems are produced primarily in the bone marrow. However, production of the Lewis antigen, blood-group-related substances secreted in saliva, and salivary polymorphic protein appear to take place in the bone marrow.

Changes in the red blood cell agglutinin appear to be related to antibody production under gene control and are presumably produced by the transformed Gm system (immunoglobulin allotype) rather then being the result of serum antibody absorption by antigens, which leaves only nonself antibodies. The transformation of the red cell enzyme EsD, PGM, ACP, PGD markers provides a useful clue for therapy for enzyme defects. RFLP analysis using DPβ, DQβ, and DRβ cDNA as a probe was carried out in a case which showed the high MLC activity although Class I and Class II on HLA typing were coincident each other. The recipient showed some specific bands which were not observed in the donor and her patients, that suggested the relationship between the specific bands and high MLC activity.

BMT between family members may lead to transformation in the gene markers, but within a pattern compatible with family inheritance patterns, and no genetic paradox will be found in later surveys of familial genetic relationships. However, in a personal identification system in forensic medicine using genetic markers as an index, the appearance of a phenotype incompatible with a blood relationship is possible after BMT from a donor who is not a blood relative. This result is similar to the inheritance pattern observed after artificial insemination by a donor, a more complete out-of-family cross.

As development of immune suppressants such as cyclosporins improves, BMT will be performed more frequently based upon HLA histocompatibility alone. Care will be required to track the transformation in personal identification and paternity testing based on genetic makers.

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FURTHER EVIDENCE FOR TRANSFORMATION OF GENETIC MARKERS IN RECIPIENTS AFTER BMT

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INTRODUCTION

The HLA compatibility has been somewhat overemphasized, even to the point of ignoring other important marker genes, including the ABO system, an essential consideration for blood transfusion. A study of the genetic makers of the bone marrow recipient after bone marrow transplantation (BMT) reveals an alteration of the genetic makers to the donor type.

Blume et al. have discussed the transformation in the MN, Rh, Kidd, and gammagroblin marker (Gm) systems as representative of the genetic marker transformation from the recipient to the donor type. Dijk et al., Wolpl et al., and Ikemoto et al. reported the transformation of genetic markers in red cells, serum protein, and red cell enzymes accompanying BMT. Mori et al. studied the red cell antigens of a recipient with an ABO incompatibility. Despite being unable to detect a transferase, these authors found ABO antigens of the donor type. The antibodies to the donor type also declined rapidly. Yam et al. concluded that deoxyribonucleic acid (DNA) restriction fragment length polymorphism (RFLP) are powerful and practical genetic markers in bone marrow transplantation studies and that further studies of mixes hematopoietec chimerism are warranted. Kitano et al. reported observations of the behavior of red cell membrane antigens of donors and recipients as an index for early recognition of transplanted bone marrow engraftment.

The following report describes that transformation of genetic markers on 17 pairs of donor and recipient with bone marrow transplantation.

MATERIAL AND METHODS

The present examine are on 17 pairs samples were obtained from the blood transfusion service of the Jichi Medical School Hospital and Tottori Central Hospital. A broad range of gene markers at 41 loci, including red blood cell markers (ABO, MNSs, Lewis, P, Rh, Duffy, Kidd, Lutheran, Kell-Cellano, Diego, and Xg), HLA systems (HLA-A, -B, -C, -DR, and -DQ), serum protein markers (Gc, Tf, Hp, Gm, Km, PLG, Bf, C2, C6, C7, C8-1, and FXIII-B), red cell enzyme markers [esterase D (EsD), acid phosphatase (ACP), glyoxalase (GLO), phosphogluconate dehydrogenase (6PGD), and phosphoglucomutase (PGM)], and salivary markers (Se, Pa, Pb, Pr, PmF, Db, PIF, Amy1) were evaluated before and after BMT over about 2 months. Blood grouping and HLA typing were used. The serum protein and red cell enzyme types were analyzed according to experimental methods described by Tamaki et al., salivary typing was determined by methods described Ikemoto et al., and flow cytometry techniques were carried out using methods described by Hashimoto et al.

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Table 2 PI*S allele frequencies in several Southern European populations

Population	Number of Subjects	Allele Frequency PI*S	References
Portugal/Madeira	151	0.1821	This study
Mainly South	1850	0.1140	Not published
Spain/Galicia	450	0.1490	Carracedo and Concheiro (4)
Barcelona	938	0.1044	Gènc et al (5)
Basques	323	0.1006	Manzano et al (6)
France /Southwest	1603	0.1095	Sesboué et Martin (7)

The highest frequency for PI*S allele have been found in the Galicia population (4) . Our study supports the European cline described for the PI*S allele from Northern to Southern European populations (8), reaching a maximum in Madeira.

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Table 1. Distribution of GC, TF and PI phenotypes and allele frequencies in Madeira

	Phenotypes	Observed Number	Expected Number	Allele Frequencies
GC System	1S	45	47.85	
	2-1S	57	51.79	GC*1S= 0.5629
	1S-1F	23	22.52	GC*1F= 0.1325
	2	12	14.01	GC*2 = 0.3046
	2-1F	11	12.19	
	1F	3	2.65	
	Total	151	151.00	
	$\chi^2 = 1.15$; df=3; $0.70 < p < 0.80$			
TF System	C1	89	88.35	
	C1C2	40	42.07	TF*C1= 0.7649
	C1C3	12	11.47	TF*C2= 0.1821
	C1B	1	0.76	TF*C3= 0.0497
	C1C2	6	5.01	TF*B = 0.0033
	C2C3	3	2.73	
	C2B	0	0.18	
	C3	0	0.37	
	C3B	0	0.05	
	B	0	0.00	
	Total	151	151.00	
	$\chi^2 = 1.03$; df= 6; $0.98 < p < 0.99$			
PI System	M1	50	47.85	
	M1M2	22	25.33	
	M1M3	18	17.45	PI*M1=0.5629
	M1S	29	30.96	PI*M2=0.1490
	M1Z	1	0.56	PI*M3=0.1027
	M2	5	3.35	PI*S = 0.1821
	M2M3	6	4.62	PI*Z = 0.0033
	M2S	7	8.20	
	M2Z	0	1.59	
	M3S	3	5.65	
	M3Z	0	0.10	
	S	8	5.01	
	SZ	0	0.18	
	Z	0	0.00	
	Total	151	151.00	
	$\chi^2 = 5.97$; df=10; $0.80 < p < 0.90$			

In the PI system, we have observed a higher allele frequency for PI*S allele (f= 0.1821) in Madeira when compared to the Continental Portuguese population (f= 0.1141) and even when compared to Southern European populations from Spain and France (f= 0.1044 to 0.1490 - table 2). Consequently, PI*M1 allele frequency is lower in Madeira.

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GC, TF AND PI POLYMORPHISMS IN MADEIRA (PORTUGAL) PI*S ALLELE AND PATERNITY TESTING

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INTRODUCTION

Madeira is an island in the Atlantic Ocean located about 500 miles off the southwest coast of Portugal, where the Portugueses, since the 15th century, contributed mainly to the present-day population.
The primary purpose of the present paper was to study the distribution of GC, TF and PI phenotypes in Madeira to obtain a estimate of allele frequencies for paternity testing purposes, with special reference to the PI*S allele frequency due to its great interest for population genetic studies.

MATERIAL AND METHODS

Serum samples from a total of 151 unrelated individuals from Madeira were collected from paternity tests.
GC, TF and PI phenotyping was carried out by isoelectric focusing with carrier ampholytes on flat-bed polycrylamide gels. GC system was studied according to the method described by Constans and Viau (1) with slight modifications. GC bands were made visible after precipitation with sulfosalicylic acid. PI system was typed according to Hjalmarsson (2). For TF phenotyping, serum samples were analysed as indicated by Budowle (3). Staining of TF and PI gels was carried out with Coomassie Blue R 250

RESULTS AND DISCUSSION

Table 1 shows the distribution of GC, TF and PI phenotypes and their allele frequencies for the population from Madeira. A good agreement was found between observed and expected number assuming a Hardy-Weinberg equilibrium.
The theoretical a priori chance exclusion values for these systems are : GC= 0.2984, TF= 0.1872 and PI= 0.3807, which indicate that these systems are useful genetic markers for paternity investigation in the population from Madeira.
The allele frequencies for GC and TF systems are not significantly different from our results (not published) of previous studies of the Continental Portuguese population, mainly from the South.

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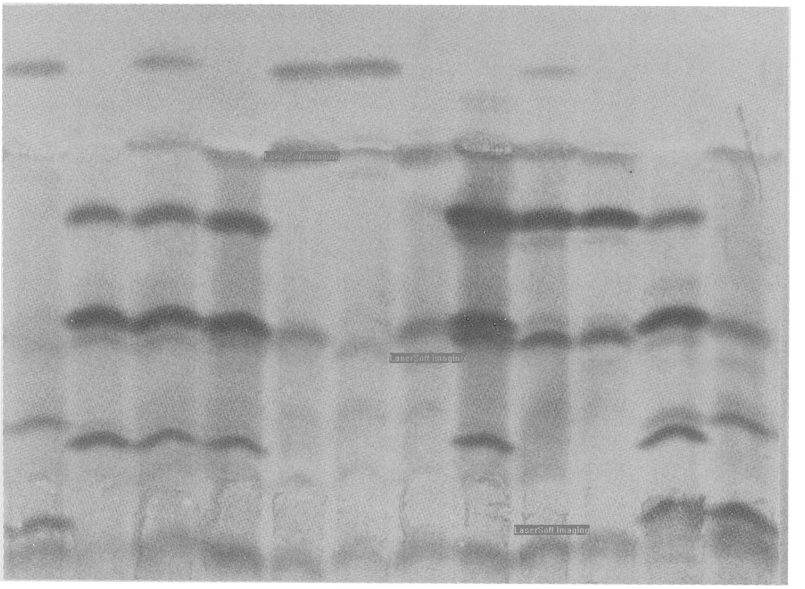
Table 2. Hp allele frequencies in several populations of Spain

Population	N	Hp*2FS	Hp*1S	Hp*1F	Hp*2SS	Hp*2FF
Basque Country residents	548	0.530	0.256	0.172	0.031	0.010
Basque Country autochtonous	301	0.547	0.262	0.136	0.042	0.013
Madrid	487	0.556	0.237	0.155	0.038	0.013
Sevilla	189	0.579	0.222	0.167	0.032	0.000

Figure 1. IEF band pattern of Hp phenotypes. From left to right: 2FF-1S, 2FS, 2FS-1S, 2FS-1F, 1F-1S, 1S, 1F, 2FS-2SS, 2SS-1S, 2SS-1F, 2FS-2FF, 2FF-1F



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RESULTS AND DISCUSSION

Figure 1 shows the isoelectric focusing band pattern of 12 different Hp phenotypes encountered in this study. The distribution of Hp phenotypes and the corresponding allele frequencies in several populations of Spain are presented in Table 1 and Table 2. In all cases, the observed phenotypes provide a satisfactory correlation to the Hardy-Weinberg equilibrium. No significant differences were found between the populations analyzed in this study.

Table 1. Observed and expected Hp phenotype frequencies in several populations of Spain

	Basque Country residents		Basque Country autochtonous		Madrid		Sevilla	
Phenotype	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
2FS-1S	154	148.96	76	86.35	119	128.54	43	48.67
2FS	150	154.00	102	89.90	159	150.80	69	63.44
2FS-1F	105	100.19	38	44.81	76	84.03	32	36.50
1F-1S	42	48.46	23	21.52	41	35.81	11	14.00
1S	36	36.02	23	20.73	31	27.39	14	9.33
2FS-2SS	17	18.02	8	13.66	20	20.59	6	6.95
1F	16	16.30	7	5.58	14	11.70	8	5.25
2SS-1S	9	8.72	9	6.56	7	8.78	2	2.67
2SS-1F	8	5.86	6	3.41	5	5.74	4	2.00
2SS	5	5.83	3	4.37	9	7.23		0.19
2FS-2FF	4	2.82	4	2.10	2	3.08		
2FF-1S	2	1.90	1	1.09	1	2.02		
2FF-1F		0.53	1	0.52	2	0.70		
2SS-2FF		0.34		0.33	1	0.49		
2FF		0.06		0.05		0.09		
Total	548	548.00	301	301.00	487	487.00	189	189.00

$\chi^2 = 2.358$; d.f. = 6; $0.90 > p > 0.80$ (Basque resident population)
$\chi^2 = 8.691$; d.f. = 4; $0.10 > p > 0.05$ (Basque autochtonous population)
$\chi^2 = 4.522$; d.f. = 6; $0.70 > p > 0.60$ (Madrid population)
$\chi^2 = 6.516$; d.f. = 4; $0.20 > p > 0.10$ (Sevilla population)
The phenotypes with expected numbers below 5 were pooled for the calculation

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HAPTOGLOBIN SUBTYPES IN SEVERAL POPULATIONS OF SPAIN

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INTRODUCTION

Population data on electrophoretic genetic markers are necessary for a statistical evaluation of forensic evidence. The aim of this study is to present the distribution of the haptoglobin (Hp) polymorphism in several populations of Spain. This could be of importance for forensic purposes as well as for subpopulation studies.

MATERIALS AND METHODS

Plasma samples were obtained of unrelated donors from Madrid, Sevilla and the Basque Country, and only those individuals whose 8 surnames and birth places of their 4 grandparents were of Basque origin were considered as autochtonous.

Isoelectric focusing, under reducting conditions, of neuraminidase-treated plasma samples was carried out using dry miniaturized (interelectrode distance 55 mm) polyacrylamide gels, as previously described (Alonso et al. 1990), rehydrated in presence of 2-mercaptoethanol and a mixture (2:1) of Pharmalyte carrier ampholytes (pH 4-6.5 and pH 6-8) followed by immunoblotting. The diversity between observed and expected results has been tested using the χ^2 test for independence and genetic equilibrium according to the Hardy-Weinberg law.

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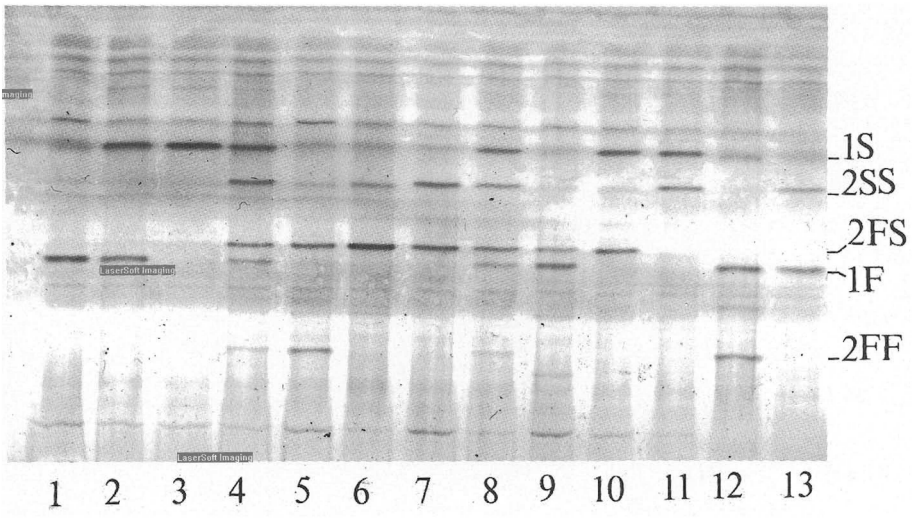

Figure 1: HP subtypes. (1) 1F, (2) 1F-1S, (3) 1S, (4) mix, (5) 2FF-2FS, (6) 2FS, (7) 2FS-2SS, (8) mix, (9) 1F-2FS, (10) 1S-2FS, (11) 1S-2SS, (12) 1F-2FF, (13) 1F-2SS

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Subtypes	Observed	Expected	χ^2	Allelic Frequencies
1F	5	4.35	0.097	HP*1F = 0.1475
1F-1S	13	13.57	0.024	
1S	10	10.58	0.032	HP*1S = 0.2300
1F-2FF	0	0.30	0.295	
1F-2FS	36	34.81	0.041	HP*2FF = 0.0050
1F-2SS	0	1.62	1.623	
1S-2FF	0	0.46	0.460	HP*2FS = 0.5900
1S-2FS	57	54.28	0.136	
1S-2SS	2	2.53	0.111	HP*2SS = 0.0275
2FF	0	0.01	0.005	
2FF-2FS	2	1.18	0.570	
2FF-2SS	0	0.06	0.055	
2FS	66	69.62	0.188	
2FS-2SS	9	6.49	0.971	
2SS	0	0.15	0.151	
Total	200	200.00	4.758	

Table 1: subtypes distribution and allele frequencies in the population sample from Tuscany

Country	N	*1F	*1S	*2FF	*2FS	*2SS	References
Norway	6668	.158	.220	.004	.578	.040	Teige '92
Sweden	564	.156	.231	.001	.571	.041	Hjalmarson '88
Denmark	2184	.151	.241	.002	.565	.040	Thymann '90
Germany							
Berlin	1275	.147	.250	.002	.575	.025	Patzelt '85
L.Saxony	1500	.153	.252	.003	.562	.029	Rothämel '89
L.Saxony	431	.153	.240	.002	.568	.036	Basler '92
Rhine-Ruhr	1035	.139	.257	.001	.583	.019	Bertrams '87
Southwest	182	.144	.254	.000	.574	.024	Zischler '87
Stuttgart	1485	.158	.217	.004	.600	.020	Härle '92
Hungary	615	.119	.221	.004	.665	.001	Hever '78
Switzerland							
Berne	1266	.126	.239	.010	.583	.042	Scherz '90
Lausanne	500	.147	.249	.003	.567	.034	Dimo-Simonin '90
France	202	.139	.245	.012	.547	.045	Shibata '82
Spain							
Madrid	250	.168	.232	.020	.552	.028	Alonso '90
Barcelona	317	.142	.238	.006	.621	.002	Moral '83
Italy							
Continental	441	.133	.241	---	.626	---	Santoro '83
Sardinia	165	.215	.245	---	.540	---	Santoro '83
Tuscany	200	.148	.230	.005	.590	.027	this paper
Greece	212	.042	.311	.005	.463	.179	Stromatias '87

Table 2: HP allele frequencies in Europe

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HAPTOGLOBIN SUBTYPES IN TUSCANY (ITALY)

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INTRODUCTION

The genetic polymorphism of haptoglobin (HP) has been first observed, through starch gel electrophoresis, by Smithies *et al* (1955), who demonstrated three common phenotypes: HP1, HP2-1 and HP2. The HP-α chain, which carries the polymorphism, has subsequently been shown to exist in several additional allelic forms ("subtypes") - 1S, 1F, 2FS, 2FF and 2SS - giving rise to three different HP1, six different HP2-1 and six different HP2 phenotypes. Only in the last few years, however, methods suitable for routine HP subtyping have been developed.
For this reason, while allelic frequencies of the "main" HP types in the various human populations are well known, the geographic distribution of the HP subtypes has still not been examined to the same extent (for a recent review see Teige *et al* 1992).
The aim of this study is to present the distribution of HP subtypes in Tuscany (Italy). Up to now, the only available data about the italian population were reported by Santoro *et al* (1983), who determined exclusively the 1F/1S subtypes.

MATERIALS AND METHODS

Sera were collected from a random sample of 200 healthy blood donors born in Pisa.
HP subtypes were determined by the method described by Scherz *et al* (1990), which includes four steps: isolation of HP by immunoprecipitation - reduction and alkilation in the presence of urea - PAGIF - protein staining.
For a cross-validation of the results, the HP "main" phenotypes were also determined, using conventional PAA electrophoresis. Control sera samples were kindly provided by Dr Krüger, from the Legal Medicine Institute of Hannover (BDR).

RESULTS AND DISCUSSION

The HP subtypes distribution and the estimated allele frequencies in our population sample are reported in table 1. The observed numbers are in good agreement with the expected distribution under the Hardy-Weinberg equilibrium (χ^2 = 4.76, df = 10, P> .90). In this sample, nine of the fifteen phenotype combinations have been found. The HP subtypes pattern, obtained by immunofixation/PAGIF technique, is shown in fig.1 (the 1F-2FF and 1F-2SS types, in the last two lanes, are control sera).
Allelic frequencies in Tuscany are consistent with those reported in other European populations (see table 2). In particular, the frequency of HP*1F allele, which is the most discriminating parameter between different ethnical groups (Teige *et al*, 1992), is included in the Caucasian population range.
The heterogeneity test between our gene frequencies and those reported by Santoro *et al* for Continental Italy was not significant (G^2 = 0.595, df = 2, P = 0.70-0.90); the same test, applied to Sardinian data was at the boundary (G^2 = 6.947, df = 2, P = 0.05-0.02).
The theoretical exclusion rate in cases of disputed paternity (32.8 %) is comparable with that of more informative classical systems (MNSs, GC and PGM1). This is a good reason to include HP system within the conventional markers to be used in combination with DNA polymorphisms for paternity testing.

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Population Genetics

Table 2: Distribution of F XIIIA phenotypes in samples from SW-Germany and N-Portugal

Phenotypes	SW-Germ. observed	exp.	N-Port. observed	exp.
1A	5	7,36	7	7,96
1B	95	96,31	73	65,23
2A	0	0,03*	3	0,72*
2B	14	12,09	7	5,75
1A1B	56	53,26	39	45,57
2A1A	1	0,97*	7	4,78
2B1A	20	18,87	20	13,53
2A1B	6	3,51*	11	13,67
2B1B	65	68,25	33	38,74
2A2B	0	1,24*	0	4,06*
4-1B	1	0,64*		
4-2B			1	0,17*
0-1B	1	0,64*		
others	0	0,83	0	0,82*
total	264		201	
chi^2		1,61		8,30
P (3 df)		>0,30		>0,95
		<0,40		<0,975

*pooled for chi^2 calculation

Table 3:

Gene frequencies	SW-Germany	Portugal
F XIIIA*1A	0,167	0,199
1B	0,604	0,570
2A	0,011	0,060
2B	0,214	0,169
4	0,002	0,002
0	0,002	

Table 3 shows the distribution of F XIIIA alleles in a sample of 260 unrelated individuals from SW-Germany and of 201 unrelated individuals from North-Portugal.

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Fig. 2: Zymogram of Transglutaminases

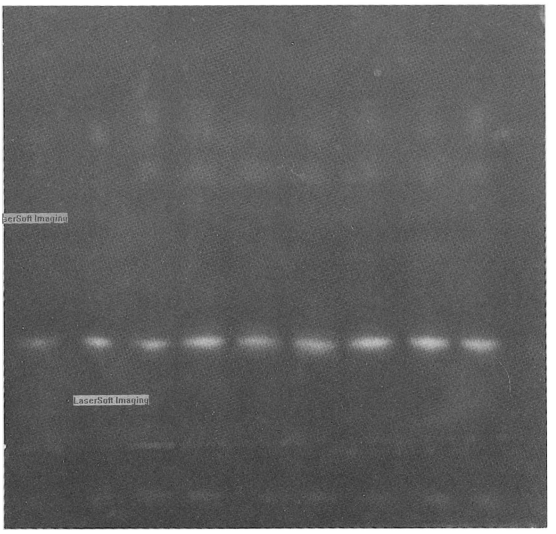

Fig. 2 shows the results after IEF and functional assay (using the transglutaminase activity of activated F XIIIA). There are two more patterns with transglutaminase activity, a result which was first discribed by Sebetan (1988), he interpreted it as additional loci of F XIIIA.

Formal Genetics

Table 1 shows the segregation in 184 families with 513 children in SW-Germany. Observed and expected segregation ratios are in good agreement: $chi^2 = 14,14$, $0,7 < P > 0,80$, $df = 11$.
Thus, by this extended formal analysis, the hypothesis proposed by Suzuki et al. (1988) is confirmed:
"Four common alleles F XIIIA*1A, 1B, 2A, 2B at an autosomal locus control the expression of 10 phenotypes"

Table 1:

matings		No.of fam.	phenotypes of children 1A	1B	1A1B	2B	2A	2A1A	2A1B	2B1A	2B1B	2A2B
1A	x 1B	2			8							
	x 1A1B	3	3		0							
	x 2B1A	2	4							1		
1B	x 1B	18		63								
	x 1A1B	33		38	49							
	x 2B	9									24	
	x 2B1A	14			14						22	
	x 2B1B	43		51							63	
	x 2A1A	2			3				3			
1A1B	x 1A1B	8	8	8	9							
	x 2B	6								8	11	
	x 2A1A	1	1		1			1	1			
	x 2A1B	4		0	2			2	4			
	x 2B1A	6	2		6					4	6	
	x 2B1B	12		10	9					10	6	
2B	x 2B1A	1				2				0		
	x 2B1B	2				3					3	
2A1B	x 2B1B	1		0					2		0	0
2B1A	x 2B1A	1	1			0				2		
	x 2B1B	6			5	5				4	5	
2B1B	x 2B1B	10		2		7					17	
Total		184	19	172	106	17		3	10	29	157	

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Subtyping of coagulation factor XIIIA by Isoelectric focussing

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Introduction

Human blood coagulation factor XIII consists of 2 dimeric molecules, F XIIIA and F XIIIB which are under separate control of loci on chromosome 1 and 6, respectively.
F XIIIA is the zymogen for factor XIIIa bearing transglutaminase activity, which forms intermolecular γ-glutamyl-ε-lysine cross-links between monomeric fibrin strands, thereby stabilizing the fibrin clot.
A genetic polymorphism of factor XIIIA with two common alleles, F XIIIA* 1,2, was first discribed by Board (1979).
Suzuki et al. (1988) demonstrated the existence of subtypes of each of this alleles, F XIIIA* 1A, 1B, 2A, 2B, by isoelectric focusing.

Material and Methods

EDTA-Plasma samples and diluted crude white cell lysates from 184 families with 513 children from SW Germany and 201 unrelated individuals from North-Portugal were analyzed. Separation was by IEF in a pH gradient from 5-8 with 3 M urea.
(Acrylamidegel-total volume:15 ml, 2,5 ml acrylamide, 1,2 ml bis-acrylamide, 0,8 ml ampholyte 5-6.5, 0,2 ml ampholyte 6-8, 1,95 g saccharose, 2,7 g urea).
Visualization was by Enzyme-Immuno-Assay with anti-human-F XIIIA (rabbit, Behring) and anti-rabbit (goat, POD con-jugated, ATAB) after blotting onto nitrocellulose.

Results and discussion

Fig. 1: Pherogram of F XIIIA subtypes

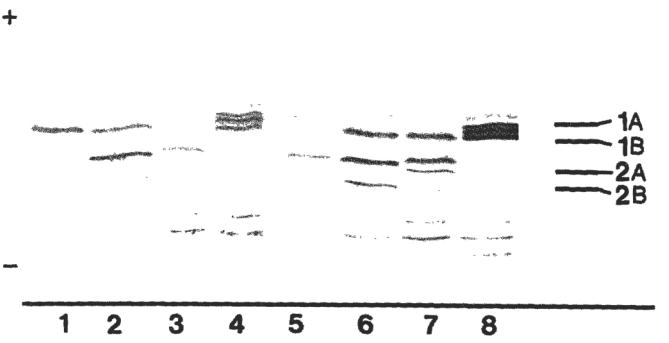

Fig. 1 shows five different phenotypes of F XIIIA
(1) 1B, (2,6) 2B1B, (3,5) 2B1A, (4,8) 1A1B, (7) 2A1B

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10. Conventional genetic markers

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TABLE 3: FAMILY DATA FROM 7 FAMILIES* WITH ALTOGETHER 9 CHILDREN:

Parents:	n	Children: 1.1,1.2	1.2,4	1.3,3	2,4	3,3	3,4	4,4
1.1,1.1x1.2,4	1	1	-	-	-	-	-	-
1.1,4x2,2	1	-	-	-	1	-	-	-
1.1,4x3,4	1	-	-	-	-	-	-	1
1.2,4x4,4	1	-	1	-	-	-	-	2
1.3,2x3.4	1	-	-	1	-	-	-	-
2,3x3,4	1	-	-	-	-	-	1	-
3,3x3,4	1	-	-	-	-	1	-	-

* We examined another 4 families where the putative father was excluded from paternity in two other systems in each case; so these families were not taken into account.

SECNF: 0.5986

SUMMARY:

- The distribution of HLA-DQα subtypes found for a South Bavarian population sample is in good agreement with the Hardy-Weinberg-equilibrium.
- The power of discrimination is 0.929.
- In comparison with other population samples the differences varied from uninconsiderable (other German or Central European populations being compared) to significant (populations from other continents being compared).
- The segregation of HLA-DQα subtypes in 7 South Bavarian families with altogether 9 children was in accordance with an assumed autosomal codominant mode of inheritence.
- SECNF being 0.5986, the HLA-DQα system can be considered to be an informative marker for paternity testing.

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RESULTS

ALLELE FREQUENCIES:

We examined 213 individuals from a South Bavarian population, which means 426 alleles. The allele distribution was as follows:

1.1:	45 x	= 10.56%
1.2:	79 x	= 18.54%
1.3:	34 x	= 7.98%
2:	57 x	= 13.38%
3:	68 x	= 15.96%
4:	143 x	= 33.57%

FIGURE 1: A COMPARISON OF OBSERVED ALLELE FREQUENCIES IN VARIOUS POPULATIONS:

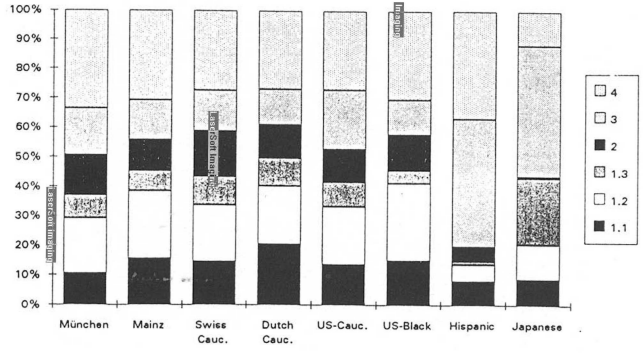

TABLE 1: OBSERVED AND EXPECTED PHENOTYPE FREQUENCIES IN OUR POPULATION SAMPLE:

Genotype	n	observed %	expected %	n	χ^2
1.1,1.1	3	1.41	1.12	2.38	0.0767
1.1,1.2	8	3.76	3.92	8.34	0.0694
1.1,1.3	5	2.35	1.69	3.59	0.2593
1.1,2	8	3.76	2.83	6.02	0.3053
1.1,3	5	2.35	3.37	7.18	0.3115
1.1,4	13	6.10	7.09	15.11	0.1378
1.2,1.2	7	3.29	3.44	7.33	0.0677
1.2,1.3	8	3.76	2.96	6.31	0.2139
1.2,2	10	4.69	4.96	10.57	0.0145
1.2,3	14	6.57	5.92	12.61	0.0719
1.2,4	25	11.74	12.45	26.52	0.0408
1.3,1.3	0	0.00	0.64	1.36	0.6370
1.3,2	1	0.47	2.14	4.55	1.3000
1.3,3	6	2.82	2.55	5.43	0.0284
1.3,4	14	6.57	5.36	11.41	0.2753
2,2	6	2.82	1.79	3.81	0.5887
2,3	9	4.23	4.27	9.10	0.0005
2,4	17	7.98	8.98	19.13	0.1117
3,3	4	1.88	2.55	5.43	0.1762
3,4	26	12.21	10.72	22.83	0.2072
4,4	24	11.27	11.27	24.00	0.0000
total:	213			213.01	χ^2 4.8938 0.995>p>0.990 df 15

The expected phenotype frequencies were calculated according to the Hardy-Weinberg-equilibrium from the allele frequencies.

The Power of Discrimination (PD) was 0.929.

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HLA-DQα PCR SYSTEM: FREQUENCIES OF A SOUTH BAVARIAN POPULATION AND FAMILY DATA

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INTRODUCTION

- The HLA-DQα locus and the analysis of amplified DNA by using non-radioactive-labelled oligonucleotide probes in dot-blot-form have first been described by Saiki et al. (1986).
- Further developement of the polymerase-chain-reaction followed (Saiki et al., 1988).
- Ultimately, Cetus Corporation, Emeryville, introduced it`s AmpliType® Type HLA-DQα kit (1989).
- The aim of our random test was to collect data about the distribution of HLA-DQα alleles and phenotypes in a South Bavarian population and to compare the obtained data with other statistics already described in relevant literature.
- Furthermore, the exploration of family data for the same population was intended, same as the determination of the single exclusion chance for non-fathers (SECNF).

MATERIALS and METHODS:

Whole blood from 213 unrelated individuals out of a South Bavarian population and 11 families with altogether 15 children.

DNA extraction: sodium dodecyl sulfate (SDS)/proteinase K digestion
phenol / chlorophorm / isoamylalcohol (24:24:1) extraction
ethanol precipitation
This is done by inserting 2 ml of whole blood.
OR:
Applicaftion of the QIAmp Blood Kit from Qiagen according to the manuacturer`s instructions in-serting 250 µl of whole blood.

DNA amplification: Amplification was carried out using the HLA-DQα Forensic DNA Amplification and Typing Kit from AmpliType, Cetus, according to the manufacturer`s instructions (AmpliType™ User Guide, 1990). It took place in the GeneAmp PCR System 9600 from Perkin Elmer running the following programme for 32 cycles:

- Denaturation at 94°C for 20 sec.
- Primer-Template-Annealing at 60°C for 10 sec.
- Primer Extension at 72°C for 20 sec.

HLA-DQα Typing: Dot blot hybridisation and colour developement was managed according to the manufacturer`s instructions.

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Distribution of DQα Allele in Marion County, FBI, and Cetus Populations

	MARION COUNTY		FBI		CETUS	
DQα Allele	Caucasian N=185	Black N=203	Caucasian N=737	Black N=589	Caucasian N=826	Black N=448
1.1	0.124	0.167	0.131	0.134	0.137	0.150
1.2	0.238	0.310	0.205	0.270	0.197	0.263
1.3	0.068	0.022	0.075	0.042	0.085	0.045
2	0.127	0.089	0.138	0.126	0.109	0.121
3	0.176	0.111	0.182	0.113	0.201	0.118
4	0.268	0.300	0.282	0.322	0.271	0.304

Distribution of DQ Alpha Alleles in Marion County, FBI and Cetus Populations

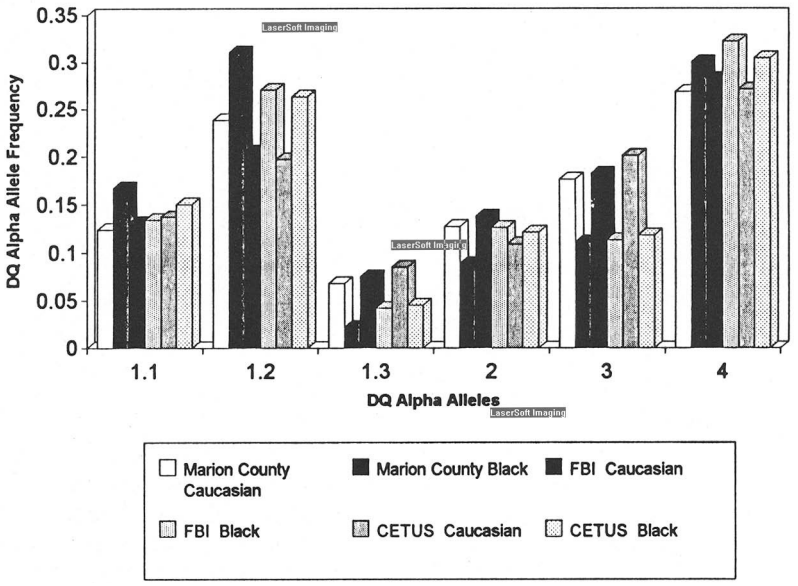

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Table 1 HLA DQ Alpha Genotype Distribution in Caucasian Population of Marion County, Indiana

HLA DQ Alpha GENOTYPE	OBSERVED (N=185)[a]		EXPECTED (N=185.3)[a]	
1.1, 1.1	4[b]	(.022)[c]	2.8	(.015)[c]
1.1, 1.2	7	(.038)	10.9	(.059)
1.1, 1.3	2	(.011)	3.1	(.017)
1.1, 2	4	(.022)	5.8	(.031)
1.1, 3	11	(.059)	8.1	(.044)
1.1, 4	14	(.076)	12.3	(.066)
1.2, 1.2	10	(.054)	10.5	(.057)
1.2, 1.3	8	(.043)	6.0	(.032)
1.2, 2	11	(.059)	11.2	(.060)
1.2, 3	15	(.081)	15.5	(.083)
1.2, 4	27	(.146)	23.6	(.127)
1.3, 1.3	0	0	0.8	(.004)
1.3, 2	3	(.016)	3.2	(.017)
1.3, 3	1	(.005)	4.4	(.024)
1.3, 4	11	(.059)	6.7	(.036)
2, 2	1	(.005)	3.0	(.016)
2, 3	13	(.071)	8.3	(.045)
2, 4	14	(.076)	12.6	(.068)
3, 3	5	(.027)	5.7	(.031)
3, 4	15	(.081)	17.5	(.094)
4, 4	9	(.049)	13.3	(.072)

a: N = Number of people typed.
b: number of each genotype.
c: frequency of each genotype.
Chi-square = 14.13; degree of freedom = 9; (0.100 < P < 0.250)

Table 2 HLA DQ Alpha Genotype Distribution in Black Population of Marion County, Indiana

HLA DQ Alpha GENOTYPE	OBSERVED (N=203)[a]		EXPECTED (N=202.6)[a]	
1.1, 1.1	3[b]	(.015)[c]	5.7	(.028)[c]
1.1, 1.2	25	(.123)	21.0	(.104)
1.1, 1.3	1	(.005)	1.5	(.007)
1.1, 2	8	(.039)	6.0	(.023)
1.1, 3	7	(.034)	7.5	(.037)
1.1, 4	21	(.103)	20.3	(.100)
1.2, 1.2	19	(.094)	19.5	(.096)
1.2, 1.3	4	(.020)	2.8	(.014)
1.2, 2	12	(.059)	11.2	(.055)
1.2, 3	13	(.064)	14.0	(.069)
1.2, 4	34	(.168)	37.8	(.186)
1.3, 1.3	0	0	0.1	(.0004)
1.3, 2	0	0	0.8	(.004)
1.3, 3	3	(.015)	1.04	(.005)
1.3, 4	1	(.005)	2.7	(.013)
2, 2	2	(.010)	1.6	(.008)
2, 3	5	(.025)	4.0	(.020)
2, 4	7	(.034)	10.8	(.053)
3, 3	1	(.005)	2.5	(.012)
3, 4	15	(.074)	13.5	(.067)
4, 4	22	(.108)	18.3	(.090)

a: N = Number of people typed.
b: number of each genotype.
c: frequency of each genotype.
Chi-square = 5.51; degree of freedom = 7; (0.50 < P < 0.75)

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DISTRIBUTION OF HLA - DQα ALLELES IN DEOXYRIBONUCLEIC ACID (DNA) FROM CAUCASIAN AND BLACK POPULATIONS OF MARION COUNTY,INDIANA, USA

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The frequency in the population of the genotype markers, found in the evidentiary sample is important in associating an individual as a donor to the crime scene. Among the genotype markers used in forensic science, the HLA-DQα and the highly polymorphic VNTR (variable number of tandem repeats) loci have the most discrimination power for eliminating a suspect (Budowle, et. al., 1990). However, in those cases where the suspect cannot be excluded as a likely contributor to the crime scene, it is essential to determine the probability of other individuals in the population which can be the potential donor of the biological evidentiary sample. For that matter, the data on the frequency of alleles at HLA-DQα and VNTR loci are required for statistical analysis. We report in this paper the results on the distribution of alleles at HLA-DQα locus in DNA from black and caucasian population of Marion County, Indianapolis, Indiana. The data on VNTR loci of these populations will be presented elsewhere.

The blood samples were collected from 203 black and 186 caucasian, all residing in Marion County, Indianapolis, Indiana. About 700μL liquid blood was frozen at -80°C for extracting DNA according to the procedure described in FBI Manual (1992). Quantitation of DNA was done spectrophotometrically by recording the absorbance at 260, 270, 280 and 300 nm.

HLA-DQα genotyping: Two μL of extracted DNA was used to amplify the specific segment of DNA by polymerase chain reaction (PCR) following the procedure recommended by Cetus (1991). The amplification was performed using Taq polymerase enzyme in a programmed Perkin-Elmer thermal cycler, with cycle setting as follows: denaturation at 94°C for 60 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds; the total cycles being 32. With each batch negative and positive controls were included to determine the authenticity of the final product. The amplified DNA was used for HLA-DQα genotyping as narrated in Cetus Amplitype kit (1991). Tables 1 and 2 depict the observed HLA-DQα alleles frequencies respectively of black and caucasian population along with the expected frequencies computed by Chi-square method based on the assumption of Hardy-Weinberg equilibrium (Mange and Mange, 1990). It is obvious from Figure 1 that HLA-DQα alleles distribution in black and caucasian population markedly differed from each other. However, despite these differences, both populations seem to be in conformity with the Hardy-Weinberg Equilibrium. The large probability of Chi-square values suggest that the deviation between the observed and expected genotype frequencies (Table 1,2) can be assigned to chance variance alone. The low proportion of homozygotes in both populations indicates that there were no significant non-random mating sub-populations within these ethnic populations and that there were no undetected alleles in these populations (Cetus, 1991).

Our data on the frequencies of HLA-DQα alleles distribution in black and caucasian are quite similar to those reported by FBI (1992) and Cetus Corporation (1991) for ethnic populations. From these data it is thus possible to compute the proportion of each genotype which could be expected in a known population.

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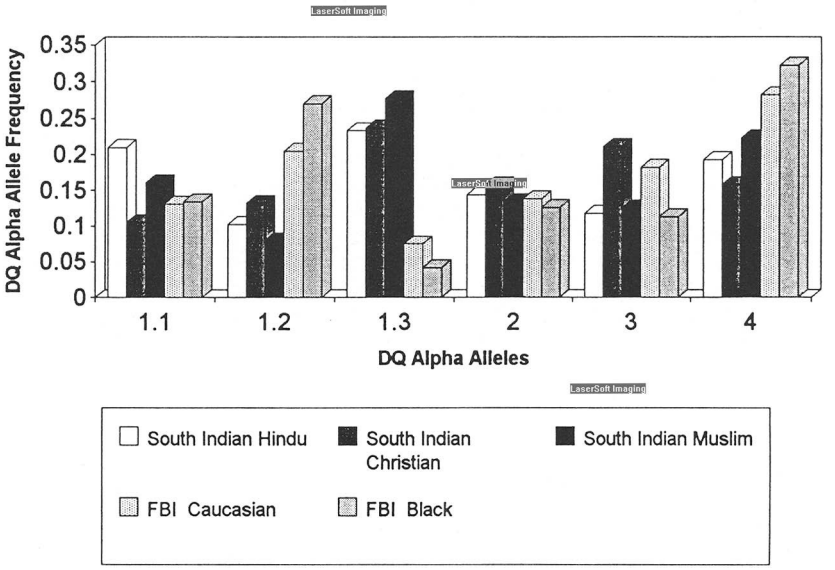
Distribution of DQα Alleles in Various Sets of South Indian Populations and FBI Populations

	SOUTH INDIAN			FBI	
DQα Allele	Hindu N=420	Christian N=19	Muslim N=56	Caucasian N=737	Black N=589
1.1	0.210	0.105	0.161	0.131	0.134
1.2	0.102	0.132	0.080	0.205	0.270
1.3	0.233	0.237	0.277	0.075	0.042
2	0.144	0.158	0.134	0.138	0.126
3	0.118	0.211	0.125	0.182	0.113
4	0.193	0.158	0.223	0.282	0.322

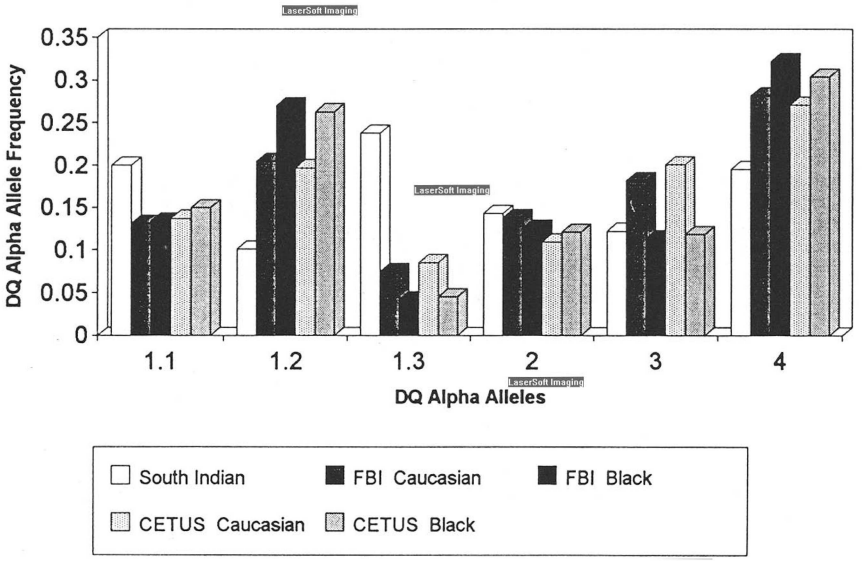
DQ Alpha Alleles in Various South Indian Sects and FBI Populations



Distribution of DQα Data from South Indian, FBI, and Cetus Populations

		FBI		CETUS	
DQα Allele	South Indian N=495	Caucasian N=737	Blacks N=589	Caucasian N=826	Blacks N=448
1.1	0.200	0.131	0.134	0.137	0.150
1.2	0.101	0.205	0.270	0.197	0.263
1.3	0.238	0.075	0.042	0.085	0.045
2	0.143	0.138	0.126	0.109	0.121
3	0.122	0.182	0.113	0.201	0.118
4	0.195	0.282	0.322	0.271	0.304

Distribution of DQ Alpha Alleles in South Indian, FBI, and Cetus Populations



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DEOXYRIBONUCLEIC ACID (DNA) HLA - DQ α ALLELE FREQUENCY DISTRIBUTION IN VARIOUS SECTS OF SOUTH INDIAN POPULATION

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Abstract

Blood samples from 495 South Indian individuals (420 Hindu, 56 Muslim, and 19 Christian) were collected and subjected to HLA-DQ α typing using the polymerase chain reaction. (Blood was collected in clot tubes and deposited directly on cotton swatches, dried at room temperature and packaged in paper envelopes and stored at -20° C until typing.) DNA typing was performed according to the FBI protocol using Cetus AmpliType kits and a Perkin Elmer Cetus thermocycler. All the 21 genotypes were observed in the Hindu population group, but because of the small sample size, some of the genotypes were not observed in the other two groups. The observed genotype frequencies for the Hindu population did not follow Hardy-Weinberg equilibrium. There was an apparent excess of homozygotes, probably due to consanguinity in this population. The Muslim and Christian population samples were too small for statistical analysis. Unlike other population groups that have been genotyped at the DQ α locus by the FBI and by Cetus Corporation (1991), Hardy-Weinberg equilibrium cannot be used to calculate the expected genotype frequencies in the South Indian Hindu population.

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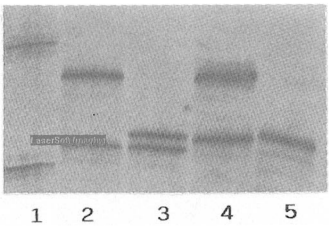
Table 1. Distribution of COL2A1 alleles in 216 Italians

Alleles	Frequencies	Statistical evaluations
1A1	0.0185	$\chi^2 = 36.57$ $0.75 < P < 0.90$ (45 d.f.)
1	0.0787	
5	0.2477	Heterozygosity = 0.810
6	0.2616	Expected heterozygosity = 0.785±0.027
7	0.0301	
8	0.0208	PD = 0.91
9	0.0185	Chance of exclusion = 0.58
10	0.2778	
12	0.0324	Italians/Germans χ^2=57.30 P< 0.0000
15	0.0139	G statistic = 60.2347 P< 0.0000

Fig. 2. Magnification of COL2A1 amplified products after Page and silver staining. Samples: (1) 123 bp weigth marker BRL; (2) type 1-6; (3) type 5-6; (4) type 1-5; (5) type 5-5. Allele 5 in lane (4) is slightly displaced with respect to the same adjacent alleles.



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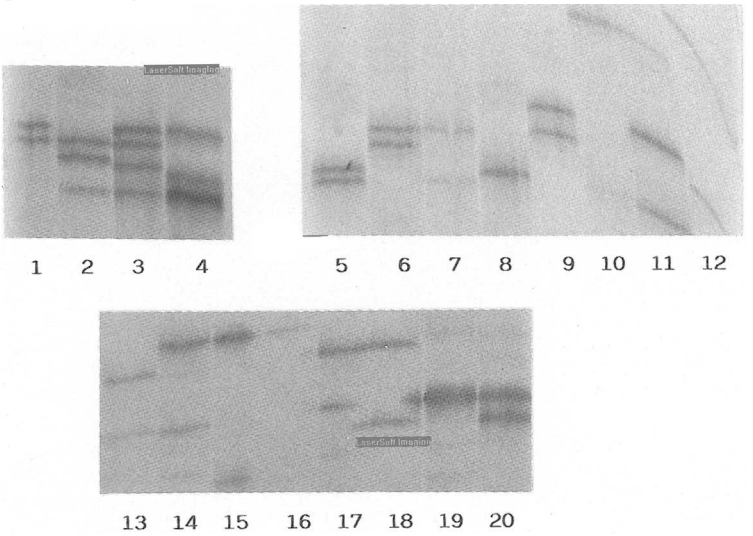
standard error for H, were h is the expected heterozygote frequency and N the number of subjects examined.
The power of discrimination (PD) was calculated using the equation proposed by Fischer (1951): $1-\sum(Pi)^2$, where Pi represents the frequency of each genotype. The exclusion chance was calculated from allele frequencies (Garber and Morris 1983).
The allelic frequencies were statistically compared with previous studies to recognize possible intraethnic variations by a computer program kindly supplied by G. Carmody (Carleton University, Ottawa, Canada).

Fig. 1. COL2A1 amplified products after PAGE and silver staining. Samples: (1) type 5-6; (2) ladder composed of alleles 6,7,10; (3) ladder composed of alleles 5,6,8,10; (4) ladder composed of alleles 5,9,10; (5) type 9-10; (6) type 6-7; (7) type 6-10; (8) type 9-9; (9) type 5-6; (10) type 1A1-9; (11) type 5-10; (12) 123 bp weigth marker BRL; (13) type 1-5; (14) type 1A1-5; (15) type 1A1-9; (16) type 1A1-1A1; (17) type 1-5; (18) type 1-6; (19) type 5-5; (20) type 5-6.



RESULTS AND DISCUSSION

Ten alleles were found in a sample of 216 Italians and named according to the Munster classification and ISFH recommendations. Table 1 shows allelic frequencies and the results of statistical analysis, which demonstrate no deviations from Hardy-Weinberg expectations and from the expected heterozygote rate. The most frequent alleles were COL2A1*10, COL2A1*6, COL2A1*5 and COL2A1*1, in line with the frequencies found in previous studies on Caucasians performed with the same procedures(Wiegand et al., pers. comm.; Prinz et al., pers. comm.). Even if the allelic frequencies follow the same trend, the specific values differ for each allele to the extent that heterogeneity does in fact occur between the Italian and German samples. The same test was not applied to the data of Berg and Olaisen because they used different analytical approaches and nomenclature.
The high PD and chance of exclusion found in this study make this system a powerful tool for forensic identification and paternity testing.
One of the most relevant problems which affect this VNTR is the presence of alleles differing from each other by few bases and not by a sequence repeat unit. In our study we found slight disalignment between some bands, which could be explained only by a difference of a few bp (Fig. 2). Such alleles can only be identified by sequencing (Berg and Olaisen, 1993) since PAGE does not distinguish clearly between similar alleles. Further studies of the analytical methods to improve the resolution of alleles and to make a new nomenclature are desirable.

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PCR TYPING OF THE COL2A1 SYSTEM: ALLELIC FREQUENCIES IN TWO POPULATION SAMPLES FROM NORTH AND CENTRAL ITALY

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Istituto di Medicina Legale e delle Assicurazioni, Università di Parma, Italy.

INTRODUCTION

The COL2A1 system is a VNTR in the 3'- flanking region of the human Type II collagen gene. It was first sequenced by Stoker et al. (1985), who pointed out the polymorphism was due to repetitive sequences of 31 or 34 bp. Mendelian inheritance and polymorphism were investigated at the same time with the polymerase chain reaction (PCR) technique by Wu et al. (1990) and Priestley et al. (1990). Using different primers these authors found four and five alleles respectively.
Further alleles were recently discovered by analysing the amplified products with the more sophisticated high-resolution polyacrylamide gel electrophoresis (PAGE) followed by silver staining. Puers et al. (1992) discovered various alleles whose sizes differed by less than the expected 31 or 34 bp, down to 3 bp. Even more recently Berg and Olaisen (1993) sequenced 19 allelic variants studied by denaturing PAGE, and demonstrated that the polymorphism is due to: 1) a different total number of copies of the core repeat units; or 2) the same total number of copies but containing a different proportion of core repeat units of 34 or 31 bp; or 3) small deletions in some core units.
Population studies to assess the number and the frequency of alleles were published by Priestley et al (1990), Puers et al. (1992), Rand et al. (1992), Berg and Olaisen (1993). Further population studies were performed by Wiegand et al. (pers. comm.) and Hou et al. (pers. comm.), the latter demonstrating allelic variations among different racial groups. The aim of this study was to investigate the polymorphism of this system to verify a) the number of alleles and b) the allelic frequency distribution in a sample of Italians, and c) to compare the results with other Caucasian samples.

MATERIALS AND METHODS

Fresh blood samples were collected from healthy unrelated donors living in Ancona (n = 117) and Parma (n = 99). The two laboratories performed the analysis independently, following the same procedures for DNA extraction (Budowle and Baechtel, 1990), amplification and identification of phenotypes.
Amplification was carried out in a thermal cycler PTC 100-60 and a Minicycler (MJ Research, USA) with primers proposed by Wu et al. (1990): 5'-CCAGGTTAAGGTTGACAGCT-3'; 5'-GTCATGAACTAGCTCTGGTG-3'
for 25 cycles at the following conditions: denaturation at 94°C 1 min; annealing at 64°C 1 min; extension at 70°C 2 min. The amplification mix contained 50 ng DNA template, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 1 U (0.2 µl) Taq polymerase (Promega Corporation, USA), 0.4 µM each primer, 200 µM each nucleotide, diluted to a total volume of 25 µl and overlayed with 30 µl oil.
Separation of amplified products was carried out in PAGE (Budowle et al. 1991) at 5% T, 3% C with PDA as cross-linker, thickness 450 µm, using a discontinuous buffer system (Allen et al. 1989). The bands were visualized with silver staining (Budowle et al. 1991), as shown in Fig. 1. Alleles were identified by side-to-side comparison with reference alleles, isolated or mixed in a cocktail obtained from the amplified products.
The results were verified by the chi-square test between observed and expected genotypes under the Hardy-Weinberg law and by comparing the observed and expected heterozygosity frequencies, the latter calculated as allelic diversity ($H = \left[1 - \sum X_i^2\right]\left[n/n-1\right]$, where X_i is the allelic frequency and n the sample size (Nei and Roychoudhury, 1974). $\sqrt{\left[h(1-h)/N\right]}$ is the formula to compute the

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Fig 1

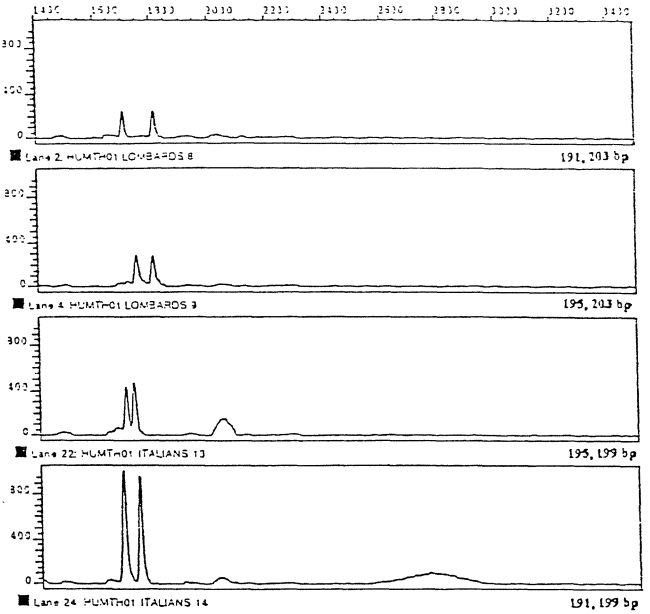
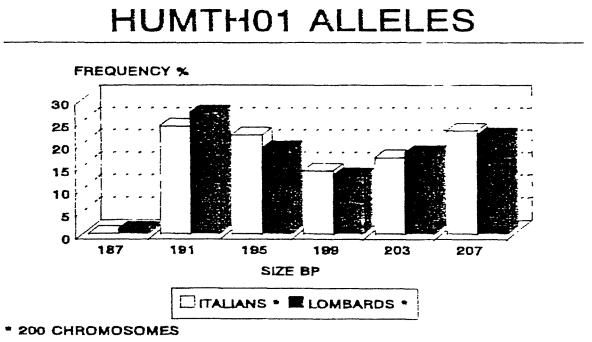


Fig 2



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Thus, electrophoretic mobility variations (from lane to lane and gel to gel) that can lead to inconsistent allele sizing are automatically normalized. Furthermore, multiple primers, each "tagged"with a distinct fluorescent dye, can be used. This allows the simultaneous analysis of multiple VNTR profiles.

This study examines the potential of automated fluorescent detection of STR alleles as a sensitive and accurate approach for DNA typing.

We report DNA typing of the tetrameric repeat (AATG) STR HUMTH01 with fluorescent primer and STR products have been run and analyzed on an automated sequencer (4). Our study have established the TH01 allelic frequencies in the Italian population on DNA extracted from blood by comparing unrelated individuals selected from northern Italy (100 samples) to the whole Italian population.

Materials and Methods

Human genomic DNA was prepared from peripheral blood by standard methods involving cell lysis, proteinase K digestion and ethanol precipitation. Microsatellite locus STRs HUMTH01 (Edwards et al. 1991) was used in this study. All oligonucleotide primers used were synthesized using a Model 380 DNA synthesizer (Applied Biosystems, F.C., CA). For the automated detection a fluorescent dye (TAMRA) was attached to the 5'- end of the forward PCR primer used for amplifying of the locus using Aminolink 2 (Applied Biosystems, F.C., CA) and HPLC purified.

We used and internal lane size standards consisted of a Pst I digest of Lamda phage, labeled with a second red dye (ROX) by a ligation method. (1). Nonfluorescent primers were synthesized using the same sequences.

PCR were carried out for fluorescence primers for 25 cycles with 50-100 ng of DNA in 50 µl reaction volume. STRs HUMTH01 locus was labelled with TAMRA- dye and amplified with single PCR.

For gel analysis with fluorescent amplification products, 0.5 µl of the internal lane standards and 2 µl of PCR reaction were combined with 3 µl of deionized formamide. Were electrophoresed in a 6% acrylamide gel (BIORAD) with 8M urea in 1x TBE.

Gels were electrophoresed for 6 hr. at 30 W constant power on an Applied Biosystems, (F.C., CA) automated DNA sequencer model 373A. The size of PCR products is determined automatically and precisely by GeneScan 672 Software (ABD, F.C., CA) using the labelled size standard in each lane.

Results

We have not found any significant alleles variation in our comparison with northern italians (100 samples Lombards) and the rest of the Italian population (100 samples) (see figure). Six alleles were observed ranging in frequencies from 0.01 to 0.27. The observed overall heterozygosity was 76%.

Sometimes extra peaks were occasionally present, but they have not caused any difficulties in typing the HUMTH01 locus.

We believe that the adoption of the fluorescent technology should give us the appropriate choice of markers to obtain population data rapidly and accuratly.

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ALLELE FREQUENCIES OF THE HUMTH01 LOCUS IN THE ITALIAN POPULATION

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Servizio Polizia Scientifica, Sez. Indagini Biologiche, Roma, Italia.

SUMMARY

Human identification through DNA analysis has been revolutionized in the last years. The technique of in vitro DNA amplification using the polymerase chain reaction (PCR) (Mullis and Faloona, 1989) has facilitated the study of genetic variations in terms of simplicity and sensitivity (Reynold et al., 1991).

Microsatellites, stably inherited short runs of tandemly repeated nucleotide sequences (STRs), are a rich source of higly polymorphic markers in Human population and easily detecte by PCR.

Very recently, a new dimension in DNA typing analysis was opened with the development of instruments for automated analysis of fluorescent amplification products.

STR systems were evaluated from Fourney R. and Fregeau G. J. (1993) for sensitivity and accurancy using the Gene Scanner and compared to other DNA typing methods currently in use. Their results strongly indicate that excellent precision and accurancy can be obtained using non-denaturing gel systems with the added advantage that the majority of STR fragments can be more rapidly resolved because of increased electrophoretic migration.

We report DNA typing of the tetrameric repeat (AATG) STRs HUMTH01 mapped to chromosomal locus 11.p. 15. 5 (Edwards A. et. al., 1991) with fluorescent STR primers. The objective of the project is to establish the TH01 allelic frequencies in the Italian population by comparing unrelated individuals selected from northern Italy (100 samples) to the whole Italian population (100 samples). We have found any significant allele variations in our comparision of northern Italians to rest of Italian population.

Introduction

A large proportion of the human genome (ca. 25%) consists of repetitive DNA sequences whose inherent polymorphic characteristic provides information for forensic identification.

In recent years, through the use of the PCR, a class of repetitive elements, microsatellites, has become the focus of attention for discrimination between individuals (2).These small stretches of DNA (less than 300 bp.) are composed of tandemly repeated monomers of 2 to 5 bp. in length and like minisatellites, are widespread throughout the genome.

Numerous STR systems have been documented to date, many of which have been used to study polymorphisms within several population databases. It is estimated that the human genome contains approximately 500.000 STRs (6000 to 10.000 trimeric and tetrameric repeats) (3) each characterized by a limited number of easily identified alleles but which collectively offer unlimited potential for a highly discriminatory test for human identification.

The automated analysis of fluorescently labeled DNA products using an instruments as Applied Biosystems Division (ABD, Foster City, CA) Model 373A with GeneScan software provides a welcome alternative to the manual processing of STRs. Automated real-time analysis allows electrophoretic information to be immediately stored. In addition, automatic allele sizing and quantitation are made possible by running an internal lane standard that is recognized by the computer to generate a calibration curve.

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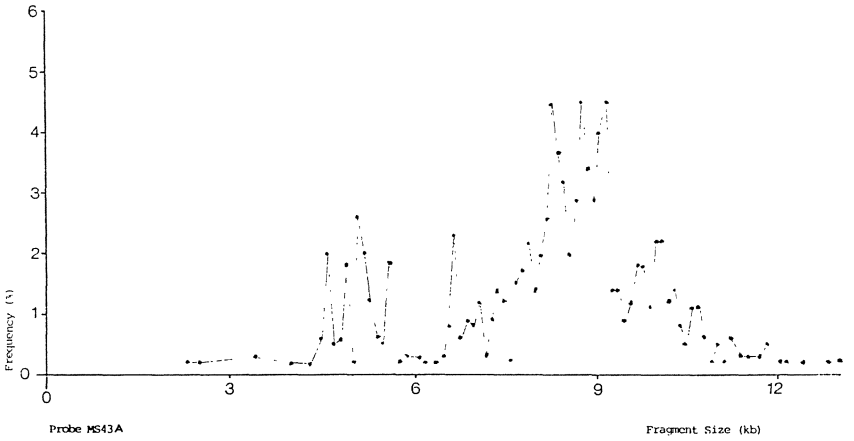

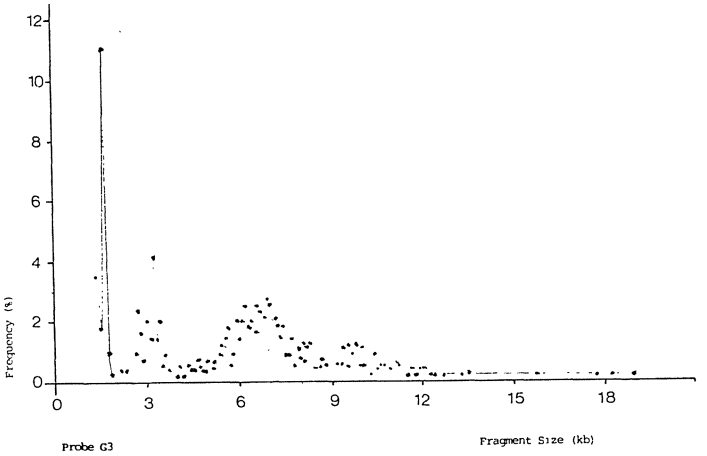

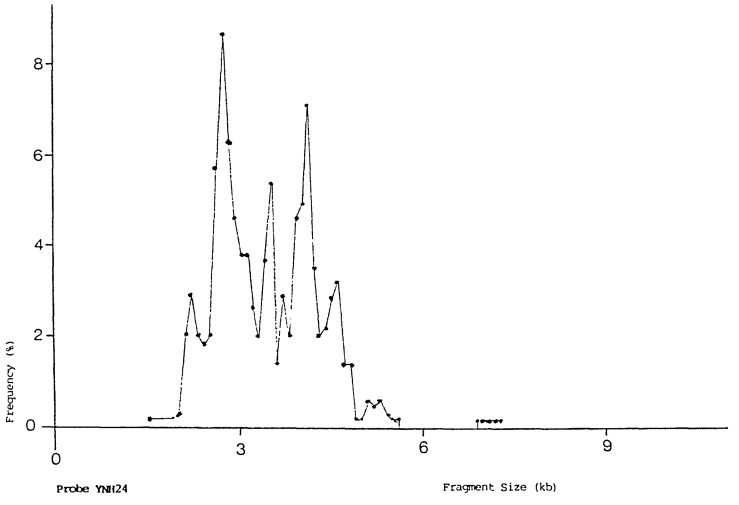

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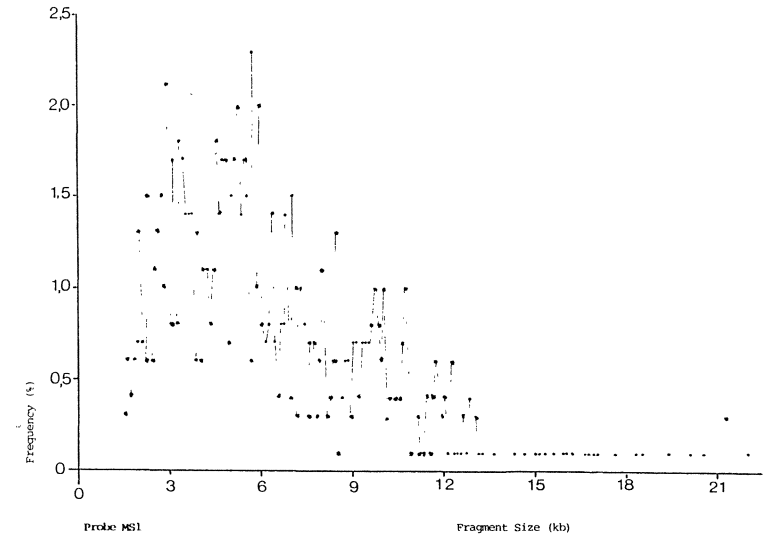

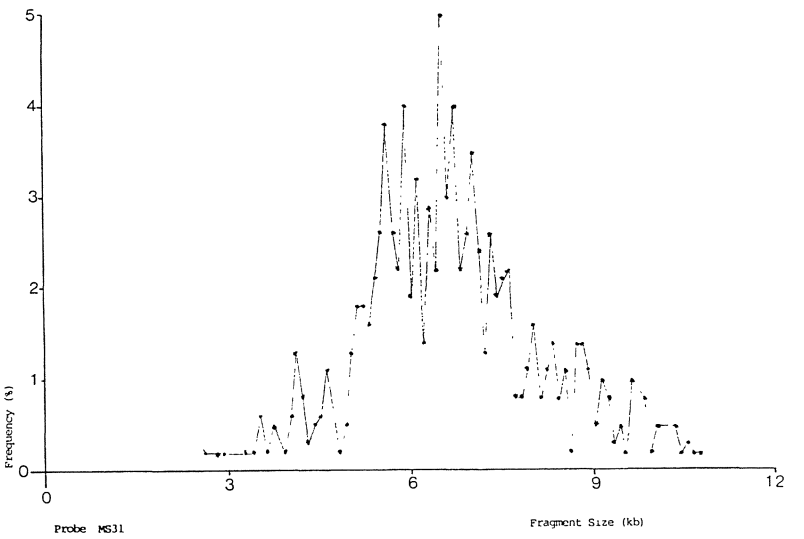

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Allele Frequency Distribution in Schleswig-Holstein for the DNA Probes MS1, MS31, MS43A, G3 and YNH24

E. Simeoni and E. Lange

Kiel, Germany

Introduction:

In cases of disputed paternity and in forensic science case work it is necessary to know whether the frequency distribution of the DNA alleles differs from other reported populations. Therefore we investigated blood samples of unrelated persons from Schleswig-Holstein. By using single locus minisatellite probes (SLP) the probability of paternity can be calculated according to ESSEN-MÖLLER. The German federal regulations say that the own frequencies should be used.

Material and Methods:

We investigated paternity cases and evaluated 355 unrelated persons.
DNA was prepared by proteolytic digestion of pelleted white blood cell nuclei, extracted by saline solution and ethanol precipitation.
5 µg DNA was digested with Hinf I (5 U/µg) and separated by electrophoresis in 0,7 % agarose gel (Pharmacia NA) (20 x 20 cm) at 30 V/33 mA for 24 hours.
The transfer was carried out by capillary blotting to a positive charged nylon membrane (Boehringer).
The membrane was hybridized with 0,6 µl/ml alkaline phosphatase labled MS43A probe (ICI). DNA fragments were detected with a chemiluminescent system CSPD (Tropix), weight marker NICE Ladder (Gibco BRL). Measurement was carried out by digitizer according to MUCHE.
The blot was stripped and hybridized with:
YNH24 (Promega 4 µl/ml), MS1 (ICI), MS31 (ICI) and G3 (ICI).

Results:

The frequency distributions of SLP fragments are similar to other published frequencies (HENKE et al, PFLUG et al, BUFFERY et al).
The reproducibility of measurement was confirmed by examining control DNA (K 562) on every gel. The calculation of fragment sizes is carried out using the next two marker lanes for which a result can be obtained.

References:

Buffery C, Burridge F, Greenhalgh M, Jones S and Willott G (1992) Frequency databases for the DNA probes MS1, MS31, MS43A, and YNH24, derived from caucasians, and afro-caribbeans in the London area.
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However, for SE33 the bimodal frequency distribution shows a marked shift of the high molecular weight alleles and we recognized a considerable number of new high frequency alleles in this range. We suppose that the enhanced electrophoretic separation that especially effects the high molecular weight alleles accounts for this phenomenon. Therefore this alignment is only speculative and sequencing studies are needed to establish a final nomenclature for SE33. For TC11, allele assignment was easily possible when comparing to the frequency values of other authors (Tab 2). Two new low frequency alleles (V04,V05) could be identified. However, we will still use our own nomenclature since we feel that a definitve nomenclature for any VNTR-marker should be based on sequencing data. Reamplifiable allelic cocktails, composed of alleles with known sequence as well as standardized electrophoretic separation conditions for each marker system are needed to unambigously type for VNTR loci. Under these prerequisites, we think that typing for STR loci by high resolution PAGE will become a reliable and effective method in forensic haemogenetics in the future.

Tab 2 TC11 Allele Frequencies

	AUSTRIA (n=206; this study)	FRG (n=110; Wiegand et al 1993)	USA (n=40; Edwards et al 1992)
V04	0.2	0.0	0.0
V05	0.2	0.0	0.0
V06	23.1	20.7	26.2
V07	16.3	18.0	8.8
V08	11.2	12.6	11.3
V09	19.2	17.1	16.2
V10	28.9	30.2	36.2
V11	1.0	1.4	1.3

Acknowledgements

The authors are grateful to B.Brinkmann and P.Wiegand for providing useful information.

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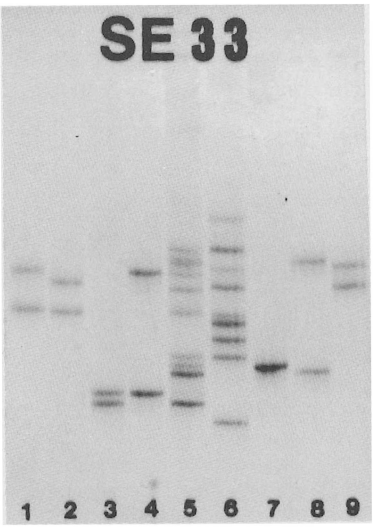
Tab 1 SE33 Allele Frequencies (n=200)

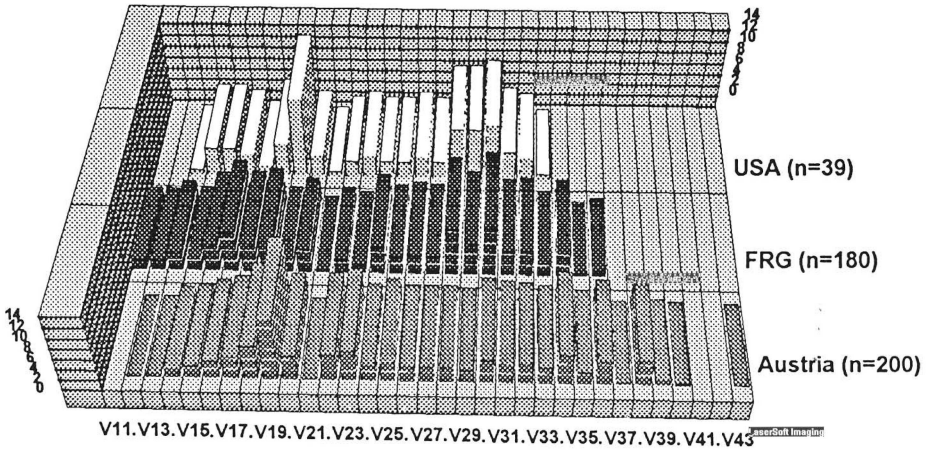
V11	0.0	V28	3.0
V12	0.2	V29	2.5
V13	0.2	V30	4.5
V14	2.0	V31	4.0
V15	2.5	V32	3.8
V16	3.5	V33	3.2
V17	4.3	V34	5.8
V18	6.5	V35	2.5
V19	11.2	V36	4.5
V20	4.7	V37	0.5
V21	0.2	V38	4.7
V22	5.0	V39	1.0
V23	5.8	V40	0.5
V24	3.0	V41	0.0
V25	4.0	V42	0.0
V26	3.0	V43	0.2
V27	3.0		

Fig 1 SE33 Typing by PAGE



Lane 5: Allelic Cocktail: V04, V07, V08, V09, V15, V19, V22, V24, V25, V27 Lane 6: Allelic Cocktail: V02, V09, V12, V14, V15, V20, V23, V27, V32 Lane 1: V16-V22, Lane 2: V15-V20, Lane 3: V04-V05, Lane 4: V05-V20, Lane 7: V08-V08, Lane 8: V07-V24, Lane 9: V19-V23

Fig 2 Comparison of SE33 allele frequencies from our study with data of Edwards et al (1992) and Wiegand et al (1993)



TYPING FOR STR-LOCI BY ELECTROPHORESIS ON REHYDRATABLE POLYACRYLAMIDE GELS: PHENOTYPE AND ALLELE FREQUENCIES OF SE33 AND TC11 IN AN AUSTRIAN POPULATION SAMPLE

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Introduction

Short tandem repeat (STR) microsatellite loci (Edwards et al 1991) are becoming increasingly important in forensic haemogenetics. Like the VNTR-AMPFLP microsatellite loci they represent highly polymorphic markers for genetic linkage studies, human identification and paternity testing and can be typed by direct high resolution polyacrylamide gel electrophoresis (PAGE). Because of their short overall size (100-500bp) STR loci may still be successfully amplified by PCR from highly degraded material (stains) when other methods fail to produce a typeable result. In order to establish procedures and references for reliable typing of the STR-loci SE33 (Polymeropoulos et al 1992) and TC11 (Edwards et al 1992) we optimized conditions for high resolution PAGE with subsequent silver staining of the amplification products and employed this method for typing a local population sample.

Material and methods

Samples

DNA from healthy unrelated individuals (Austrian blood donors; SE33: n=200, TC11: n=206) was extracted from peripheral blood samples by a salting out procedure (Miller et al 1988).

PCR, PAGE, siver stainig

Details on the amplification, electrophoresis and detection of the samples are described elsewhere in this issue(Schwartz et al: Simple and rapid Typing for VNTR polymorphisms using high resolution electrophoresis of PCR products on rehydratable polyacrylamide gels).

Allele assignment

In initial studies, the samples were compared to each other and to a molecular weight standard to identify the different alleles and to establish the optimal electrophoresis conditions. After that phase, allele cocktails (2 for SE33, 2 for TC11) were produced by coamplification of genomic DNA of selected individuals. In comparison to these allelic ladders all samples were typed using a provisional local nomenclature. After calculation of allele frequencies this nomenclature was aligned with the nomenclature suggested by Wiegand et al (1993) for SE33 and Edwards et al (1992) for TC11. Until confirmation we will use a capital V (Vienna) to indicate that direct comparison is still lacking.

Statistics

For both systems the heterozygosity index (HI) and the mean exclusion chance (MEC, Krueger et al 1968) was calculated. For testing for Hardy-Weinberg equilibrium, alleles had to be grouped together because of the small sample size and the high number of possible genotypes.

Results and discussion

Under optimized conditions, 30 alleles for SE33 and 8 alleles for TC11 were readily distinguishable. The mean range of separation between the smallest and the longest observed alleles was 23mm for SE33 (Fig.1) and 8mm for TC11. Allele frequencies obtained from our study are listed in Tab 1 and Tab 2. The phenotype frequencies (data not shown) showed good agreement with Hardy-Weinberg-expectations when using the approach of grouping alleles together in order to obtain expected phenotype frequency values of n>5. This method was repeated several times with different allelic groups and no siginificant deviation was observed. The Heterozygositiy Index was 94.8% for SE33 and 78.8% for TC11. The calculated MEC values give a combined exclusion chance of 97.7% (94.5% for SE33, 58.3% for TC11). The distribution of alleles was compared to the results of other workers after provisional alignment of the alleles as described and showed good overall agreement (Fig.2,Tab.2)

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Tab 1 Allele Frequencies (%) for 4 VNTR Marker loci

ApoB (n=201)		COL2A1 (n=204)		D1S80 (n=200)		YNZ22 (n=206)	
27	0.7	01	1.0	V16	0.0	01	6.3
28	0.5	02	9.3	V17	0.0	02	13.1
29	3.5	03	0.0	V17M	0.0	03	13.8
30	0.5	04	1.5	V18	23.1	04	30.8
31	7.7	05	12.0	V18M	0.2	05	2.9
32	1.2	06F	3.2	V19	0.9	06	3.6
33	0.7	06S	24.8	V20	3.7	07	1.9
34	0.2	07F	9.3	V21	0.2	08	4.1
35	25.4	07S	3.4	V21M	0.9	09	8.0
36	0.7	08	30.4	V22F	4.2	10	8.7
37	33.8	09	3.9	V22S	0.4	11	2.7
38	0.0	10	1.2	V23	1.1	12	1.9
39	3.5	11	0.0	V23M	0.0	13	1.2
40	0.5			V24	35.5	14	0.5
41	2.2			V24M	0.0	15	0.2
42	0.5			V25	4.4		
43	0.0			V25M	0.0		
44	0.5			V26	1.3		
45	0.2			V26M	0.2		
46	0.5			V27	0.7		
47	6.0			V28	6.4		
48	1.2			V28M1	0.2		
49	8.2			V28M2	0.2		
50	0.0			V29	4.0		
51	1.0			V30	1.5		
52	0.0			V31	7.9		
53	0.5			V32	0.2		
				V33	0.2		
				V34	0.0		
				V35	0.0		
				V36	1.3		
				V37	0.7		
				V38	0.0		
				V39	0.0		
				V40	0.2		
				V41	0.0		
				V42	0.2		

Tab 2 Heterozygosity Index (HI) and Mean Exclusion Chance (MEC) for 4 VNTR marker loci

	ApoB	COL2A1	D1S80	YNZ22
HI	80.1%	81.0%	80.3%	84.1%
MEC	64.0%	64.7%	63.9%	76.5%

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provisionally designated V22F and V22S. No significant deviations from Hardy-Weinberg equilibrium were observed for any locus when using the approach of grouping together alleles to obtain expected phenotype frequency values of n>5. Then Heterozygosity Indices and the Mean Exclusion Chances are listed in Tab 2. The Combined Exclusion Chance for all 4 systems was calculated to 0.9892 which is of course sufficiently high enough to suggest the only use of these 4 VNTR AMPLFP systems for routine forensic paternity and identity testing. However, there is still a lot of work to be done (typing of larger population samples of different races, segregation studies, sequencing of alleles) until VNTR-AMPLFP markers can completely replace conventional marker systems.

Acknowledgements
The authors are grateful to B.Brinkmann and P.Wiegand for providing allelic cocktails for D1S80, YNZ22 and for other useful information.

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PHENOTYPE AND ALLELE FREQUENCIES OF 4 VNTR-AMPFLP´S IN AN AUSTRIAN POPULATION SAMPLE

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Introduction

Microsatellite loci (VNTR, Variable Number of Tandem Repeats), a highly polymorphic system of non-coding marker loci dispersed throughout the whole human genome with constant rates were introduced to paternity and identity testing by Jeffreys et al (1988). Initially, typing has been carried out by Restriction Fragment Length Polymorphism (RFLP). Since this method has its limitaions and moreover, is time-consuming and difficult to perform and to analyze, typing by Amplification Fragment Length Polymorphism (AMPFLP, Budowle et al 1991) represents a major improvement to the typing of VNTR-loci. Since this is a simple and reliable technique and sufficently high exclusion chances can easily be achieved, it will presumably replace conventional systems for paternity and identity testing in the near future. Population and family studies that are necessary to select and to introduce new marker systems to forensic haemogenetics have already been performed by a number of workers(see Lit.). In this paper, we present the allele and phenotype frequencies of an Austrian population sample typed for the VNTR-loci ApoB, COL2A1, D1S80 and YNZ22.

Material and methods

Samples

DNA from healthy unrelated individuals (Austrian blood donors; ApoB: n=201, COL2A1: n=204, D1S80: n=200 YNZ22: n=206) was extracted from peripheral blood samples by a salting out procedure (Miller et al 1988).

PCR, PAGE, siver stainig

Details on the amplification, electrophoresis and detection of the samples are described elsewhere in this issue (Schwartz et al: Simple and rapid Typing for VNTR polymorphisms using high resolution electrophoresis of PCR products on rehydratable polyacrylamide gels).

Allele assignment

For D1S80 and YNZ22 allele assignment was achieved by direct comparison to allelic cocktails provided by B. Brinkmann and P. Wiegand. For COL2A1 and ApoB the samples were compared to each other and to a molecular weight standard first to identify different alleles and to establish a provisional nomenclature which was converted to the nomenclature published by other authors after all observed alleles were provisionaly assigned and allele frequencies were known. For D1S80, we decided to use still a local provisional nomenclature because of the high number of interalleles observed. This nomenclature just uses a capital V (Vienna) to assign provisional status.

Statistics

For both systems the heterozygosity index (HI) and the mean exclusion chance (MEC, Krueger et al 1968) was calculated. For testing for Hardy-Weinberg equilibrium, alleles had to be grouped together because of the small sample size and the high number of possible genotypes.

Results and discussion

With all systems, typing was easily possible after optimizing the electrophoretic separation protocol. The allele frequencies are listed in Tab 1. The allele assignment was based on direct comparison with allelic cocktails from other workers (Wiegand and Brinkmann) for D1S80 and YNZ22 and on comparison of the obtained with published allele frequencies for ApoB and COL2A1. Comparison of allele frequencies showed similar distribution for all systems and populations studied, taking into account the high number of individual alleles and the relatively small sample sizes. New alleles could be identified for D1S80 (n=5), ApoB(n=9) and YNZ22 (n=3). Furthermore, we observed two distinct variants of the D1S80-22 allele which were

5. SEQUENCE ANALYSIS AND STABILITY OF THE VARIANTS

Allelic sequences at FABP and THO1 loci were analysed to determine if there was sequence microheterogeneity within the length defined alleles.

FABP

Representative samples of FABP*10 (n=7) and FABP* 13 (n=6) were sequenced.
In every case no variation is seen in the repeat units nor in the flanking sequences. As noted elsewhere the individual exhibiting each of the three alleles was sequenced. The sequences there also identical, variation only observed in number of repeats.

THO1

Sequence analysis of the *10-1 allele in caucasians (n=4) shows a deletion of a T in a core repeat unit. The sequences of the *9 and *10-1 *11 alleles can be represented thusly:

*9 *****$[CATT]_9$*****
*10-1 *****$[CATT]_3$CAT $[CATT]_6$*****
*11 *****$[CATT]_{11}$*****

Sequence analysis of the 10-1 allele in Maoris (n=4) shows the same base deletion at the same site. Thus the *10-1 allele in caucasians and Maoris is identical at the sequence level.

6. IMPLICATIONS FOR ALLELIC STABILITY AT THO1

The common sequence of the *10-1 allele in caucasians and Maoris indicates that a mutation giving rise to its existence predates the splitting of these two populations. Moreover these sequence data provide evidence of allelic stability at the THO1 locus. Genetic variation at STR loci is believed to be generated by sequence slippage during strand replication in meiosis and or unequal crossing over. Both processes would be expected to yield x-1 variants of the other alleles, *10-1 alleles with the deletion site in different repeat units, and a true *10 allele.

That none of these products were observed in this diverse population sampling indicates that neither process occurs at significant frequency. This observation is consistent with the mutation rate estimated by Edward *et al* of 3.5 x 10^{-5}[4]. It is to be expected that as the number of individuals tested at the THO1 locus increases, that some of these variants may appear at low frequency. When observed these rare variants can be used to estimate the age of the THO1 polymophisim.

7. CONCLUSIONS

1. All three New Zealand populations are in Hardy-Weinberg Equilibrium at both loci.
2. The heterozygosity levels found are comparable with other reported populations.
3. The sequence deletion of the *10-1 repeat common to caucasian and Maori indicates an ancient origin of the allele.
4. Non templated single base addition can be removed by Klenow at certain loci.

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The THO1 patterns show single bands for each allele. Each allelic band sizes one base longer than the nominal allele size based on sequence; the addition of an extra base at the end of each PCR product appears to be virtually complete. All "THO1*10" alleles sized as *10-1; no true THO1*10s were observed.

4. POPULATION STUDIES

Table 1 **FABP ALLELE FREQUENCIES (%)**

Allele	NZCau	Am-Ind	Maori	Samoa
8	-	-	-	-
9	-	2	1	4
10	50	55	47	67
11	12	8	3	3
12	5	-	2	-
13	27	29	28	27
14	6	8	19	-
15	-	-	-	-
N	47	49	57	51
Het (%)	62	59	60	47
DP (ca%)	78	76	77	67

Table 2 **THO1 ALLELE FREQUENCIES (%)**

Allele	NZCau	Maori	Samoa
5	-	-	-
6	25	25	6
7	23	27	47
8	10	7	18
9	9	13	15
10-1	34	28	15
11	-	-	-
12	-	-	-
N	46	69	34
Het (%)	83	71	71
DP (ca%)	94	85	85

There was no evidence of interloci allelic association.

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STUDIES ON THE HUMFABP AND HUMTHO1 POLYMORPHISMS

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1. INTRODUCTION

Genetic variation at loci containing short tandem repeats (STR) have been demonstrated to be highly informative for purposes of human identification and for the study of population structure [1, 2]. We describe here studies on two STR loci and their expression in three New Zealand populations.

HUMFABP is the gene for human intestinal fatty acid binding protein [3]; it is located on chromosome 4 at q31. STR variation is found in an ATT trinucleotide repeat at the 3' end of intron 2. Eight alleles, defined by sequences containing 8-15 repeats, were identified in the survey by Edwards et al of four US populations (Euro-americans, Afro-americans, Mexican-americans, and Asian-americans).

HUMTHO1 is the gene for human tyrosine hydroxylase [4]; it is located on chromosome 11 at p15. STR variation is found in a CATT tetranucleotide repeat in intron 1. The Edwards survey identified eight alleles defined by sequences contain 5-12 repeats. One of these alleles is anomolous: the THO1*10 allele contains a single base deletion in one of the repeat units and is referred to as the 10-1 allele.

FABP and/or THO1 typing has been performed on over 250 individuals representing US and New Zealand populations. The three New Zealand populations studied are:

Maori The original peoples of new Zealand; samples were collected from unrelated individuals from North and South island.

Samoan The original peoples of the Samoan islands (Polynesia) located approximately 3000 km northeast of New Zealand. Samples were collected from Samoans residing in New Zealand.

Caucasian Migrants to New Zealand from Europe, mostly of British extraction. Samples were collected from unrelated individuals in the Christchurch area of the South Island.

2. METHODS

The amplification and typing conditions used were those defined by Edwards et al [1,2] with the exception of one primer being end labelled and amplification products run on 5% M13 sequencing gels. Allelic Bands were revealed with autoradiography.

3. STR TYPING RESULTS

The use of the M13 sequence as a sizing ladder allows allelic band sizes to be assigned with single base resolution; any deviations in allele size are readily noted. Under the electrophoretic conditions used, no differences in allele band mobility were noted between PCR products end-labelled on either primer.

FABP typing patterns typically show a major and minor band for each allele; the two bands differ in length by a single base. The absolute band sizes indicate that the larger band is one base longer than the expected sequence; we demonstrated this experimentally. The double banding does not introduce ambiguity into typing although it can confound the typing of mixtures as might be encountered in case samples.

We have encountered one individual, an Hispanic , who appears to possess three FABP alleles; the nominal type would be 10, 13, 14. The sequences of each of the allelic PCR products have been determined. The sequences of the flanking regions in each are identical and the STR regions differ only in repeat number. This is consistent with a gene duplication on one chromosome.

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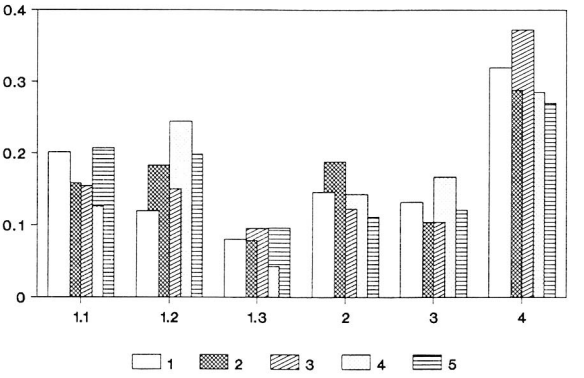
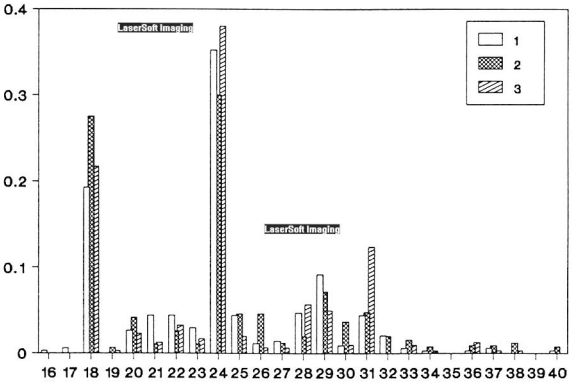
FIG. 1.- COMPARISON BETWEEN CAUCASIAN POPULATIONS: DQA1



1: Andalusia, Spain.
2: Spain (region unspecified), Lorente et al., 1992.
3: Catalonia, Spain, Gene et al., 1992.
4: USA, McClure and Schart, 1991.
5: Holland, Kloosterman et al., 1992.

FIG. 2.- COMPARISON BETWEEN CAUCASIAN POPULATIONS: D1S80



1: Andalusia, Spain.
2: USA, Cetus Corporation, 1990.
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RESULTS

TABLE I DQA1 ALLELE FREQUENCIES

ALLELE	FREQUENCY	95% LCL	95% UCL
1.1	0.2011	0.1590	0.2432
1.2	0.1207	0.0864	0.1550
1.3	0.0805	0.0519	0.1091
2	0.1466	0.1094	0.1838
3	0.1322	0.0965	0.1679
4	0.3190	0.1655	0.4725

N = 174 individuals
H-W equilibrium (15 degrees of freedom) $X^2 = 15.9912$
Homoqygosity (n = 36) : 0.207
Heterozygosity (n = 138) : 0.793
Expected heterozygosity : 0.800 ± 0.095
Power of discrimination = 0.984
All but 1.2, 1.2 genotype were encountered in the population sample.

TABLE II D1S80 ALLELE FREQUENCIES

ALLELE	FREQUENCY	95% UCL
16	0.0030	0.0089
17	0.0059	0.0142
18	0.1923	0.2352
20	0.0266	0.0441
21	0.0444	0.0668
22	0.0444	0.0668
23	0.0296	0.0480
24	0.3521	0.4041
25	0.0444	0.0668
26	0.0118	0.0236
27	0.0148	0.0279
28	0.0473	0.0704
29	0.0917	0.1231
30	0.0089	0.0191
31	0.0414	0.0631
32	0.0207	0.0362
33	0.0059	0.0142
34	0.0030	0.0089
36	0.0030	0.0089
37	0.0059	0.0142
40	0.0030	0.0089

N = 166 individuals
H-W equilibrium (36 degrees of freedom) $X^2 = 201.9817$
Homoqygosity (n = 30) : 0.177
Heterozygosity (n = 139) : 0.822
Expected heterozygosity : 0.820 ± 0.0008
Power of discrimination = 0.972
57 different genotypes and 21 alleles were observed in the population sample.

According to the results of statistic calculations, a divergence of genotype frequencies from Hardy-Weinberg expectation was found ($X^2 = 201.9817$ is much higher than tabulated value for 36 freedom degrees). This anomaly can be due to the high polymorphism of the population which probably requires a larger sample set, or another statistic strategy to be undertaken for calculations. Nevertheless Hardy-Weinberg equilibrium can be accepted in a population which is known to be at equilibrium for many other genetic characters (Bertranpetit, 1993, personal communication).

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HLA DQA1 AND D1S80 POLYMORPHISMS IN THE POPULATION OF ANDALUSIA (SOUTHERN SPAIN).

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INTRODUCTION

We report here the results of a survey of HLA DQA1 and D1S80 polymorphisms studied by the polymerase chain reaction (PCR) in blood samples from people living in Andalusia (Southern Spain). A population data base for both loci has been established for purposes of forensic identity testing.

MATERIAL AND METHODS

Population Sample: Blood was obtained from 174 unrelated Caucasian individuals living in our region, selected from casework.
Samples of 3 μL liquid blood were deposited on clean sterile cotton tissue (20x20 mm) in a Petri dish, dried at room temperature. Once dried, cotton pieces were wrapped in aluminium foil and stored at -20°C until use.

DNA extraction: DNA for PCR analysis was isolated from blood stains by chelating resin extraction (Imidoacetic Acid, Sigma) according to Cetus Corporation technique (AmpliType User Guide, Version 2).
Extracted DNA was quantified spectrofluorometrically in a DNA fluorometer, TKO, Heofer Scientific Instruments.
Fluorescence reagent: Bis-benzimide (Hoescht 33258, Sigma).
Amplification and typing: Perkin Elmer Cetus DNA Thermal Cycler 480
DQA1: Amplitype HLA DQA1 Forensic DNA Amplification and Typing kit (Perkin Elmer). PCR product was detected by hybridisation with allele specific oligonucleotide probes
D1S80: The D1S80 DNA Amplification Reagent Set (Perkin Elmer) was used, PCR product was detected by flat bed SDS-PAGE followed by silver staining (gel: 8-18% SDS PAA; gel buffer: 0.12 M Tris-Acetate pH 6.4; electrode buffers: anode: 0.3 M Tris-Acetate,4 g/L SDS, pH 6.4, cathode: 0.08 M Tris, 0.8 M Tricine, 4 g/L SDS, pH 7.1; Electrophoretic conditions: 400V, 100 mA, 30 W, 50-55 min).

STATISTICS
- Allele frequencies: calculated from numbers of each genotype in the sample set.
- Expected numbers of genotype frequencies were calculated under the assumption of Hardy-Weinberg expectation and possible divergences calculated by standard Chi-square test.
- Heterocygote frecuency (=allele diversity): (Nei and Roychoudhury, 1974)
$h = n\,[1 - \Sigma(n_i/n)^2]\,/(n-1)$, where: n_1, n_2 ... n_i = allele counts, n = number of chromosomes; Standard error: $\sqrt{h\,(1-h)/N}$, where h = expected heterozygote frequency, N = number of individuals.
- Lower and upper intervals of 95% confidence (LCL, UCL) for individual allele and genotype frequencies data were calculated taking into account a normal distribution or exactly calculated assuming a binomial distribution, depending on n_i values. (Domenech-Massons, 1990).
- Comparison between the different Caucasian populations was performed by pairwise comparisons in the Chi-squared test of homogeneity.
- Power of discrimination: $1 - \Sigma\,(P_i)^2$ (P_i = frequency of each genotype).

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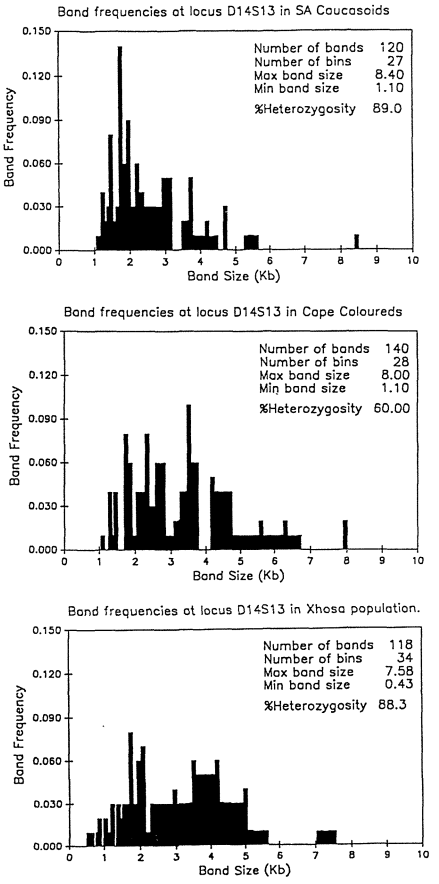
Distribution of D14S13 alleles in the South African Caucasoid, Cape Coloured and Xhosa populations of Southern Africa.



RESULTS & DISCUSSION

These ethnic groups demonstrate differences in their allele distributions as may be seen from the histograms. The size range for all the population groups studied was between 0.7kb and 9 kb and no single allelic form was present in an exceptionally high frequency although the most common allelic species differed in the populations as shown in Table 1.

The allele size range and frequency distribution of the four populations appeared similar at the D2S44 locus. At the D10S28 locus the range of allele sizes was greater in the Xhosas than in the Caucasoids with the Cape Coloureds midway between the two. The size range and distribution of alleles of the Khoi-San was similar to that of the Caucasoids. At the D14S13 locus the allele size range was similar for the three populations studied but the distribution was not, with the Xhosas having larger fragments whereas the Caucasoids had a preponderance of smaller sized fragments. As expected, the size range and frequency distribution of the fragments observed in the Cape Coloureds at this locus reflected features of both the South African Caucasoid and Xhosa populations.

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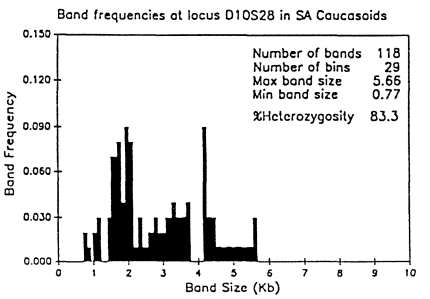
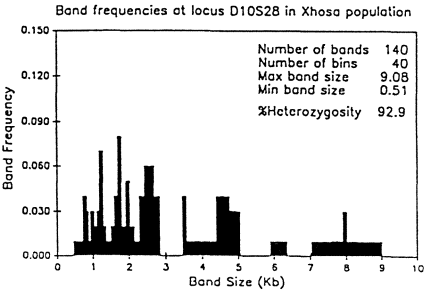
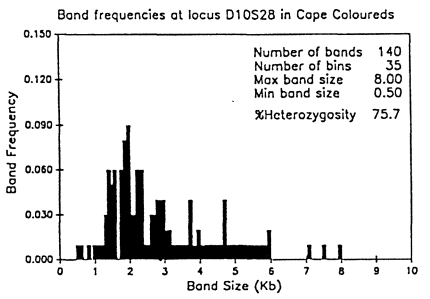
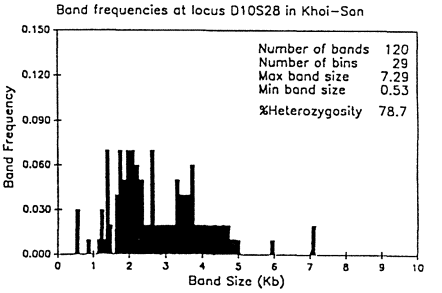
Distribution of D10S28 alleles in the South African Caucasoid, Cape Coloured, Khoi-San and Xhosa populations of Southern Africa.

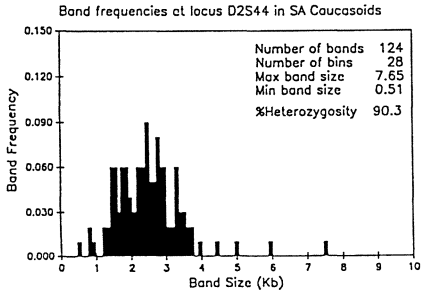


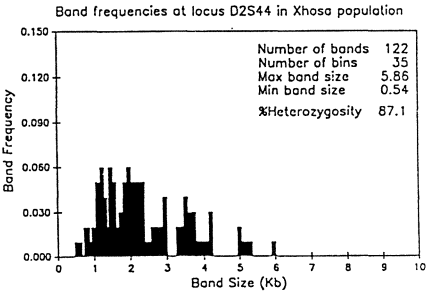


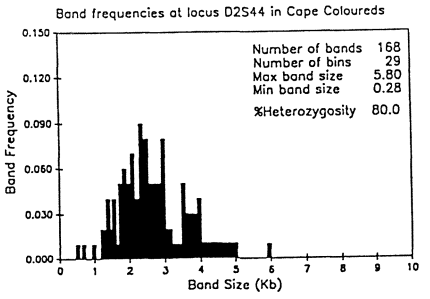


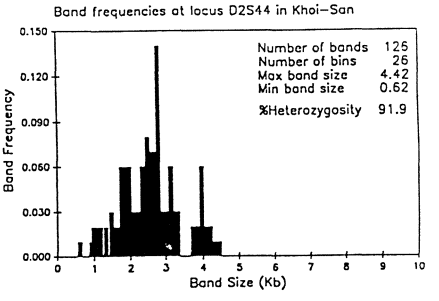


Distribution of D2S44, alleles in the South African Caucasoid, Cape Coloured, Khoi-San and Xhosa populations of Southern Africa.









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ALLELE FREQUENCIES AT THE D2S44, D10S28 AND D14S13 LOCI IN FOUR SOUTHERN AFRICAN POPULATIONS

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Polymorphic DNA arising from variations in the number of short tandem repeats accounts for many of the hypervariable regions detected in the human genome (Nakumura et. al., 1987). For this preliminary study we have analysed 3 such loci and have determined the size range and frequency distribution of the VNTR alleles in the South African Caucasoid, Cape Coloured, Xhosa and Khoi-San populations.

The South African Caucasoids originate from a small genetic pool of Dutch, German and French ancestors with an admixture of approximately 7% Southern African and Asian genes (Botha et al., 1972). The Cape Coloureds are an anthropologically distinct population group possessing Western European, Southern African and Asian genes in significantly different proportions from those seen in South African Caucasoids. The Xhosa group are representative of the Nguni-speaking tribes. The Khoi-San are descended from people of the latter Stone Age and reside in Namibia.

METHODS

Genomic DNA was prepared from peripheral blood samples by the salting-out method (Miller et. al., 1988). The DNA was digested with the restriction enzyme Hae III, Southern blotted and hybridised to the VNTR probes YNH24 (D2S44), TBQ7 (D10S28) and CMM101 (D14S13) (Promega). A previously standardised protocol was applied (Reavis et.al., 1992) and, in order to obtain reliable band frequencies the data were analysed using a digitised video-image system developed in this laboratory. Allele sizes (kb) were calculated from a curve fit (least squares method) of band size (kb) versus log [mobility]. Binning databases (dBaseIV, Borland) for each population group were created from which the histograms of the allele distributions were generated. A fixed-bin approach was used in which the bin increments were 6%.

Table 1. Predominant allelic species seen in the different populations

	Genetic Locus		
Population Group	D2S44	D10S28	D14S13
Caucasoid	2.41 - 2.56	1.91 - 2.02	1.70 - 1.80
Cape Coloured	2.27 - 2.41	1.91 - 2.02	3.42 - 3.63
Khoi-San	2.71 - 2.87	2.02 - 2.15	Not done
Xhosa	1.91 - 2.02	1.70 - 1.80	1.70 - 1.80

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Discussion

The PCR has provided a simple and rapid method for studying many polymorphisms, including VNTR. Since optimized PCR-based protocols readily detect one or two bands in DNA samples from homozygotes and heterozygotes, the allele frequency estimates were obtained simply by gene counting. Thus, the excess of single-banded phenotypes observed at the locus D1S80 should be regarded as indicating an excess of homozygotes in our sample of Tuscans. As a likely explanation it should be considered that the expected number of heterozygotes in the HWE hypothesis depends on observed allelic frequencies with relatively high sampling errors. On the other hand, the highly significant difference between the allele frequency distribution of D1S80 and that of some Caucasian databases (Budowle et al. 1991; Spinella, 1993) is apparently due to the presence of different "private" alleles in the different samples. However, it could be put forward that comparisons of data gathered through different methodologies may be misleading. For instance, the use of either ultra-thin horizontal or standard vertical PAGE vs. agarose gel analysis could have introduced a serious bias in the allocation of individual alleles at the 16-bp VNTR locus D1S80. It seems to us that previous PCR databases of VNTR loci reflected the binning attitude of the RFLP era. Moreover, the development of allelic ladders has undoubtedly improved the resolution of discrete alleles at the D1S80 locus. These two latter considerations may help to explain why the number of alleles is lower in early works.

The results of the present study provide an estimate of genic diversity in a well defined population. Other regionally founded VNTR databases could be useful in assessing the extent of population substructure.

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Results

Allele frequency distributions and data analysis are shown in Figure 1 and in Table 1, respectively.

D17S30. Twelve different alleles were observed in 100 unrelated Tuscans. The length of the alleles ranged from 160 to 930 bp. Their designation #1 through #12 reflects the number of repeats.

Out of 78 possible genotypes, given 12 observed alleles, 37 were detected in 100 individuals. Genotypic frequencies were consistent with the assumptions of the HWE. Allelic counts were homogeneous to those found in a Caucasian sample reported by Batanian et al. (1990). The marginal departure from HWE in the heterozygosity test accounts for the slight heterozygote excess found at this locus.

D1S80. Twenty-four different alleles were found in 99 DNA samples (one less than those originally included in the present study, because of an accidental loss before amplification).

The length of the alleles ranged from 387 to 803 bp. Their designation #15 through #41 reflects the number of repeats. The frequency distribution of the D1S80 alleles is multimodal: the most common allele is #24; two additional modes are located approximately 6 repeat units both left- and rightward; a much less frequent mode could also be present 6 repeat units further.

A large excess of single-banded phenotypes is present as judged by the heterozygosity test ($p<10^{-6}$). However, the genotypic distribution was in accordance with HWE expectations. Heterogeneity test was higly significant when comparing the allele frequencies with published Caucasian databases (e.g., Budowle et al. 1991) or with Italian data (Spinella, 1993).

Table 1. Synopsis of the statistical approaches

		D1S80 (n=99)	D17S30 (n=100)
TESTS FOR HWE			
GENOTYPIC DISTRIBUTION			
Standard pooling strategy[a]	X^2	2.54	6.18
	d.f.	3	8
	p	<0.5	<0.75
Chi-squared with small numbers (Smith, 1986)	*z*	1.47	0.38
	p	<0.07	<0.34
HETEROZYGOSITY TEST	obs	71	91
	exp	90.2	83.8
	X^2	45.65	3.86
	p	$<10^{-6}$	<0.05
ALLELIC DISTRIBUTION			
Heterogeneity test[b] (Smith, 1986)	*z*	7.63	0.67
	p	<0.001	<0.25
COMPARISONS BASED ON MUTATION-DRIFT MODELS			
E(*h*) = expected heterozygosity		0.92	0.85
k = observed number of alleles		24	12
E(*k*) (Ewens, 1972)		32.5	20.7

[a] Genotypes with expectations less than 5 were pooled.

[b] Comparisons were made with data from Budowle et al. (1991) for D1S80 and from Batanian et al. (1990) for D17S30.

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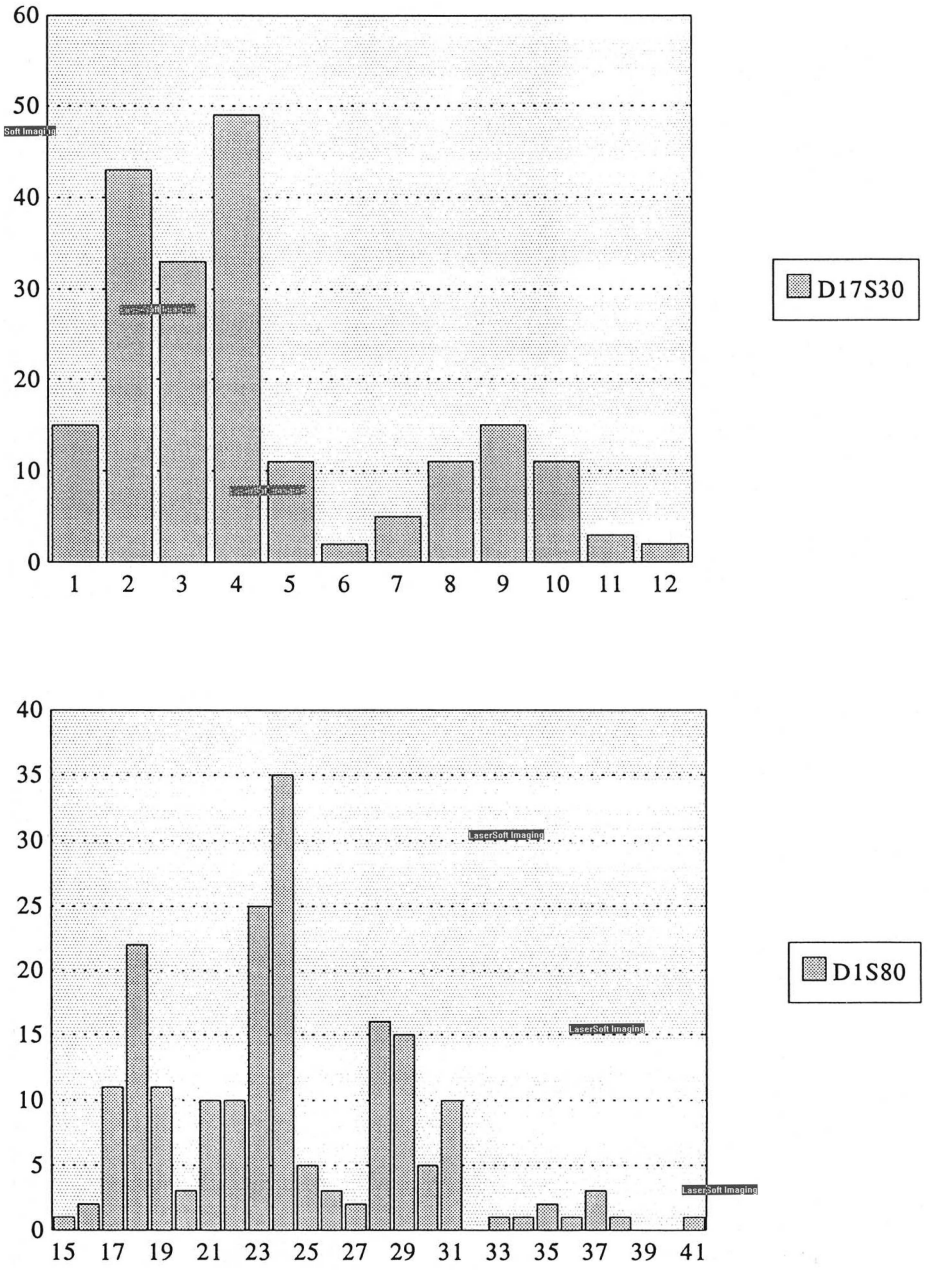

Figure 1. Frequency distribution of D17S30 and D1S80 alleles.

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GENETIC SUBSTRUCTURE OF THE ITALIAN POPULATION AT THE VNTR LOCI D1S80 AND D17S30: THE TUSCAN REGION

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Introduction

Hypervariable loci within the human genome are useful tools in several disciplines. Because of their high informational content, they resurrected genetic linkage studies in families (Nakamura et al. 1987) and served extensively in forensic science (Reynolds et al. 1991). It has been estimated that each human genome is heterozygous at $3x10^6$ nucleotide sites. Consequently, it is theoretically possible the unique identification of individuals from their DNA.

However, data concerning the extent of ethnic variation at such highly polymorphic loci as VNTR is still limited. In fact, ethnic groups defined on the basis of geography or national boundaries can still harbor substantial genetic variation even among their subpopulations (Lewontin and Hartl, 1992).

The present study describes the population genetic characteristics of the D1S80 and the D17S30 VNTR loci in a well defined population. We performed PCR-based DNA typing to address whether the genotype distribution at these loci were in accordance with their Hardy-Weinberg equilibrium (HWE) expectations. Several approaches were pursued and compared one each other to evaluate statistically our sample of Tuscan ancestry. Moreover, the allele frequency distributions were compared with published Caucasian databases to assess the presence of population substructure.

Materials and Methods

Subjects. DNA samples from 100 unrelated individuals were tested. They were residents of the Florence area and all their four grand-parents were born in the Tuscan region.

PCR conditions (GeneAmp PCR System 9600).

Locus	D1S80	D17S30
$MgCl_2$	1.5mM	1.2mM
Input DNA	100ng	10ng
Cycles	25	30
Denaturation	20s 94°C	20s 94°C
Annealing	20s 67°C	20s 56°C
Extension	30s 72°C	60s 72°C

Electrophoresis. PCR products of the D1S80 locus were run by vertical PAGE (8% T, 3.3% C, 1mm thick) and stained with ethidium bromide. Alleles at the D17S30 locus were studied after 2% agarose gel electrophoresis and ethidium bromide staining.

Statistics. Tests for HWE from the genotype distributions were carried out by computing the near normally distributed variable z defined as the deviation of $V\bar{x}^2$ from its expected value m divided by its standard error $V\bar{v}$ (Smith, 1986). The same method was also applied to a contingency table to test for heterogeneity between the allele distributions of our Tuscan population and those of published Caucasian databases.

Additional tests for HWE were conducted by using the summary statistics total heterozygosity as well as by studying the congruence between the observed and expected levels of heterozygosity and number of alleles (Chakraborty et al. 1988).

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The most frequent alleles are the 18 and the 24 repeats with the same frequency (0.3084). All other alleles have frequencies less than 0.1 . Table 2 gives the distribution of the observed and expected genotypes found in our population. In total 60 genotypes were observed.
The frequency distribution of the alleles in the North of Portugal is slightly different to that found in other Caucasian populations (Budowle et al, 1991; Thyman et al, 1993;Schnee-Griese et al, 1993), even when compared with very close geographical populations such as NW Spain and Central Portugal (Lareu et al, in press). In our population the allelic frequencies of the two most common alleles are equal, while in those there is a prevalence of allele 24.
The theoretical a priori chance exclusion value (C.E.) is 0.6174. From genotype data, the power of discrimination (P.D.) according to the equation suggested by Fisher (1951) is 0.9327.

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There were no differences in results obtained by the two typing methods used in this work.

Table 1. Frequencies of the D1S80 alleles found in the population of the North of Portugal

Allele	Frequency	Allele	Frequency
14	0.0022	26	0.0088
17	0.0066	27	0.0088
18	0.3084	28	0.0374
19	0.0088	29	0.0881
20	0.0176	30	0.0066
21	0.0264	31	0.0573
22	0.0352	32	0.0110
23	0.0066	34	0.0066
24	0.3084	36	0.0154
25	0.0308	37	0.0088

Total 20

As there are many alleles and thus a great number of genotypes, for chi-square calculation the alleles were pooled into five groups (Budowle et al, 1991). We found that there was no significant deviation from the Hardy-Weinberg equilibrium ($x^2 = 4.4736$; df = 10; $0.90 < P < 0.95$).

Table 2. Distribution of observed and expected D1S80 genotypes from 227 individuals

Genotype	Observed	Expected	Genotype	Observed	Expected	Genotype	Observed	Expected
14-18	1	0.3084	19-19	1	0.1762	24-30	1	0.9251
17-20	1	0.0528	20-21	1	0.2114	24-31	7	8.0176
17-24	1	0.9251	20-24	3	2.4670	24-32	1	1.5418
17-34	1	0.0198	20-29	2	0.7048	24-34	1	0.9251
18-18	21	21.5859	21-21	1	0.1586	24-36	1	2.1586
18-19	2	1.2335	21-24	3	3.7004	24-37	1	1.2335
18-20	1	2.4669	21-29	1	1.0573	25-28	2	0.5242
18-21	4	3.7004	21-31	1	0.6872	25-29	2	1.2335
18-22	4	4.9339	22-22	1	0.2819	25-31	3	0.8018
18-23	1	0.9251	22-24	6	4.9339	26-31	1	0.2291
18-24	48	43.1718	22-28	1	0.5991	26-37	1	0.0352
18-25	1	4.3172	22-31	1	0.9163	27-29	1	0.3524
18-27	1	1.2335	22-36	2	0.2467	28-29	3	1.4978
18-28	8	5.2423	23-31	2	0.1718	28-31	1	0.9736
18-29	13	12.3348	24-24	21	21.5859	29-31	1	2.2907
18-30	1	0.9251	24-25	6	4.3172	29-32	1	0.4405
18-31	8	8.0176	24-26	2	1.2335	29-36	2	0.6167
18-32	3	1.5412	24-27	2	1.2335	29-37	1	0.3524
18-34	1	0.9251	24-28	2	5.2423	30-36	1	0.0625
18-37	1	1.2335	24-29	13	12.3348	31-36	1	0.4009

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STUDY OF D1S80 LOCUS POLYMORPHISM IN THE NORTH OF PORTUGAL

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INTRODUCTION

The Polymerase Chain Reaction (PCR, Saiki et al, 1985) can enhance the detection and offers a viable alternative to RFLP (restriction fragment lenght polymorphism) analysis of known VNTR loci (Budowle et al, 1991) particularly in forensic situations where limited amounts of DNA are available.
The Locus D1S80, a VNTR locus, is a very useful genetic marker system for forensic DNA analysis. The polymorphism has been localized in the short arm of chromosome 1 and consists of VNTR repeats each of them about 16 bp long (Nakamura et al, 1988; Kasai et al, 1990). This work presents data for the locus D1S80 from the North of Portugal.

MATERIAL AND METHODS

Blood samples were collected from 227 unrelated individuals from paternity tests. DNA was extracted by a method previously described by Singer-Sam and col. (1989) using a chelating resin. Some of the samples were extracted using a phenol - chloroform procedure.
Amplification of D1S80 (pMC118) was achieved using the primers described by Kasai et al (1990). The primers were obtained from Cetus and used under conditions recommended by the manufacturer. Amplification was done by the method described by Budwole et al (1990, 1991) with slight modifications, according to Cetus recommendations.
Typing was performed using Phast Gels 10-15 (Pharmacia LKB) and silver staining with the conditions described in Barros et al (1992) or runing an 2.5% agarose (FMC) gel in TBE buffer at 4V/cm for 6-7 hours and visualized by staining with ethidiumbromide using the same ladder (Cetus).

RESULTS AND DISCUSSION

In this study we have found 20 D1S80 alleles (Table 1).

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When compared to other Caucasian data (Helmuth et al 1990; Gené et al, 1992; De Stefano et al 1992) the allelic frequencies of the most and fewest frequent alleles are quite similar, but other variants present some differences even in populations geographically very close, such as NW Spain and Central Portugal (Lareu et al, in press). Relatively to other ethnic populations these differences are significantly larger.

ACKNOWLEDGEMENTS: We thank Cetus Corporation for supplying us two HLA DQα Amplitype Kits.

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Table 1. Allele and genotype frequencies of the HLA DQA1 system in the population of North of Portugal

	Observed		Expected	
Genotypes	n	%	n	%
1.1 - 1.1	13	4.00	9.65	2.97
1.1 - 1.2	12	3.69	17.23	5.30
1.1 - 1.3	7	2.15	10.34	3.18
1.1 - 2	12	3.69	19.64	6.04
1.1 - 3	20	6.15	17.75	5.46
1.1 - 4	35	10.77	27.74	8.53
1.2 - 1.2	10	3.08	7.69	2.37
1.2 - 1.3	10	3.08	9.23	2.84
1.2 - 2	18	5.54	17.54	5.39
1.2 - 3	15	4.61	15.84	4.87
1.2 - 4	25	7.69	24.77	7.62
1.3 - 1.3	2	0.61	2.77	0.85
1.3 - 2	14	4.31	10.52	3.24
1.3 - 3	14	4.31	9.51	2.93
1.3 - 4	11	3.38	14.86	4.57
2 - 2	11	3.38	9.99	3.07
2 - 3	20	6.15	18.06	5.56
2 - 4	28	8.61	28.23	8.69
3 - 3	6	1.85	8.16	2.51
3 - 4	22	6.77	25.51	7.85
4 - 4	20	6.15	19.94	6.13
	Total 325			
1.1 = 0.1723	**2** = 0.1754		x^2 = 15,66	
1.2 = 0.1538	**3** = 0.1585		df = 15	
1.3 = 0.0923	**4** = 0.2477		P > 0,40	

The theoretical a priori chance exclusion value (C.E.) is 0.6399 from genotype data, the power of discrimination (P.D.) according to the equation suggested by Fisher (1951) is 0.9427. The allelic diversity value (h) as described by Nei and Rouchoudhoury (1974) is 0.8209 (Table 2).

Table 2. Statistical parameteres of HLADQA1 system.

CE	0.6399
PD	0.9427
h	0.8209
Heterozygosity	0.8092

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THE DISTRIBUTION OF HLA DQA1 ALLELES IN THE POPULATION OF THE NORTH OF PORTUGAL

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INTRODUCTION

DNA profiling has rapidly become a routine technique in forensic laboratories worldwide since its introduction in 1985. One of the PCR strategies that have been developed for forensic application is the dot-blot analysis of the human leucocyte antigen (HLA) DQα Locus (Saiki et al, 1986; Westwood et al 1990), now redesignated as DQA1.
The DQA1 typing system has been used to study many populations (Helmuth et al, 1990; Sullivan et al, 1992; Lareu et al, in press). We report here the allele and genotype frequencies distribution of a Portugal subgroup population, North of Portugal.

MATERIAL AND METHODS

Blood samples were taken from 325 unrelated individuals of routine paternity cases. DNA was extracted by a method previously described by Singer-Sam and col. (1989) using a chelating resin. Some of these samples were extracted using a phenol-chloroform procedure. PCR amplification was performed using HLA DQα Amplitype Kit (Cetus) and the protocols provied by the manufacturer. The cycling reaction was done in a programmable heat block (TC 480 Perkin Elmer - Cetus Thermal Cycler).
Typing of HLA DQA1 alleles was achieved using allele specific oligonucleotide probes (ASO probes) and reverse dot blot methodology (Cetus Amplitype User Guide).

RESULTS AND DISCUSSION

The genotype and allele frequencies distribution indicates that there is no deviation from the Hardy-Weinberg equilibrium.
The allele frequencies range from 0.0923 (allele DQA1 * 1.3) to 0.2477 (allele DQA1 * 04) (Table 1).

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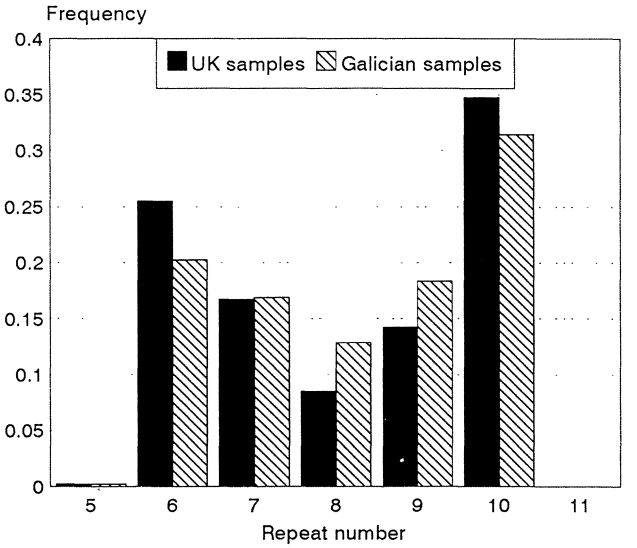

Fig 3: HUMTH01 allele frequencies

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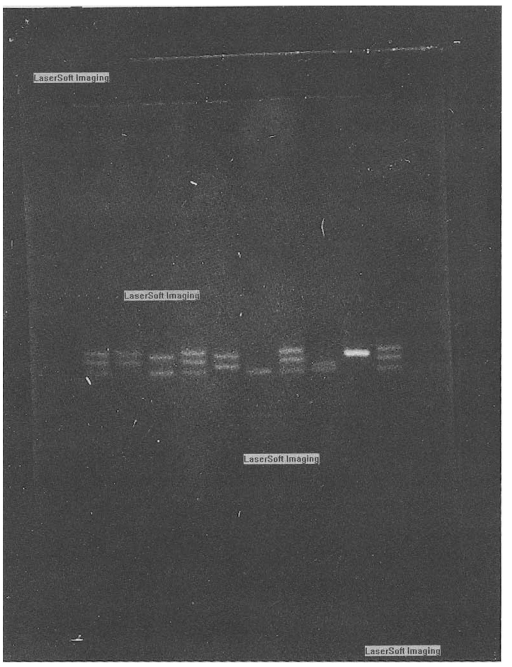
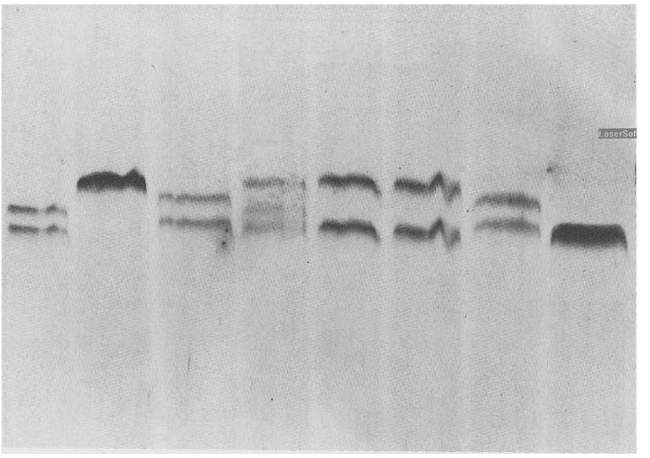


Lane	1	6.8.10 ladder
	2	8.10
	3	6.9
	4	6.8.10 ladder
	5	7.9
	6	6.6
	7	6.8.10 ladder
	8	6.7
	9	9.9
	10	6.8.10 ladder

Fig1: HUMTH01 genotypes obtained with agarose electrophoresis



Lane	1	10.10
	2	7.9
	3	6.10
	4	6.10
	5	6.8.10 ladder
	6	7.9
	7	6.6
	8	8.10

Fig2: HUMTH01 genotypes obtained with polyacrylamide electrophoresis

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Genotype	UK observed	UK expected	Galicia observed	Galicia expected
10.10	25	24.15	21	20.74
9.10	17	19.81	23	24.20
8.10	13	11.81	18	16.97
7.10	27	23.28	25	22.31
6.10	32	35.44	24	26.71
9.9	6	4.06*	6	7.06
8.9	2	4.84*	10	9.90
7.9	7	9.55	13	13.02
6.9	18	14.53	19	15.58
8.8	1	1.44*	4	3.47*
7.8	6	5.69	6	9.13
6.8	11	8.67	12	10.93
7.7	5	5.61	6	6.00
6.7	17	17.08	14	14.37
6.6	12	13.01	8	8.60
5.9	1	0.14*	0	
5.7	0		1	0.17*
Other 5 types	0	0.86*	0	0.83*

Table 1: Population survey of UK (agarose) and Galician (polyacrylamide) Caucasians

Chi-square 3.39, df=9, p>0.05 (UK); 3.93, df=7, p>0.05 (Galicia) (*classes pooled)

Heterozygosity = 0.755 (0.764 expected) (UK); 0.786 (0.785 expected) (Galicia)

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Gels were run at 80V for 5 hours. Tank buffer (at 4°C) was replaced three times during run. Ethidium bromide (0.005%) concentration, was added to buffers.

Polyacrylamide gels, cross-linked with bisacrylamide and polymerized with TEMED/ammonium persulphate, were made to a concentration of C=10, T=3.3 using 33mM tris-sulphate pH9.0 gel buffer. 17cm wide x 22cm long x 0.5mm gels, with 5mm x 1.5mm x 0.25mm wells, 2cm from end, were cast on glass-supported Gelbond (FMC Bioproducts). 1cm thick electrodes were prepared from 2% agarose in 0.52M tris-borate buffer, pH8.5. 4µl samples were pipetted into the wells and run at 15°C at a constant 450V, 25mA for 2½ hr after a 45 min pre-run. Gels were silver stained using a standard protocol (Allen R et al., BioTechniques, 7, 736-744, 1989).

A seven allele ladder, supplied as part of a European DNA Profiling (EDNAP) collaborative exercise, was successfully amplified, but problems were encountered in obtaining clearly defined bands for all seven alleles. Therefore, for routine genotyping, a 6.8.10 allele ladder, constructed by combining authenticated heterozygote and homozygote controls, was run at regular intervals across the gel.

RESULTS

Typical electrophoretic results are shown in Figs 1 and 2. Genotype frequencies are listed in Table 1. Fig 3 shows the allele frequencies. Fifty samples, tested in duplicate using both electrophoretic techniques, showed no discrepancies in genotype assignment. There was no evidence of departure from Hardy-Weinberg equilibrium or allele dropout. The discrimination power of the HUMTH01 locus was estimated as 90.3% (UK) and 91.8% (Galicia).

50% of the UK false family trios and 56% of the Galician false family trios, showed an exclusion of paternity. Expected exclusion rates were 53.7% and 57.0% respectively. The 80 meioses observed in the mother-child pairs showed normal segregation with no examples of mutation or non-Mendelian inheritance.

CONCLUSIONS

HUMTH01 is an informative polymorphism for which population statistics are readily established. The use of a 6-8-10 allelic ladder proved a simple and effective control for routine typing. Agarose electrophoresis, using a high-sieving gel such as MetaPhor, provides a simple and rapid technique for separating STRs and has the advantage of greater consistency between gels, when compared with polyacrylamide gels. Polyacrylamide electrophoresis, followed by silver staining, is, however, more sensitive than agarose at detecting low yield of product after amplification. Used in combination with other similar STR loci, HUMTH01 would provide a powerful extension to the analytical tools being used in forensic science today.

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Investigation of the STR locus HUMTH01 using PCR in Caucasian samples from England and Galicia, NW Spain

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INTRODUCTION

Short tandem repeat (STR) loci consist of variable numbers of tandemly repeated units (VNTRs) which are typically dimeric, trimeric, tetrameric or pentameric in length. The loci are widespread throughout the genome and normally display a discrete number of alleles. Unlike other types of fragment length polymorphism, they can often be successfully amplified from degraded, low molecular weight DNA, since loci are shorter. With such characteristics STR loci would appear to be ideal candidates for use in forensic analyses.

HUMTH01 is an STR locus, found within intron 1 of the tyrosine hydrolase gene located at chromosome 11p15.5 - p15. It consists of variable numbers, from 5 to 11, of AATG repeat units, giving 7 alleles ranging in size from 187 - 211 base pairs using the listed primers.

A survey of unrelated Caucasians, from the UK and from Galicia (NW Spain), was performed and the populations compared. The genotyping technique used on the UK samples employed agarose electrophoresis, whilst the samples from Galicia were tested using polyacrylamide electrophoresis. The genetic stability of HUMTH01 and its applicability as a polymorphic marker in paternity analysis was investigated by constructing false family trios.

MATERIALS AND METHODS

Blood samples from 200 UK and 210 Galician Caucasians were used. Some of the samples were from paternity cases. Mother-child pairs from these cases were used to construct a total of 80 false family trios (30 from the UK and 50 from Galicia).

DNA was extracted directly from whole blood or bloodstains using a chelating resin (Sigma Chemical Co) following the procedure developed by Singer-Sam et al. (Amplifications, 3, 11, 1989) with extracts being used in the PCR reaction mix. Amplification was performed using the 24mer primers described by Edwards et al. (Am J Hum Genet, 49, 746-756, 1991) and synthesized by the phosphoramidite method in a 380A DNA synthesizer and purified through OPC columns (Applied Biosystems). The primer sequences were:

5' GTG/GGC/TGA/AAA/GCT/CCC/GAT/TAT 3' (AATG strand)
5' ATT/CAA/AGG/GTA/TCT/GGG/CTC/TGG 3' (TTAC strand)

A 30 cycle PCR reaction was used: denaturation at 94°C, 45 sec; annealing at 60°C, 30 sec; elongation at 72°C, 30 sec. In a total reaction volume of 25µl, 10µl of DNA extract was amplified with 0.25µMx2 primers and 200µMx4 nucleotides with 1 unit of Taq1 DNA polymerase. Reaction buffer (10x) consisted of 100mM tris-HCl pH8.3, 500mM KCl, 15mM MgCl and 0.01% gelatin. The quality of amplification was assessed on an agarose minigel.

Agarose gels (8cm x 10.5cm) were prepared using 4.5% MetaPhor (FMC Bioproducts) in 40ml 1xTAE, poured at 70°C. The gel and 1xTAE tank buffer were cooled to 4°C before the run and 20µl samples loaded into 1mmx5mm wells with 5x loading buffer.

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Figure 2 : distribution of D1S80 alleles in a french caucasian random sample (n=216) and in a sample of aircrash victims (n=170)

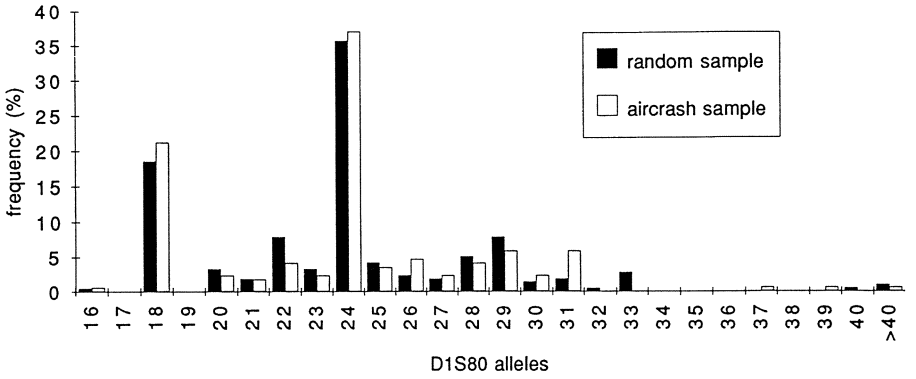

Table 2 : observed D1S80 genotypes in a french caucasian random sample of 108 individuals and in a sample of 85 aircrash victims .

OBSERVED GENOTYPES	Aircrash Sample	Random Sample	OBSERVED GENOTYPES	Aircrash Sample	Random Sample
16-23	1	0	22-25	0	3
16-24	0	1	22-26	1	0
18-18	3	4	22-28	1	0
18-20	0	2	22-29	0	2
18-22	1	4	22->40	0	1
18-23	2	2	23-24	1	2
18-24	16	13	24-24	10	12
18-25	3	1	24-25	2	4
18-26	1	1	24-26	2	0
18-28	2	3	24-27	3	2
18-29	2	1	24-28	3	6
18-30	1	1	24-29	4	8
18-31	1	1	24-31	5	2
18-32	0	1	24-34	0	4
18-34	0	1	24-39	1	0
18-37	1	0	25-31	0	1
20-24	3	3	26-26	1	1
20-30	0	1	26-29	1	1
20-31	1	0	26->40	1	1
20-40	0	1	27-29	0	2
21-23	0	1	28-29	1	2
21-24	1	3	29-30	1	1
21-25	1	0	29-31	1	0
21-27	1	0	29-34	0	1
22-22	1	0	30-31	1	0
22-23	0	2	31-31	1	0
22-24	2	5			

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Figure 1 : distribution of HLA-DQ alpha alleles in a french caucasian random sample (n=166) and in a sample of aircrash victims (n=168)

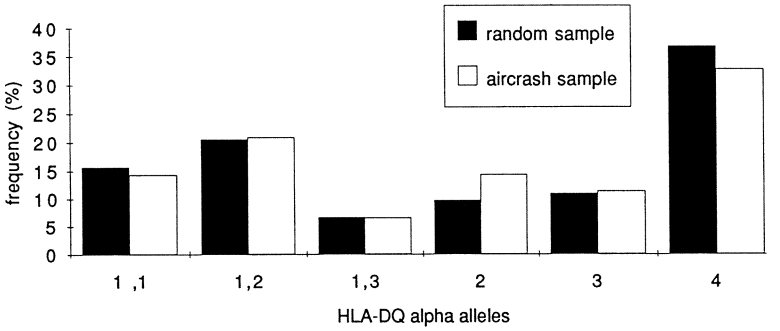

Table 1 : observed HLA-DQα genotype frequencies (%) in a frenc caucasian random sample of 83 individuals and in a sample of 84 aircrash victims.

OBSERVED GENOTYPES	RANDOM SAMPLE n	%	AIRCRASH SAMPLE n	%
1.1-1.1	1	1.20	2	2.38
1.1-1.2	9	10.84	4	4.76
1.1-1.3	1	1.20	--	--
1.1-2	2	2.40	6	7.14
1.1-3	1	1.20	3	3.57
1.1-4	11	13.25	7	8.33
1.2-1.2	4	4.82	3	3.57
1.2-1.3	4	4.82	3	3.57
1.2-2	3	3.60	7	8.33
1.2-3	1	1.20	3	3.57
1.2-4	9	10.84	12	14.28
1.3-1.3	--	--	--	--
1.3-2	1	1.20	2	2.38
1.3-3	2	2.40	1	1.19
1.3-4	3	3.60	5	5.95
2-2	--	--	1	1.19
2-3	4	4.82	3	3.57
2-4	6	7.23	4	4.76
3-3	1	1.20	3	3.57
3-4	8	9.64	3	3.57
4-4	12	14.46	12	14.28
Heterozygotes	n = 65	78 %	n = 63	75 %
Homozygotes	n = 18	22 %	n = 21	25 %
Allelic diversity (h)		0.78		0.79
Power of discrimination (PD)		0.91		0.92

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D1S80 locus : 17 and 19 different D1S80 alleles were observed for the aircrash sample and the french caucasian random sample respectively. As observed previously (2) alleles 18 and 24 were highly represented (fig. 2). No significant differences appeared between both population samples as far as the allele distribution was concerned ($X^2 = 12.2$; df = 13 ; $0.5 < p < 0.9$). The observed (table 2) and expected D1S80 genotypes compared by the chi-square test showed conformity to HW expectations for the french caucasian random sample ($X^2 = 20.76$; df = 13 ; $0.05 < p < 0.1$) and for the aircrash sample ($X^2 = 11.89$; df = 9 ; $0.2 < p < 0.3$) and there were no significant differences between both population samples ($X^2 = 2.63$; df = 6 ; $0.5 < p < 0.9$).

Conclusion

HLA.DQ-Alpha and D1S80 PCR typing proved to be a very usefull tool for solving complex problems of identification. The application of the PCR method for the identification of the victims of the aircraft accident which occured in Strasbourg in 1992 resulted in the typing of 85 unrelated individuals. The population data showed that the sample gathering the victims of the aircrash could be considered as a sample of individuals taken at random in the french caucasian population.

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HLA.DQ-ALPHA AND D1S80 DATA FROM A FRENCH CAUCASIAN POPULATION RANDOM SAMPLE VERSUS A SAMPLE OF AIRCRASH VICTIMS.

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Introduction

On January 20, 1992, an airbus A320 crashed in Strasbourg area : among the 90 passengers and the 6 crew, 87 died and 9 survived. Identification of the victims was investigated using conventional methods (dental records, physical and X rays examinations) and DNA fingerprinting. Then, the complete reconstitution of most heavy mutilated bodies was performed by DNA typing at the HLA.DQ-Alpha (1) and D1S80 (2) loci for all the human fragments. The HLA.DQ-Alpha and D1S80 data collected for 84 and 85 victims respectively were compared to the data obtained for a sample of individuals taken at random from the french caucasian population. Allele and genotype frequencies at the HLA.DQ-Alpha and D1S80 loci were determined by polymerase chain reaction (PCR) amplification.

Material and methods

HLA.DQ-Alpha locus : the random sample included 83 unrelated individuals randomly selected from the french caucasian population and the DNA was extracted from blood samples. Concerning the sample of aircrash victims only 84 into 87 victims could be typed at the HLA.DQ-Alpha locus and the DNA was extracted from 82 muscle samples and two bone samples. The amplification of the DNA (2 ng) and the identification of the alleles were performed using the Amplitype^{TM} HLA.DQ-Alpha forensic DNA Amplification and Typing Kit from Cetus (Cetus Corporation, CA, USA). PCR thermal cycles were carried out as indicated in the protocol of the kit and run in a Perkin-Elmer thermocycler.

D1S80 locus : the random french caucasian population sample numbered 108 randomly selected individuals and the aircrash sample was constituted of 85 victims. The amplification of the DNA (10 ng) was performed using the D1S80 Forensic DNA Amplification Reagent Set from Cetus (Cetus Corporation, CA, USA). PCR thermal cycles were carried out as indicated in the protocol of the kit. D1S80 alleles were identified on 1.5 % SeaKem GTG agarose gels after ethidium bromide staining.

Results

HLA.DQ-Alpha locus : the 6 different HLA.DQ-Alpha alleles (1.1 - 1.2 - 1.3 - 2 - 3 and 4) were identified for the french caucasian random sample and for the sample of aircrash victims. As observed in previous studies (3,4), the most frequent HLA.DQ-Alpha allele was allele 4 and the least frequent HLA.DQ-Alpha allele was allele 1.3 (fig. 1). The HLA.DQ-Alpha allele distribution determined from the aircrash sample was similar to that obtained with the french caucasian random sample (X^2 = 2.89 ; df = 5 ; $0.5<p<0.9$).The distribution of the HLA.DQ-Alpha genotypes (table 1) was in Hardy-Weinberg (HW) equilibrium for the french caucasian population sample (X^2 = 14.58 ; df = 15 ; $0.3<p<0.5$) as for the aircrash sample (X^2 = 14.8 ; df = 15 ; $0.3<p<0.5$). Population data comparisons were established by pooling all classes with less than 3 events and showed that the french caucasian random sample and the aircrash sample were similar : no significant differences appeared (X^2 = 12.34 ; df = 11 ; $0.3<p<0.5$).

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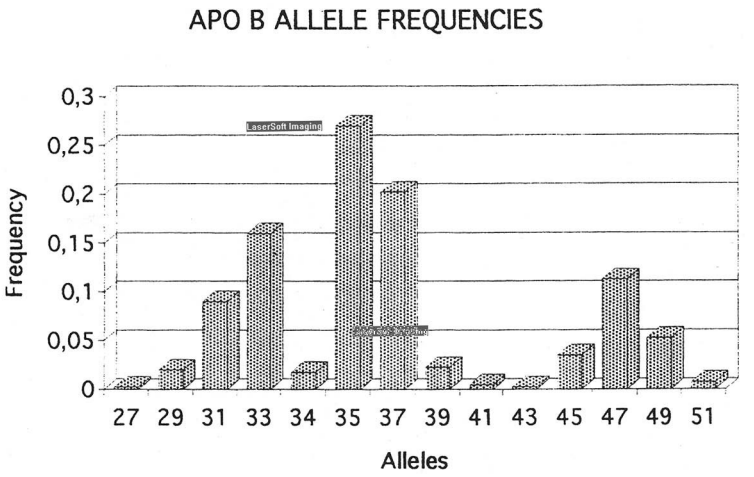

Fig. 1

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Non denaturing page makes a good separation of the alleles without disturb the migration of the DNA fragments and of the molecular weight marker. Depending on the percentage of T, some first bands of less molecular weight of the marker may be lost, but the resolution is better than agarose gel electrophoresis. This may be important for a correct typing especially when a complete allelic ladder is not available.

Table 1. APO B 3' HVR genotypes

Genotype	observed	(expected)	frequency
27-35	1	(0.27)	0.005
29-29	1	(0.08)	0.005
29-33	1	(1.28)	0.005
29-35	2	(2.16)	0.01
29-37	1	(1.62)	0.005
29-41	1	(0.04)	0.005
29-47	1	(0.90)	0.005
31-31	3	(1.62)	0.015
31-33	4	(5.76)	0.02
31-35	13	(9.72)	0.065
31-37	6	(7.29)	0.03
31-41	1	(0.18)	0.05
31-47	6	(4.05)	0.03
33-33	8	(5.12)	0.04
33-35	19	(17.28)	0.095
33-37	13	(12.96)	0.065
33-39	2	(1.44)	0.01
33-45	4	(2.24)	0.02
33-47	3	(7.20)	0.015
33-49	2	(3.36)	0.01
34-35	3	(1.89)	0.015
34-47	3	(0.78)	0.015
34-49	1	(0.37)	0.005
35-35	10	(14.58)	0.05
35-37	23	(21.87)	0.115
35-39	3	(2.43)	0.015
35-45	4	(3.78)	0.02
35-47	15	(12.15)	0.075
35-49	5	(5.67)	0.025
37-37	10	(8.20)	0.05
37-39	3	(1.82)	0.015
37-45	1	(2.83)	0.005
37-47	7	(9.11)	0.035
37-49	5	(4.25)	0.025
37-51	2	(0.60)	0.01
39-47	1	(1.01)	0.005
43-47	1	(0.11)	0.005
45-47	2	(2.83)	0.01
45-49	3	(0.73)	0.015
47-47	2	(2.53)	0.01
47-49	1	(2.36)	0.005
47-51	1	(0.33)	0.005
49-49	2	(0.55)	0.01

Allele frequency	
27	0.0025
29	0.02
31	0.09
33	0.16
34	0.0175
35	0.27
37	0.2025
39	0.0225
41	0.005
43	0.0025
45	0.035
47	0.1125
49	0.0525
51	0.0075

Heterozigosity (n=164) = 0.82

Hardy Weinberg equilibrium (chi square 10.61, df =9, $0.20 < P < 0.30$)

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APO B POLYMORPHISM IN THE DISTRICT OF BOLOGNA (ITALY)

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INTRODUCTION

VNTRs are high polymorphic segments, called micro and minisatellites and due to the degree of allelic variability and the Mendelian inheritance they are useful markers in forensic application. Their polymorphism is attributed to allelic variation in the number of tandemly repeated DNA sequences. One such hypervariable region is APOB 3' HVR, located 75 bp downstream from the second polyadenilation signal at the 3' end of human APOB, wich maps to human chromosome 2 (Knott et al 1986). The polymorphism is due to the repeat of two structurally related sequences "x" and "y" of 15 bp in lengh (Boerwinkle et al. 1989, Ludwig et al 1989).
Initially the polymorphism was studied by Southern blotting with APOB gene probes, recently it is detected by PCR with flankig primers to amplify HVR sequences, and the products were separated according to size by electrophoresis on agarose gels (Boerwinkle et al 1989, Deka et al 1992) or on denaturing and non denaturing polyacrilamide gels (Ludwig et al 1989, Rand et al 1992).
On the basis of different criterion to evaluate repeat elements, Boerwinkle (1989) proposed a nomenclature at odd alleles (29-51) corresponding to even alleles (30-52) of Ludwig et al (1989), who also observed two alleles with an odd number of repeats: 25 and 35. Recently, in a personal communication to Deka (1992), Boerwinkle , sequencing PCR amplified alleles, has modified his first terminology.
Really, HVR alleles differing in length may not represent the total allelic polymorphism of this locus because of sequence microheterogeneity undetected by means of electrophoresis.

MATERIALS AND METHODS

DNA was extracted from blood of 200 blood donors unrelated, residents in the district of Bologna. DNA was isolated using the fast protocol Genomix (Kontron) .
30 ng of genomic DNA were taken for PCR.DNA amplification was performed using the primers described by Boerwinkle et al (1989) : 5' ATGGAAACGGAGAAATTATG-3' and 5' CCTTCTCACTTGGCAAATAC-3'. Genomic DNA was amplified in a total volume of 50 ul containing 10mM Tris-HCl pH 8,8,1,5 mM MgCl2, 50mM KCl, 0,1% Triton X-100 ,100uM each dNTP, 0,2 uM each primer, 2,5 U Taq DNA-polymerase (Promega).Amplification was carried out using the thermal cycler Perkin Elmer 480 with the following condition: denaturation 1' at 95°, annealing and extension 6' at 58°. A total of 26 cycles was carried out. The hot start tecnique was employed before the first thermal cycle.
Gel electrophoresis of amplified DNA fragments was performed using 2% agarose gel or horizontal polyacrilamide gel, 750 um thick, 6% T, 3% C with PDA as cross-linker (Budowle et al 1991). For page a discontinuous buffer system was used (Allen et al 1989) and the separation distance was 18 cm. Sample were applied to the gel surface via a silicone applicator mask (Serva, Heidelberg). Separation was carried out at 500 V and stopped after 1h 30'. DNA bands were visualized by silver stain (Bio Rad) and phenotyping was obtained by comparison with the 100 bp ladder (Pharmacia) and with an internal ladder of some alleles. The gel image was recorded by a video camera and the analysis was performed by UVP's GelBase software using UVP's System 5000.

RESULTS AND DISCUSSION

All DNA samples could be typed for APO B 3' HVR locus. In the present study we have applied to the alleles the nomenclature of Boerwinkle (1989) . According to Chakraborty R. (1992), the sample size of this study may be adequate for estimates of allele and genotype frequencies. The alleles show large differences in size, ranging from 27 to 51 repeats. In table 1 allele designation and frequencies are reported together with the index of heterozigosity . 14 different alleles were observed , 4 of which occuring at a frequency greater than 0.10. To this regard the distribution of alleles shows three peaks at repeats 33,35,37 and another peak at repeat 47. This confirms the bimodal pattern of allele frequency distribution at this locus (Deka et al 1992) (Fig.1). The total sample meets H-W expectations for APO B 3' HVR locus. Any class with less than 4 observations was pooled.

1.3 to 1.7% error in the overall experiment). Following the experiment, an electronic archive was established and distributed to all participants, who agreed to store their future data into updated versions of the archive. The archive is presently near to filing a representative group of individuals from five Italian regions.
Like other previous experiments of the kind, this initiative has shown that standardization may be achieved among a relatively large number of laboratories. Further trials will follow, dealing with creation of frequency data, harmonization of procedures and quality controls.

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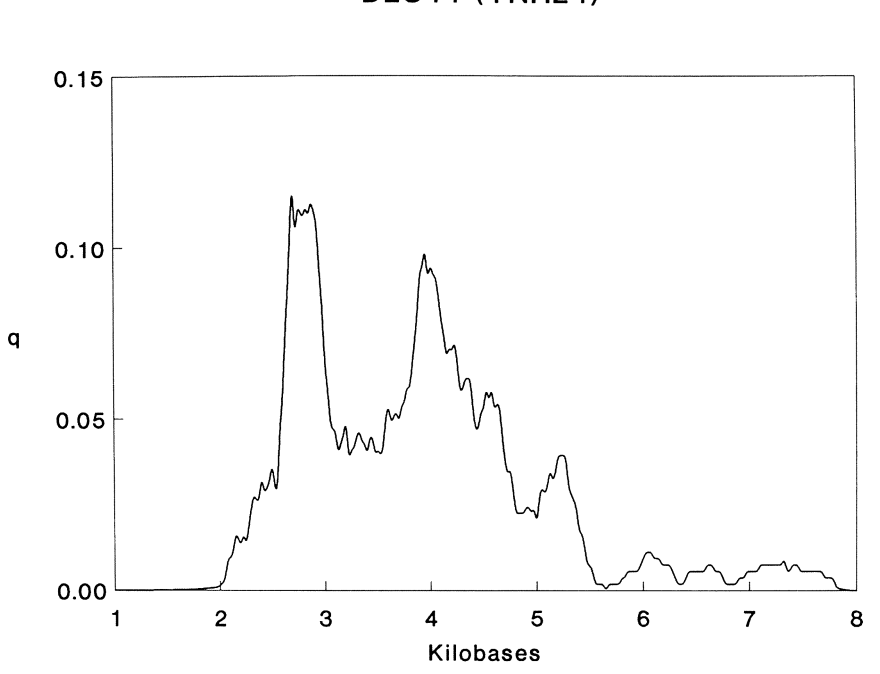
Figure 1
The general profile of frequencies emerging from the present collaborative experiment is sketched in this figures. As shown two major peak of frequency are present at 2.8 and 4.0 Kb, with a 0.12 frequency value for the most common allele. The use of the proposed common protocol and the creation of a permanent archive of frequencies let the majority of Italian Laboratories involved in DNA analysis to reach a satisfactory level of standardization.



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GARDA1 (D2S44 SBA) - HARMONIZATION OF PROTOCOLS AND A COLLABORATIVE DATABASE

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By initiative of the Italian working group in Forensic Hemogenetics (G.E.F.I.), a collaborative experiment was promoted, aimed at standardizing protocols and procedures of Southern Blot Analysis (SBA) for addressing single locus probes (SLPs). In 1991, sixteen laboratories throughout Italy agreed to adopt the protocol proposed by the EDNAP network (1) and to produce compatible data. The procedure adopted in the experiment was also recognized as the official GEFI protocol for releasing evidentiary expertises in courtrooms.

MATERIALS AND METHODS

Each partecipating laboratory agreed to process at least 45 blood samples of random individuals and one genomic control (K562, Promega). Standard extraction procedures were used, then the DNA digestion was carried out with tenfold Hinf I excess and the separation of restriction fragments was achieved by an 0.8% submarine agarose gel electrophoresis. It was intended that all participants had to perform at least five electrophoretic run. Every gel contained the same molecular weight ladder (1Kb ladder, BRL) and the genomic control. Hybridization was carried out with freely chosen procedures, but using the same D2S44 probe batch under stringent conditions. Autoradiograms were sent to a coordinating laboratory where they were processed using a semi-automatic procedure so as to create a common database. Keys of access to the database were several error measurements reflecting intra- and inter-laboratory variance units.

RESULTS AND DISCUSSION

The general profile of frequencies emerging from the collaborative experiment is shown in Fig.1. This figure shows that, according to other Caucasian populations, Italians have two major peaks of frequency at 2.8 Kb and 4.0 Kb. The average heterozygosity of the system was 99.5%. A total of 651 individuals were typed, and most laboratories contributed more autoradiograms than the minimum figure required to comply with the experiment. All laboratories were found to share comparable measurement errors in ascribing molecular weight to bands (0.25-1.75% error in intralaboratory experiments; from

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Cigarette butts are routinely analyzed in our laboratory. A minimal amount of DNA (i.e. 1 ng) is obtained in about 50% of samples, making possible the DNA amplification in, at least, one system. Then genotype frequency will depend on the number of system used.

Formalin treated or paraffined samples are not suitable for southern blotting. We succeed in DNA amplification for such samples. The extraction procedure is the major part of the process : a combination of classic extraction (proteinase K, phenol-chloroform) and further purification steps (Chelex^r, Centricon^r , dialysis) gives the best results.

DNA amplification is an attractive technique which seems simple, quick and inexpensive. However several problems are critical : unequal allele amplification in some systems, presence of inhibitors, allele marker standardisation, contaminations and low discrimination power.

In our laboratory a few cases have been resolved by DNA amplification but technique credibility has not been estimated since no trial has been judged with results obtained by PCR. In our opinion, today, Southern blotting must be the reference procedure for Forensic DNA typing. However the Polymerase Chain Reaction could be used in case of failure of the other techniques.

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Catalog N°808-0056

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D1S80 :

Allele	Frequency
16	0.0 %
17	0.4 %
18	26.3 %
19	1.5 %
20	1.9 %
21	2.2 %
22	6.3%
23	1.1 %
24	30.7 %
25	6.3 %
26	2.6 %
27	1.5 %
28	5.2 %
29	2.9 %
30	1.9 %
31	6.7 %
32	1.9 %
>32	0.0 %

YNZ22 :

Allele	Frequency
1 (lower)	6.6 %
2	24.1 %
3	9.3 %
4	25.9 %
5	4.8 %
6	3.7 %
7	0.0 %
8	5.2 %
9	5.9 %
10	6.3 %
11	4.8 %
12	2.6 %
13 (upper)	0.8 %

Von Willebrand Factor :

Allele	Frequency
1 (138 bp)	9.7 %
2 (142 bp)	10.5 %
3 (146 bp)	20.2 %
4 (150 bp)	31.9 %
5 (154 bp)	18.9 %
6 (158 bp)	7.9 %
7 (162 bp)	0.9 %

ApoB :

Allele	Frequency
1 (lower)	6.8 %
2	0.4 %
3	6.1 %
4	27.7 %
5	37.5 %
6	3.8 %
7	2.3 %
8	0.7 %
9	4.5 %
10	6.1 %
11	3.4 %
12 (upper)	0.7 %

Discussion :

Southern blotting is, for DNA typing the routine technology. However southern blotting could be unsuitable in case of a low amount of DNA or partially degraded DNA. In a such situation the Polymerase Chain Reaction (PCR) is the only technique possible for typing DNA.

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FRENCH POPULATION DATA OF 6 AMPFL'S

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Introduction :

The Polymerase Chain Reaction (PCR) is a powerful tool for DNA typing. This technique is useful because of its sensitivity, speed and ability to type few or partially degraded DNA molecules. In order to apply PCR routinely, population data is needed to estimate genotype frequencies. We present here the results obtained for French caucasian people in seven systems. In addition, we point out some applications.

Materials and methods :

DNA was extracted from blood samples and amplified in a Cetus thermocycler as previously described : D1S80 (1), ApoB (2), YNZ 22 (3), COL 2A1 (4), TC11 (5), von Willebrand Factor (6), HLADQα (7). PCR products were analyzed in horizontal polyacrylamide gel (1) (D1S80, ApoB, YNZ22, COL2A1, TC11), in vertical polyacrylamide gel (von Willebrand Factor) or using non radioactive detection (HLADQα) (7).110 unrelated caucasian people were analyzed for the population data.

Results :

HLADQα :		TC11:	
Alleles	Frequency	Allele	Frequency:
1.1	16.4 %	1 (lower)	23.8 %
1.2	17.3 %	2	15.2 %
1.3	8.0 %	3	12.9 %
2	20.8 %	4	17.5 %
3	13.2 %	5	30.2 %
4	24.3 %	6 (upper)	0.4 %

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TABLE 2 Number of alleles (K), gene diversity (gene D), and PIC of the STR loci studied in various Caucasian populations.

	Norwegians (N=300)			Caucasians[1] (N=55)			North[2] Europeans	Whites[3]
Locus	K	gene D	PIC	K	gene D	PIC	gene D	gene D
HUMTHO1	6	0.76	0.75	5	0.77	0.76		0.76
HUMFES/FPS	6	0.66	0.60	5	0.67	0.62		
HUMVWA31	8	0.81	0.78	7	0.81	0.79		
HUMF13A1	13	0.74	0.70	7	0.72	0.68	0.73	

1 Colin P. Kimpton(1993). 2 Wilson J. Wall(1993). 3 Al Edwards(1991)

TABLE 3 Matching Probability (pM) of STR Loci

Locus	Norwegians	Caucasians[1]
HUMTHO1	0.09	0.09
HUMFES/FPS	0.18	0.19
HUMVWA31	0.07	0.07
HUMF13A1	0.10	0.13

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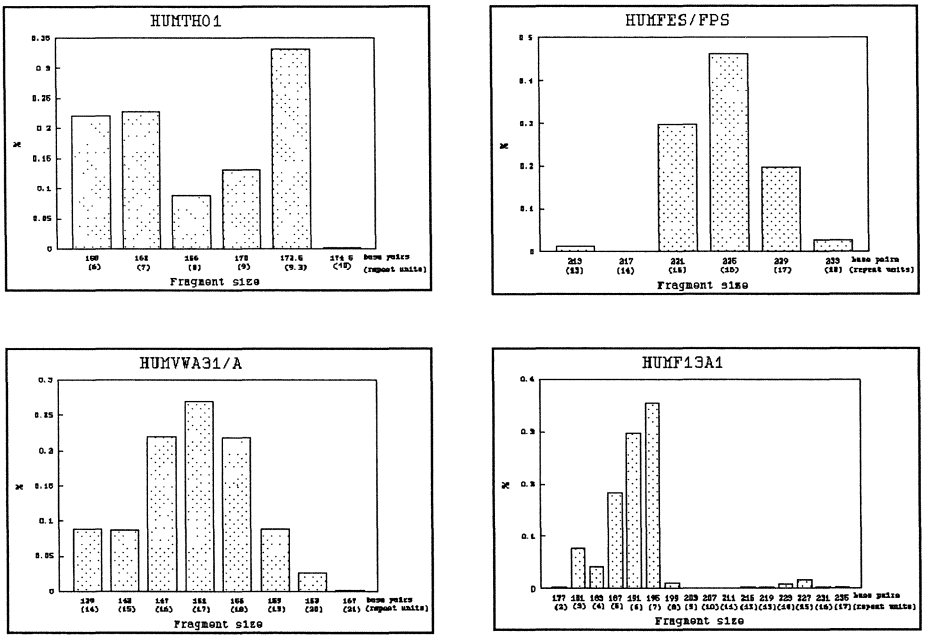

FIGURE 1 Allele frequency histograms.

THO1	tot. obs.	Allele freq.	FES	tot. obs.	Allele freq.
158	132	0.220	213	8	0.013
162	137	0.228	217	1	0.002
166	53	0.088	221	179	0.298
170	78	0.130	225	277	0.462
173.5	199	0.332	229	118	0.197
174.5	1	0.002	233	17	0.028
VWA			F13		
139	53	0.088	177	1	0.002
143	52	0.087	181	46	0.077
147	132	0.220	183	25	0.042
151	162	0.270	187	110	0.184
155	131	0.218	191	178	0.297
159	53	0.088	195	212	0.354
163	16	0.027	199	6	0.010
167	1	0.002	215	2	0.003
			219	2	0.003
			223	5	0.008
			227	10	0.017
			231	2	0.003
			235	1	0.002

TABLE 1 Allele frequency data.

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Four STRs in 300 Norwegians

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INTRODUCTION

The tetrameric short tandem repeat loci HUMTHO1, HUMFES/FPS, HUMVWA31 AND HUMF13A1 were studied. Amplification was performed according to EDNAP recommendation in a Perkin Elmer 9600, and fragments analysed in an ABI Model 373 Sequencing System using Genescan 672 software.

MATERIAL

DNA was prepared from whole blood. Blood samples were obtained from 300 unrelated Norwegians (150 males, 150 females) involved in paternity cases. Alternating male/female individuals were chosen from consequtive cases, and ascertainment of nationality was based on typical Norwegian names.

METHODS

HUMTHO1/HUMFES/FPS and HUMVWA31/HUMF13A1 were amplified in two separate duplex PCR reactions. The STR-PCR products from both reactions were loaded in one single lane on a 24 cm denaturating 6% polyacrylamide gel. The internal standard ROX2500 was included in each sample for size determination.

Hardy-Weinberg was tested using the Chi-square test. Groups with expected values below 5 were excluded.
The polymorphic information content, PIC, was calculated using the formula of Botstein et al. (1980).
The Matching Probability, pM, was calculated from the allele frequency data using the formula of Jones (1972).

RESULTS AND DISCUSSION

The genotype distributions are in accordance with Hardy-Weinberg expectations, ($P>0.6$ for all loci).

Allele distributions, PIC, and pM values are similar to those found in other Caucasian population materials (Colin P Kimpton 1993).

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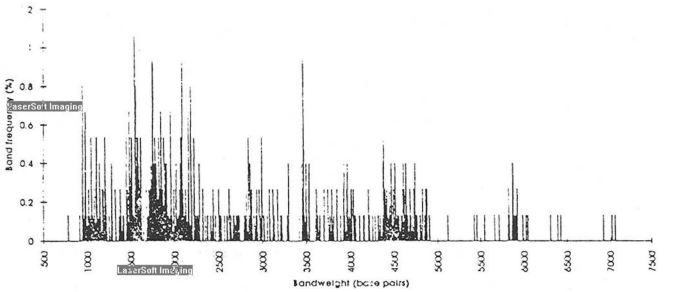

Figure 2: Raw data plot for FSS RC1 TBQ7 paternity data calculated based on a 5 base pair 'bin' for 374 profiles including 27 one-banded individuals.

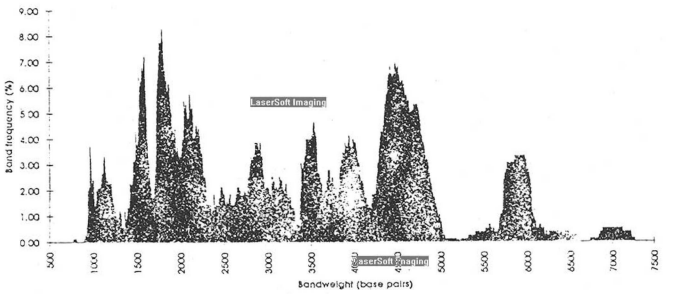

Figure 3: 2.8% sliding plot for FSS RC1 TBQ7 paternity data calculated at a 5 base pair interval for 374 profiles including 27 one-banded individuals.

A statistical analysis of the TBQ7 data to assess the practical effects of between probe dependence was conducted along similar lines to the experiments described in Evett et al [7]. TBQ7 data was compared to the data generated by the probes YNH24, MS31, MS43a, and pMLJ14. Although the value of the exercise may be limited by the relatively small database there was no evidence to suggest that the assumption of independence is invalid for any of these probe combinations.

Conclusions

The wide allele distribution (700-7000bp), the high percentage heterozygosity (93.5%) and the relatively low percentage paternal mutation rate (1.2%), indicated that the VNTR probe TBQ7 (Promega Corporation) is an extremely informative probe for use in paternity testing.

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Results and Discussion

The chemiluminescent DNA probe TBQ7 (Promega Corporation) was found to detect a highly variable locus with the distribution of allele sizes ranging from 700-7000bp.

The heterozygosity was calculated as 92.8% which concurs with a more limited study of Bragg et al [2]. The heterozygosity of this probe was compared to published data on allele frequency distributions of four other VNTR loci [3] and the data presented in Table 1.

Two paternal and four maternal mutations were detected in this study giving a TBQ7 paternal mutation rate of 0.012 (2/167), and a maternal mutation rate of 0.021 (4/192). The paternal mutation rate for TBQ7 was compared with published data on paternal mutation rates for other VNTR loci [5] and the results are shown in Table 2.

PROBE	ALLELE SIZE RANGE (Kbp)	HETEROZYGOSITY %
TBQ7	0.7 - 7.0	92.8
MS1	1.2 - >20	96.1
MS31	1.8 - 15	91.8
MS43a	2.6 - >20	87.8
YNH24	1.6 - 9.2	88.3

TABLE 1 COMPARISON OF ALLELE SIZE RANGES AND PERCENTAGE HETEROZYGOSITY FOR FIVE SINGLE LOCUS PROBES [3]

PROBE	MATERNAL MEIOSES			PATERNAL MEIOSES		
	TOTAL	MUTATED	% MUTATED	TOTAL	MUTATED	% MUTATED
TBQ7	192	4	2.08	167	2	1.2
MS1	1116	45	4.03	591	36	4.16
MS31	1127	2	0.18	600	13	1.48
MS43a	1123	1	0.09	594	5	0.57
G3	1115	2	0.18	589	6	0.69
YNH24	471	1	0.21	344	0	0.00

TABLE 2 MEIOSES AND MUTATION RATES REPORTED AT 6 VNTR LOCI [1][5][4]

Sensitivity Experiments

The sensitivity of TBQ7 was tested with concentrations of 1ug to 50ng K562 genomic DNA. 150-200ng DNA was the limit of detection at about 1200bp. [6][3]

Hybridisation of K562 genomic DNA produced two alleles. Calculations based on 196 measurements showed the mean size of the two bands generated as 1748bp (sd=10) and 1178bp (sd = 8.5).

On occasion during this study the TBQ7 profiles generated showed more than two bands. Analysis of 20 such profiles that showed more than two bands showed that the difference in molecular size between the upper and 'true' lower band was 203bp (range 179-229).

Statistical analysis

The raw data was generated by analysis of the DNA profiles of 374 unrelated individuals, ie mothers and fathers of unrelated family trios. Binning the data into 100bp bins allowed the comparison of the TBQ7 data and that generated for MS1, MS31, MS43 and YNH24 [1].

A 2.8% sliding window fit was applied to the raw data shown in Figure 2. This involved calculating the frequency for each allele at 5 bp intervals based on a +/- 2.8% bin. The resulting plot is shown in Figure 3.

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A UK CAUCASIAN DATABASE FOR THE TBQ7 (D10S28) LOCUS DERIVED FROM BLOOD SAMPLES SUBMITTED FOR PATERNITY ANALYSIS

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SUMMARY

The purpose of this study was to assess the potential value of the probe TBQ7, (locus D10S28), obtained from the Promega Corporation, for use in paternity testing.

The data was derived from DNA profiles obtained from blood samples submitted for parentage testing, and received by the FSS between April 1992 and January 1993. Only samples from Caucasian individuals were used in this study.

Raw data from DNA profiles derived from 374 unrelated Caucasian individuals is presented. The bands obtained ranged in size from about 700-7000bp. Twenty seven one banded individuals (OBI) were observed.

The raw data was smoothed using a 2.8% sliding window fit and a maximum frequency of approximately 0.08 observed. The heterozygosity was calculated to be 92.8%.

As the data was derived from individual DNA profiles obtained during parentage testing we were able to estimate the paternal and maternal mutation rates of 1.2% and 2.1% respectively.

METHODS

The DNA profiles were prepared using standard FSS protocols.

Hybridisation

The membranes used in this study had been previously hybridised with a series of single locus DNA probes, MS43a (D12S11), MS31 (D7S21), YNH24 (D2S44), and pMLJ14 (D14S13).

The membranes were incubated at 50oC for 20 minutes in hybridisation buffer containing 2.5ul TBQ7/ml buffer (Promega), and 0.2ul MW100/ml buffer (Cellmark).

Development and Detection of the DNA profiles

The membranes were dipped into Lumiphos (Cellmark Diagnostics) and the excess enzyme substrate removed by squeezing the membranes between two acetate sheet.

The autoradiographs were developed for 1-2 days at 37oC. Band sizes were determined by means of a video based image analysis system, (Foster and Freeman Ltd).

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RESULTS AND DISCUSSION.

Figure 1 illustrates 10 HLA-DQA1 different phenotypes (all of them carrying the HLA-DQA1*4 allele) as analyzed by PCR followed by a combined SSO/RFLP method of detection. As can be seen, the analysis of HLA-DQA1 homoduplexes after Rsa I and Fok I digestion by discontinuous polyacrylamide gel electrophoresis under non-denaturing conditions allowed, according to the polymorphic patterns described by Ju et al. (1991) (Table 1), a clear distinction of the HLA-DQA*0401, *0501 and *0601 alleles. It is necesary to point out, for a correct interpretation of the electrophoretic patterns, that the electrophoretic mobility of the Rsa I and Fok I digests is determined not only by size but also by conformational features. For instance, the 230 bp fragment obtained after Rsa I digestion of the HLA-DQA1*0103 allele showed lower mobility than the 230 bp fragments of the HLA-DQA1*0601 and HLA-DQA1*0201 alleles (Fig. 1, compare lanes 3, 4 and 5). In the same way, the 245 bp fragment obtained after Fok I digestion of the HLA-DQA1*0102 and *0103 alleles showed lower mobility than the 245 bp fragment of the HLA-DQA1*0301 (Fig. 1, compare lanes 1, 3 and 8).

The allele frequencies for HLA-DQA1 in a population sample of 206 unrelated individuals living in Madrid and in a population sample of 211 unrelated individuals autochthonous from the Basque Country are shown in table 2. No evidence of deviations from the Hardy-Weinberg equilibrium was observed in this two populations when the observed and expected genotypes numbers were compared by means of the Chi-squared test (Table 2). The allele frequencies of these two spanish population samples were compared by a 2-way RxC contingency table to determine whether significant differences could be observed. The data showed significant differences between the two spanish populations analyzed (Chi-square=17.11; P=0.017; df=7). The major differences in allele distributions were obsreved for the HLA-DQA1*0501 allele (Madrid: 29.8%, Basque Country: 18.2%) and the HLA-DQA1* 0201 allele (Madrid: 16.7%, Basque Country: 21.6%).

On the other hand, the comparison of the HLA-DQA1 allelic data presented here with those reported in other Caucasians populations [5-10] showed that the Basque Country (autochthonous) data were significantly different from the rest of the Caucasian populations, while the Madrid (resident) data only show significant differences when compared to the Finland, British and USA data.

ACKNOWLEDGEMENTS

The authors are grateful to Angela Zamora for the photographic work.

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	ALLELES							
	*0101	*0102	*0103	*0201	*0301	*0401	*0501	*0601
Rsa I	201, 39	201, 39	230	230	201	198	198	230
Fok I	245	245	245	196, 49	245	196	242	196

Table 1. Electrophoretic patterns (in base pairs) of HLA-DQA1 alleles after Rsa I and Fok I digestion . (Adopted from reference 1).

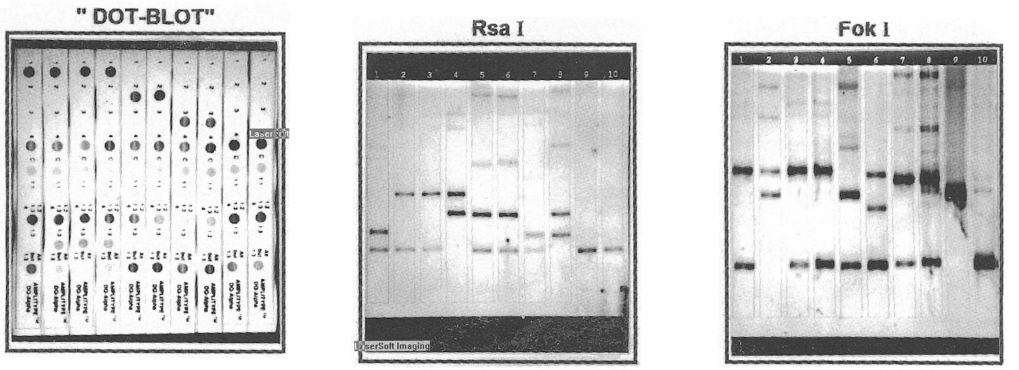

Fig. 1. HLA-DQA1 phenotypes analyzed by PCR and a combined SSO/RFLP method of detection samples: 1. HLA-DQA1*0102/*0401, 2. *0103/*0501, 3. *0103/*0401, 4. *0103/*0601, 5. *0201/*0501, 6. *0201/*0401, 7. *0301/*0501, 8.*0301/*0601, 9. *0501/*0501 and 10. *0401/*0401.

	ALLELES							
	*0101	*0102	*0103	*0201	*0301	*0401	*0501	*0601
Madrid	0.160	0.153	0.087	0.167	0.109	0.022	0.298	0.002
B. C.	0.187	0.173	0.102	0.216	0.104	0.033	0.182	0.002

	PD	h	Heterozy.	X^2 (df)	p
Madrid	0.936	0.816	81.07%	25.69 (18)	0.10<p<0.20
B. C.	0.949	0.835	79.15%	23.27 (20)	0.20<p<0.30

Table 2. HLA- DQA1 allele frequencies from 206 individuals resident in Madrid and from 211 autochthonous individuals from the Basque Country (B. C.). The power of discrimination (PD), allelic diversity value (h), heterozygosity and chi-square (X^2) are also shown.

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HLA-DQA1 SUBTYPING BY PCR FOLLOWED BY A COMBINED SSO/RFLP METHOD OF DETECTION. DISTRIBUTION OF ALLELES AND GENOTYPES IN TWO SPANISH POPULATIONS.

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INTRODUCTION.

The HLA-DQA1 locus has become a well defined genetic marker of routine use in forensic haemogenetics. Forensic HLA-DQA1 genotyping has been greatly facilitated by the development of a PCR based Kit (Amplitype HLA-DQalpha, Perkin-Elmer, Cetus Corporation). However this method allows to distinguish only 6 of the 8 alleles identified in the HLA-DQA1 locus.
In this article we present a method for the genetic characterization of the HLA-DQA1 locus by employing PCR coupled with hybridization to sequence-specific oligonucleotide probes, for the detection of the 6 most common alleles, followed by an allele-specific restriction fragment length polymorphism analysis (endonucleases Fok I and Rsa I) for HLA-DQA1*4 subtyping [1].
The method has been applied to determine the HLA-DQA1 allele and genotype frequencies in two spanish populations.

MATERIALS AND METHODS.

Blood Samples and DNA extraction

DNA was extracted from blood of 206 unrelated individuals living in Madrid and 211 unrelated autochthonous individuals from the Basque Country using the chelex resin extraction approach [2].

Amplification and "Dot-Blot" Typing

The amplification of the HLA-DQA1 locus by PCR using biotinylated primers and the analysis of the alleles by hybridization to filter strips carrying immovilized allele specific oligonucleotide DNA probes were performed using the Amplitype HLA-DQalpha Forensic DNA Amplification and Typing Kit (Perkin-Elmer Cetus).

Endonuclease digestion and electrophoresis

All samples carrying the allele 4 were digested using two restriction enzymes: Rsa I and Fok I [1]. The digested fragments were separated by vertical discontinuous polyacrylamide (11%T, 3%C) gel electrophoresis using the Tris-chloride/Tris-glycine buffer system [3]. After electrophoresis the digested DNA fragments were detected by silver stain [4]. The specific electrophoretic patterns of the different HLA-DQA1 alleles after Rsa I and Fok I digestions are shown in table 1 (according to reference 1).

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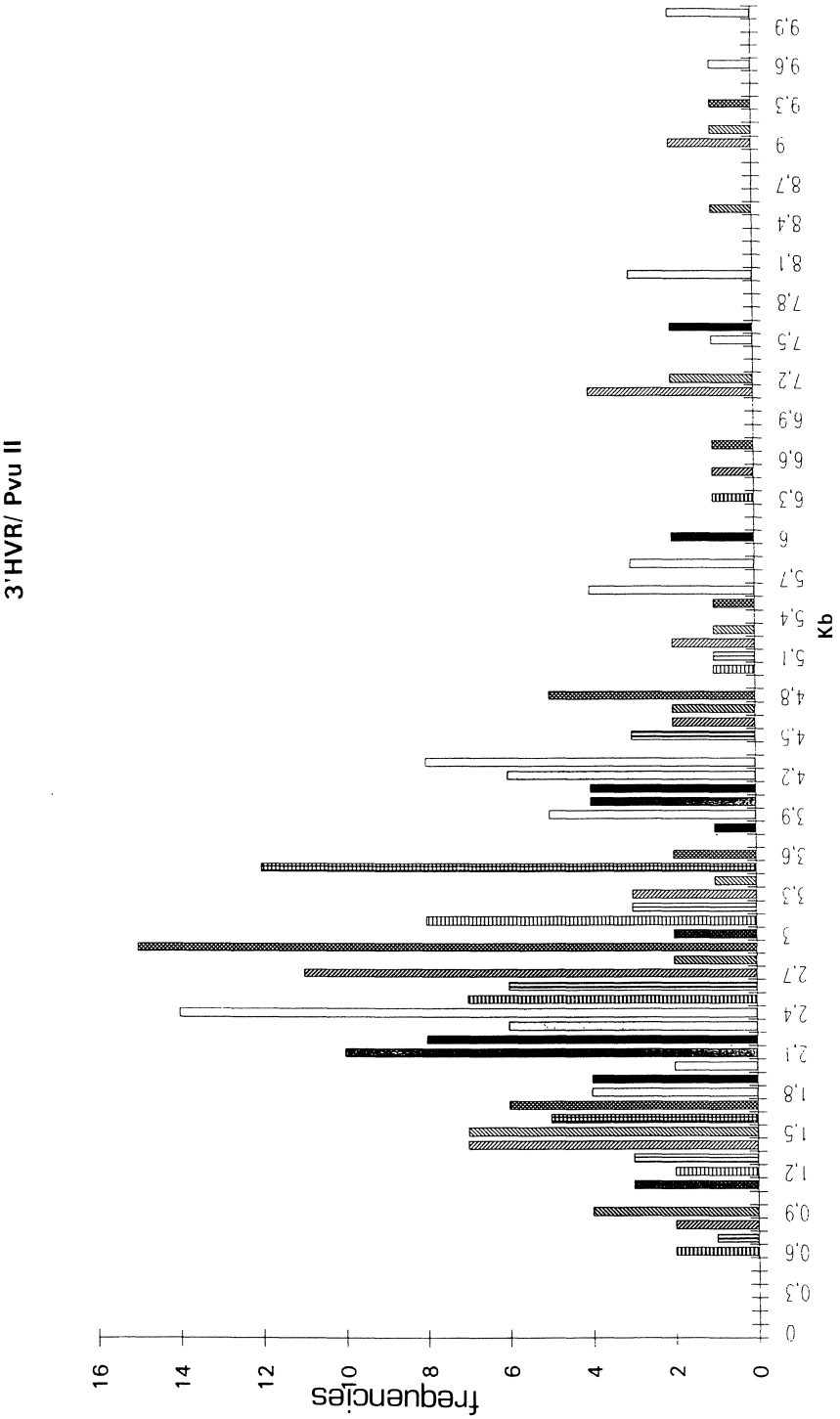

Figure 1. Allelic distribution in the population studied.

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the filters washed in stringent baths; allelic classes are determinated by at least 100 pb of electrophoretic resolution.

Results: Figure 1 shows allelic repartitions in frequencies of the 62 alleles obtained from a sample of n = 120 unrelated individuals ($2n$ chromosomes = 240). The most frequent heterozygous genotype is at 2.9-2.4 kb, with a frequency = 0.033. Calculations show that these diverse alleles are in Hardy-Weinberg equilibrium. Heterozygosity is of 90%. The discriminating power (2) of the HVR probe is $Dp = 1-\Sigma^{P2}j$, j representing allelic frequencies of each genotype = 1-0.0276 = 0.973, an excellent performance.

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Study of allele frequencies distribution of the 3'-HVR marker near α-globin gene in the french population

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The human genome contains some hypervariable regions (HVR), resulting from various combinations, between individuals in populations, of short portions of DNA tandemly repeated. The 5'-HVR sequence is located at 8 kb, in the indicated sense, of the α-globin gene on the short arm of chromosome 16. The corresponding probe was used in France in some cases of paternity disputes (1). We report here a study concerning polymorphisms of these sequencies in a French control population.

Material and methods: DNA were extracted from venous blood of a french collection of unrelated individuals, by digestion with proteinase K and phenol-chloroform extraction ; 10 µl of DNA from each sample was restricted with *Pvu*II. Restriction fragments obtained were separated on 1% agarose gel, and transfered on nitrocellulose filters. After labelling with ^{32}P, the probe obtained was hybridized with filters, and

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ACTBP2 is a very polymorphic locus. The degree of polymorphism is such that ACTBP2 can be a very informative tool for laboratories interested in human identification. However, the complexity of ACTBP2 necessitates careful designation of alleles, so interlaboratory comparisons can be meaningful. Further work should be performed in order to classify alleles in this system before it is adopted for forensic identification. The potential of a two base pair difference in size between two alleles requires that a laboratory show that it has achieved two base pair resolution; otherwise a binning strategy for allelic data may be necessary. We have amplified ACTBP2 alleles with different sets of primers such that the amplified products for the same allele differ in length by two bases. When such amplified alleles are co-electrophoresed in the same lane, they can be resolved (data not shown).

Future developments for ACTBP2 should focus on producing a common allelic ladder which can be used in a variety of laboratories. Additionally, comparison of different electrophoretic systems may have to be compared for typing efficacy of ACTBP2.

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60 C x 10 sec	+	64 C x 10 sec
72 C x 60 sec		72 C x 60 sec

3. Gel Electrophoresis Conditions: according to Lorente, et al [4].

5. Silver Staining: according to Budowle, et al [5].

6. Allele numbers were assigned after comparison with an allelic ladder.

RESULTS & DISCUSSION

SE33 was highly polymorphic in our Spanish population sample (Fig. 1). We have observed 22 different allele categories, with frequencies ranging from 0.013 (alleles 2, 12 and 22) to 0.125 (allele 7); all the other alleles have frequencies less than 0.096. The observed heterozygosity was 89.2%

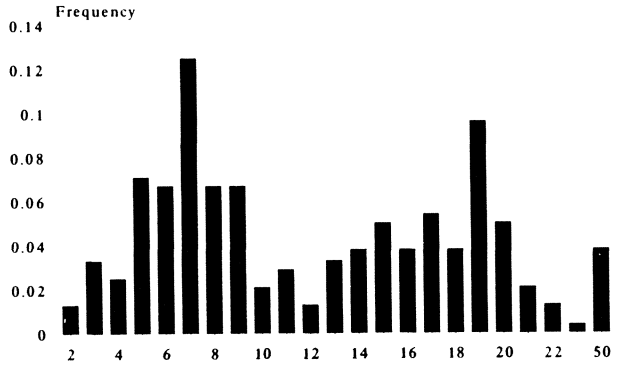

Figure 1. Histogram of ACTBP2 allele/bin frequency distribution for 120 Spanish Caucasians.

As a convention for naming alleles, the ladder bands were given an even number designation (i.e., 2-22). If an allele migrated the same distance as a band in the ladder, the allele was designated with a corresponding even number. If the allele fell between two bands of the ladder, it was given the appropriate odd number designation.

A test for independence for ACTBP2 can be problematic because of the allele designation strategy used. The even numbered alleles generally represent discrete data, while the odd numbered allele frequencies are derived from binned data. This is complicated further by the limited resolution and measurement error of the electrophoretic system. Regardless, the exact test [6] was used for a test of allelic independence. The data suggest that .....

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SPANISH POPULATION DATA FOR THE SHORT TANDEM REPEAT ACTBP2 (SE33)

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INTRODUCTION

Short tandem repeat (STR) loci can exhibit a high degree of polymorphism. One of these STR loci, called ACTBP2 (or SE33) is highly polymorphic and has been reported to be rather robust for forensic analyses [1].

For ACTBP2 to be useful for identity testing, some population data are desirable. This paper describes frequency data for ACTBP2 alleles from a Spanish sample population.

MATERIALS AND METHODS

Population: 120 unrelated Spanish Caucasians from Andalucía.

DNA isolation: organic extraction [2]

DNA quantitation: slot-blot + probe D17Z1 [3]

Primer sequences: 5′-AATCTGGGCGACAAGAGTGA-3′
5′-ACATCTCCCCTACCGCTATA-3′

ANALYTICAL CONDITIONS

1. PCR reaction (final volume: 50 µl)
5 ng DNA
1 µl dNTPs each (0.2 µM concentration each)
5 µl 10X Taq polymerase buffer
1 µl Primer A (from 12.5 µM stock)
1 µl Primer B (from 12.5 µM stock)
0.5 µl Taq polymerase (2.5 IU)
Qs H_2O to 50 µl

2. Thermal Cycler (Perkin Elmer 9600)

Two different sets of cycle programs were linked for a total of 26 cycles as follows:

# 1: 10 cycles.	# 2: 16 cycles.
94 C x 10 sec	90 C x 10 sec

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Table 1c shows that eight out of the ten Negroid subgroup comparisons yielded significant differences. It can be seen from Table 1d that none of the ethnic-groups included in any subgroup differed significantly from any other member of the same subgroup.

a)

	1
1. Caucasoids (378)	-
2. Negroids (928)	**0.49**

b)

	1	2	3
1. European gentiles (230)	-	0.33	**0.52**
2. Ashkenazi-Jews (70)		-	**0.52**
3. Asian Indians (78)			-

c)

	1	2	3	4	5
1. Nguni (298)	-	.26	**.35**	**.42**	**.43**
2. Sotho-Tswana (170)		-	.26	**.51**	**.51**
3. Venda-Lemba (144)			-	**.57**	**.64**
4. Herero-Dama (150)				-	**.39**
5. Khoisan (166)					-

d)

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Xhosa (40)	-	.52	.42	.36	.43	.54	.40	.48	.59	.47	.42	.47	.50
2. Zulu (102)		-	.35	.32	.36	.33	.42	**.46**	**.58**	**.52**	**.58**	**.66**	**.55**
3. Swazi (76)			-	.39	.55	.43	.51	**.46**	**.48**	**.40**	**.48**	**.60**	**.48**
4. Tsonga (80)				-	.40	.47	.50	**.49**	**.52**	**.43**	**.49**	**.53**	**.45**
5. Sotho (92)					-	.41	.42	**.47**	**.56**	**.54**	**.47**	**.55**	**.50**
6. Tswana (38)						-	.43	.57	.43	**.62**	**.62**	**.72**	**.56**
7. Pedi (40)							-	.57	.58	**.65**	**.67**	**.67**	**.56**
8. Lemba (84)								-	.54	**.57**	**.62**	**.75**	**.64**
9. Venda (60)									-	**.67**	**.73**	**.93**	**.71**
10. Herero (88)										-	.35	**.45**	**.44**
11. Dama (62)											-	**.46**	.44
12. Nama (74)												-	.35
13. San (92)													-

Table 1. Hellinger distances between various groups (significant distances are in boldface and underlined). The figure given after the name of each group, reflects the number of alleles in the sample. (a) A comparison of pooled Negroid and Caucasoid samples. (b) Caucasoid subgroups compared. (c) Negroid subgroups compared and (d) Negroid ethnic-groups compared.

From Table 1 and Figure 1, it can be seen that Hellinger distances between Negroid peoples who live relatively close to each other e.g. ethnic-groups belonging to the Nguni and Sotho-Tswana subgroups, tended to be insignificant. This may be indicative of past and/ or present gene flow between such groups. In contrast, Hellinger distances between ethnic-groups who live far apart e.g. the Zulu and Herero, or between those who belong to different linguistic groups e.g San and Dama, tended to be significant. The fact that the Xhosa group did not differ significantly from any other ethnic-group may be due to the small sample size (20 individuals) and/or admixture. Jenkins *et al* (1970) presented evidence indicating that the Xhosa have more Khoisan admixture than any other Bantu-speaking population.

CONCLUSION

Various southern African ethnic-groups have significantly different apoB 3'VNTR allele frequency distributions. This should be taken into account when using apoB 3' VNTR data for forensic purposes.

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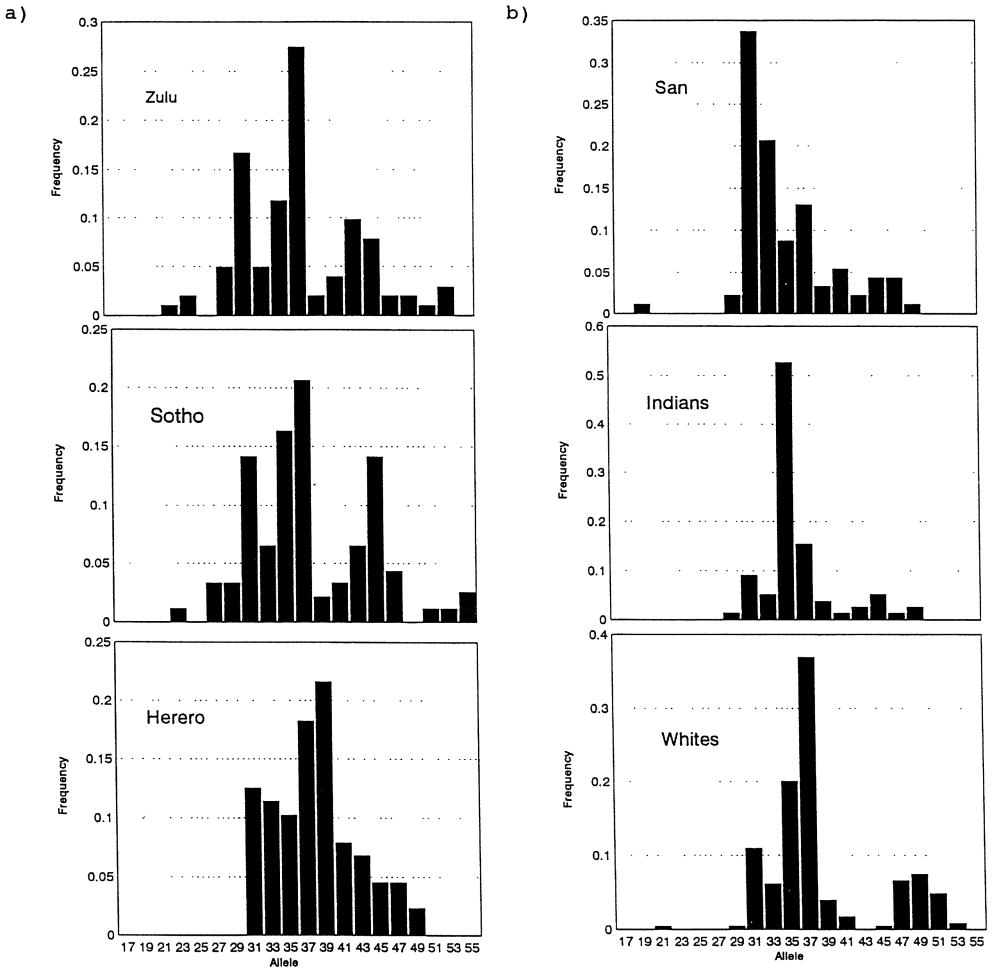

Fig. 2. Allele frequency distributions of representative ethnic groups: (a) Negroid groups. (b) Khoisan and Caucasoid groups.

The Hellinger distance between the pooled Negroid and Caucasoid samples is shown in Table 1a; the distance was found to be significant. The Caucasoid sample was divided into a European gentile, an Ashkenazi-Jewish and an Asian Indian subgroup and these subgroups compared to each other (Table 1b). The two main Caucasoid groups viz. the European gentiles and Asian Indians have significantly different allele frequency distributions. The Ashkenazi-Jewish sample did not differ significantly from the European gentile subgroup but did differ from the Indian group.

An attempt was then made to determine whether significant differences were apparent among the major Negroid subgroups. The following subgroups were constituted by pooling the data from various ethnic-groups on the basis of linguistic affiliation and geographic proximity (the ethnic groups of each subgroup are given in parenthesis):

1. Nguni (Zulu, Xhosa, Swazi and Tsonga)
2. Sotho-Tswana (Sotho, Tswana and Pedi)
3. Lemba-Venda (Lemba and Venda)
4. Herero-Dama (Herero and Dama)
5. Khoisan (Nama and San).

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APOB 3' VNTR POLYMORPHISM IN SOUTHERN AFRICAN POPULATIONS

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INTRODUCTION

Inter-ethnic differences in allele frequency distributions of VNTR containing loci exist and should be taken into account when using such systems for forensic purposes. The aim of the present study has been to determine whether significant differences in apoB 3' VNTR allele frequency distribution are detectable between southern African Negroids and Caucasoids and between subdivisions of these groups.

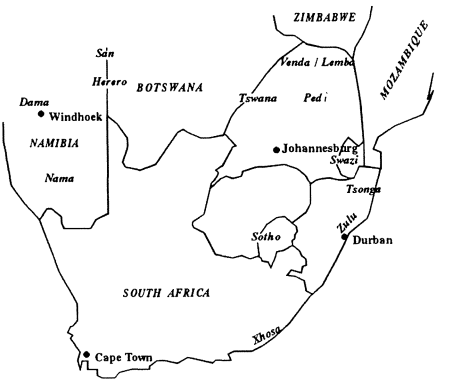

Fig. 1. A simplified representation of the approximate locations of the non-Caucasoid populations studied. The European Caucasoid and the Asian Indian populations were sampled in Johannesburg.

MATERIALS AND METHODS

The apoB 3' VNTR alleles of 653 individuals from 16 southern African populations were amplified by the polymerase chain reaction (Boerwinkle *et al* 1989) and sized by electrophoresis in agarose gels (Marques 1992). Each of the 16 population samples was checked for possible deviation from Hardy-Weinberg equilibrium using a bootstraping method described by Devlin and Risch (1992a). The allele frequency distributions of the pooled Negroid and Caucasoid groups, subgroups and ethnic-groups were compared by calculating the Hellinger distance, H, (Devlin and Risch 1992b) between them.

$$H = \sqrt{\left(\sum_{i=1} \sqrt{\pi_i^{(1)}} - \sqrt{\pi_i^{(2)}} \right)^2}$$

where $\pi_i^{(1)}$ and $\pi_i^{(2)}$ are the frequencies of allele "i" in the two populations being compared. The significance of each Hellinger distance was then assessed by combining the data from the two samples being compared and creating two pseudo-samples (with the same number of alleles as the original samples) by selecting alleles at random from the pooled sample. The Hellinger distance between the two pseudo-samples was then calculated and compared to the true Hellinger distance. This process was repeated up to 500 times. If the simulated distance exceeded the true distance in fewer than 5% of trials, the difference between the actual population samples was judged to be significant.

RESULTS AND DISCUSSION

The genotype frequencies of all the ethnic-group samples were consistent with Hardy-Weinberg expectation (results not shown). The allele frequency distributions of some of the ethnic-groups sampled are shown in Figure 2.

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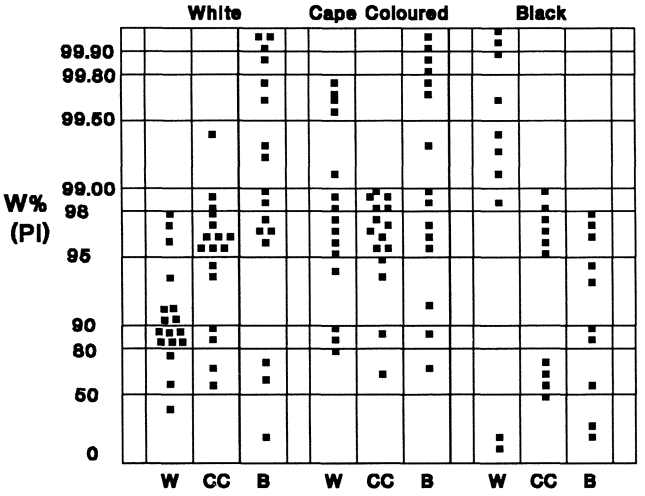

Figure 1. **43 HLA unexcluded men: PI re-calculated.**

Figure 1: The PI, calculated using own race and substituting other races in HLA unexcluded men.
The lower PI in the white group (80 - 91) is again consistent with previous findings in whites and is due to antigen sharing between alledged father and mother, mainly HLA-A1, A2 and A3 or the presence of blanks (6). The PI of false fathers not excluded by HLA, in whites, CC and blacks are all below 98.99%, but increases to levels approaching real fathers if the wrong gene frequencies are used.

Conclusion:

Although it has been the impression that the CC fall genetically midway between white and black, it is quite clear from the data in the middle column in Figure 1, that the CC group has acquired a genetic identity of their own, even if they fall midway between black and white. In our casework, it is appropriate to compare gene frequencies in other races, especially when the PI is extremely high due to unusual genes in the putative father and these genes are common in other populations in the same region. These findings will have to be verified using actual casework.

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Table 1 :

EXCLUSIONS PER INDIVIDUAL SYSTEM IN FALSE TRIOS IN RANK ORDER[1]							
WHITE (n = 74)		CAPE COL. (n = 244)		BLACK (n = 15)		TOTAL[2] (n = 333)[3]	
SYSTEM	%	SYSTEM	%	SYSTEM	%	SYSTEM	%
HLA	94.17	HLA	97.56	HLA	96.63	HLA	96.48
DBP	32.75	DBP	44.57	PGM1	28.92	DBP	33.06
PGM1	30.57	PGM1	31.43	BF	22.47	PGM1	30.43
MNS	26.21	MNS	25.82	MNS	22.39	MNS	25.15
Rh	25.41	BF	25.38	ABO	20.98	BF	23.30
ACP1	22.61	Rh	23.57	HP	19.19	Rh	22.37
BF	20.87	HP	19.15	GLO	19.09	HP	19.52
HP	20.39	ABO	17.97	Rh	16.42	ABO	18.67
ABO	16.83	ACP1	17.44	DBP	16.40	ACP1	18.02
GLO	14.55	GLO	15.67	Fy	15.09	GLO	16.38
ESD	11.40	Fy	15.57	ACP1	14.68	Fy	13.51
C3	9.09	C3	11.65	C3	10.96	C3	10.74
Fy	8.03	ESD	7.80	CA2	7.54	ESD	7.39
TF	4.62	CA2	2.19	ESD	3.17	CA2	3.17
Kell	3.24	TF	1.92	TF	2.74	TF	2.89
CA2	0	Kell	0.65	Kell	0	Kell	1.18

[1] January 1989 - July 1993.
[2] White = 309; Cape Col. = 616; Black = 268; Total = 1193.
[3] Cases with only HLA and Blood Groups.

Table 2 :

	CASES EXCLUDED IN GROUPED SYSTEMS IN FALSE TRIOS[1]							
Grouped Systems	WHITE	%	CAPE COL.	%	BLACK	%	TOTAL[2]	%
HLA	221	94.04	359	96.51	243	96.43	823	95.81
Blood Groups	156	66.38	207	55.65	145	57.54	508	59.14
Enzymes + Proteins	191	81.28	306	82.26	184	73.02	681	79.28
HLA + Bl. Gr.	230	97.87	365	98.12	249	98.81	844	98.25
HLA + Enz. + Prot.	233	**99.15**	370	**99.46**	251	**99.60**	854	**99.42**
Bl. Gr. + Enz. + Prot.	213	90.64	338	90.86	228	90.48	779	90.69
HLA + Bl. Gr. + Enz. + Prot.	235	100	371	99.73	252	100	858	99.88

[1] Cases where only HLA and Blood Group results were available were excluded from this analysis.
[2] White = 235; Cape Col. = 372; Black = 252; Total = 859.

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False Paternity Trios in white, black and Cape Coloured populations.

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Introduction:

The populations of the Western Cape region are mainly white, black (Xhosa) and the Cape Coloured (CC). The CC population are of mixed genetic background, from white, black, Khoisan and Oriental (Malay) origins. The CC is situated at some genetic mid-point between white and black groups for most genetic systems tested (1,2).

In parentage testing, one is often faced with results that reflect the presence of unusual genes for the declared race of the accused man, but genes that are not unusual for another man in the same region, but from a different race.

Methods:

False trios within the three races were created to test the power of exclusion (PE) in the 18 loci used in paternity casework. Unexcluded fathers (n = 1193) were moved down in the database according to race and date tested (January 1989 to July 1993). In 333 cases, no serum proteins or red cell enzymes were done, and they were matched within their own groups.
The PE of cases combined into three groups viz: i) HLA, ii) blood-groups and iii) red cell enzymes + serum proteins, was determined.
The Paternity Index (PI) of false fathers not excluded by HLA was calculated for the HLA system only and expressed as W% and then recalculated using the gene frequencies of the other two races.

Results:

Table 1: PE of individual systems.
The PE of HLA (94.17%) in whites was similar to those reported for American whites (94.4% and 93.5%; 4,5). The PE for HLA in blacks (96.63%) was higher than the 92.2% reported for American blacks (5).

In all HLA unexcluded false-father cases, the child had one or more blank antigens. In addition, all the white children except one, had one or more of the common white HLA-A1, A2 and A3 antigens as previously found in HLA non-exclusions (4).

The PE of the MNS, Rh, ACP1, ESD and Kell systems decreases from white to black to CC and the PE of the BF, HP, ABO, GLO and Duffy systems increases from white through CC to black, similar to findings in actual case work in the same groups (2,3). DBP is the most useful system after HLA and ranks 2nd in white and CC, but 9th in blacks.

Table 2: The combined PE in false trios.
The combined PE is 99.88% using HLA, blood groups as well as enzymes and proteins. A very high PE (> 99%) is reflected in all three race groups testing only enzymes, proteins and HLA.

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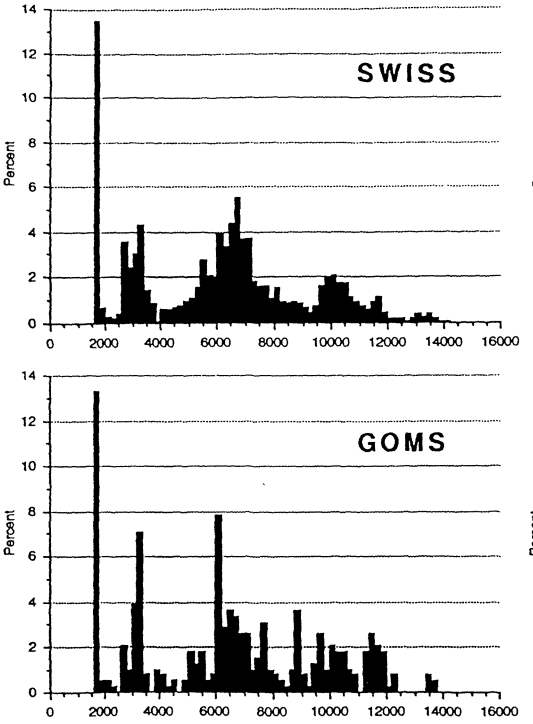

Fig. 1: Frequency distribution of probe G3

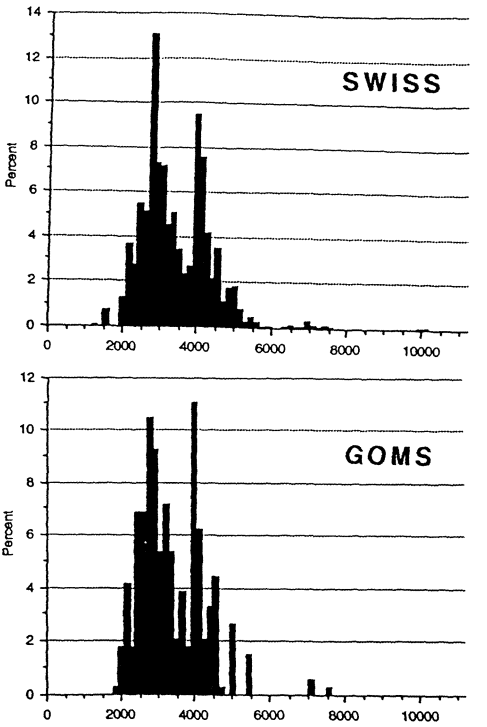

Fig. 2: Frequency distribution of probe yNH24

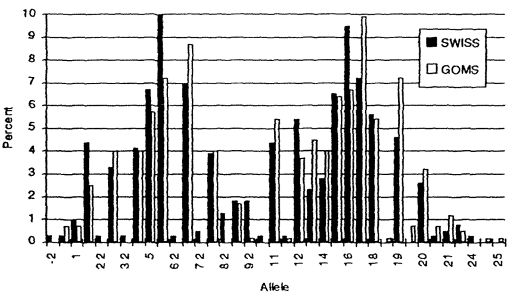

Fig. 3: Distribution of the SE33 alleles

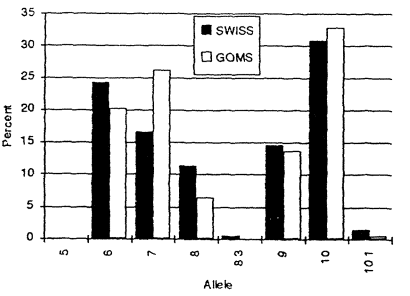

Fig. 4: Distribution of the HUMTHO1 alleles

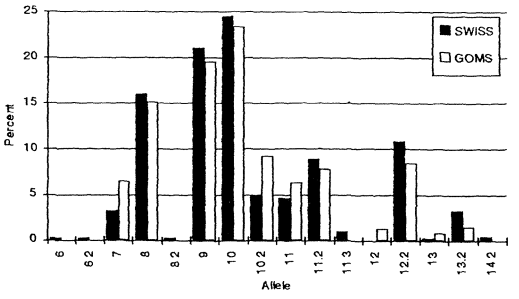

Fig. 5: Distribution of the D21S11 alleles

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results exhibit the same kind of pattern of frequency distribution for the mountain population as for the large Swiss sample. Both population samples show an equally high degree of polymorphism and no reduction of the number of the alleles in the mountain population of Goms could be observed. The differences observed are not forensically relevant.

b) STR systems SE33, HUMTHO1 and D21S11
The mountain population of Goms was also investigated for the 3 STR systems SE33, HUMTHO1 and D21S11 and compared with a general Swiss population sample of at least 195 individuals. The observed allele frequencies are shown in figure 3 - 5. In contrast to the RFLP loci the STR systems showed some remarkable differences in the distribution of the allele frequencies. In the mountain population the most common alleles of SE33 are allele 7 and 16 while in the Swiss population the most common alleles are the alleles 6 and 17. The systems HUMTHO1 and D21S11 also show differences in the allele frequency of some common alleles. For all 3 examined STR systems the observed number of alleles is greater in the general Swiss population than in the mountain population. Almost all rare alleles obtained in the Swiss population could not be found in the mountain population of Goms.

4. Discussion

This study showed that for the 4 investigated RFLP it can be excluded that the degree of polymorphism is reduced in smaller and apparently isolated populations. The frequency distribution is also comparable to those reported for other Caucasian populations [5]. The 3 examined STR sytems show indeed some noticeable differences in the distribution of the allele frequencies but the differences are also of no practical forensic significance.

Acknowledgments: The authors thank R. Fatzer, E. Garbely, K. Schluep and M. Stadler for excellent technical assistance.

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RFLP AND STR POLYMORPHISMS IN A SWISS MOUNTAIN POPULATION

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1. Introduction

In the Swiss Alps the inhabitants live to some extent in geographically separated regions and can form relatively isolated poulation groups. It has been assumed that such mountain populations would have different allele distributions from those observed in the general Swiss population. In order to determine if such differences exist approximately 200 unrelated individuals of a mountain region in the Rhone Vallely called Goms were examined. The 4 RFLP loci D12S11, D7S21, D7S22 and D2S44 and the 3 STR systems SE33, HUMTHO1 and D21S11 were analyzed and compared with a general Swiss Population sample.

2. Methods

High molecular DNA was isolated from 250 µl EDTA blood by Proteinase K / Phenol / Chloroform extraction.

a) RFLP: 1 - 2 µg DNA was digested with the restriction enzyme Hinf I. Electrophoretic separation was performed in 1% agarose gels (Sigma type II) in 1 x TBE buffer without ethidium bromide at room temperature for about 60 hours at 20 V. After Southern Blotting onto nylon membranes (Amersham Hybond N) hybridisation was done with the single locus probes MS43a, MS31, G3 and yNH24 using random primer oligolabelling [3] or the non-isotopic chemiluminescence detection described by Dimo-Simonin et al. [1]. Band lengths were measured with a BioImage Visage System of Millipore. Sizes of band fragments were calculated by the local reciprocal logarithmic approach of Elder and Southern [2].

b) STR systems SE33, HUMTHO1 and D21S11
The PCR-products of the 3 STR systems SE33 (5'->3' AAT CTG GGC GAC AAG AGT GA; ACA TCT CCC CTA CCG CTA TA) [6], HUMTHO1 (5'->3' GTG GGC TGA AAA GCT CCC GAT TAT; GTG ATT CCC ATT GGC CTG TTC CTC) [4] and D21S11 (5'->3' GTG AGT CAA TTC CCC AAG; GTT GTA TTA GTC AAT GTT CTC C) [7] were determined and detected on an ABI 373A automated DNA sequencer (ABI GeneScan 672). The Genescanner enabeled the simultaneous analysis of the 3 fluorescently labeled STR products and the automatically calculation of the allele length.

PCR reaction mixture: 10 ng template DNA, 5 µl 10x PCR buffer (GeneAMP, Perkin Elmer Cetus), 8 µl 1.25 mM dNTP's, 1 µl 12.5 µM of each primer (one primer labeled with a fluorescent dye), 1.5 U AmpliTaq DNA Polymerase (Perkin Elmer Cetus), 4 µl 0.2% BSA, sterile H_2O was added to a final volume of 50 µl; the reaction mixture was overlayed with a drop of oil.

Amplification conditions (Thermocycler: Biometra Triothermoblock):
SE33 and HUMTHO1: 94°C - 45 sec., 60°C - 30 sec., 72°C - 30 sec; 30 cycles.
D21S11: 94°C - 45 sec., 55°C - 30 sec., 72°C - 30 sec.; 30 cycles.
SE33 and HUMTHO1 were coamplified.

3. Results

a) RFLP loci D12S11, D7S21, D7S22 and D2S44
DNA samples of at least 500 unrelated Swiss individuals were investigated for the 4 RFLP loci D12S11, D7S21, D7S22 and D2S44. In collaboration with the Institute of Anthropology of the University of Zürich we could also examine a population sample of a relatively isolated mountain region in the Swiss Alps. The mountain region is called Goms and is part of the upper Rhone Valley of the Canton Valais. Approximately 200 unrelated individuals of this mountain region were analyzed; the frequency distributions obtained for the probes G3 and yNH24 are shown in figure 1 - 2. The

Table 1: Allele frequencies for SE33, D21S11, HUMTHO1, D1S80 and DQα.

Allele	SE33 Frequency	Allele	D21S11 Frequency	Allele	HUMTHO1 Frequency	Allele	D1S80 Frequency
-2	0.003	6	0.003	5	0.002	17	0.007
-1	0.003	6.2	0.003	6	0.243	18	0.261
1	0.01	7	0.033	7	0.166	19	0.002
2	0.044	8	0.16	8	0.114	20	0.03
2.2	0.003	8.2	0.003	8.3	0.005	21	0.017
3	0.033	9	0.211	9	0.146	22	0.05
3.2	0.003	10	0.246	10	0.309	23	0.025
4	0.041	10.2	0.048	10.1	0.015	24	0.328
5	0.067	11	0.046			25	0.07
6	0.1	11.2	0.089			26	0.017
6.2	0.003	11.3	0.01			27	0.022
7	0.069	12.2	0.109			28	0.032
7.2	0.005	13	0.003			29	0.047
8	0.039	13.2	0.033			30	0.017
8.2	0.013	14.2	0.005			31	0.05
9	0.018					32	0.007
9.2	0.018					33	0.002
10	0.003					34	0.007
11	0.044					36	0.002
11.2	0.003					37	0.002
12	0.054						
13	0.023						
14	0.028						
15	0.065						
16	0.095						
17	0.072						
18	0.056						
19	0.046						
20	0.026						
20.2	0.003						
21	0.005						
23	0008						
24	0.003						

Allele	DQ α Frequency
1.1	0.134
1.2	0.17
1.3	0.086
2	0.167
3	0.119
4	0.324

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genotypes; 90 genotypes were represented only once. The power of discrimination for SE33 is 0.989.

HUMTHO1: DNA samples of 197 individuals were amplified and typed for the system HUMTHO1. We found five common alleles with frequencies between 11.4% and 30.9% and one rare allele (allele 5) with a frequency of 0.2%. There also exist two variants designated as 8.3 and 10.1, which differ by just one base from the main alleles 9 and 10, respectively (see table 1).We obtained 20 different genotypes; the most frequent genotype was 6/10 with 13.8%. The power of discrimination is 0.92.

D21S11: Among 200 examined individuals 15 different alleles were detected. In the Swiss population there are 9 common alleles (3.3% and 24.6%) and 6 rare alleles with frequencies between 0.3 and 1%. The allele frequencies are listed in table 1. We found 42 genotypes; the most frequent genotype is the homozygote type 10/10 with 8.2%. The power of discrimination for this system is 0.956.

HLA DQα: A sample of 227 individuals were analyzed for the HLA DQα locus. All 6 described alleles and 21 genotypes were found. We obtained allele frequencies between 8.6% and 32.4% (see table 1). The most common allele in the Swiss population is HLA DQα 4. The observed heterozygosity rate is 80.2 % and the power of discrimination 0.93.

D1S80: DNA samples of 201 Swiss individuals were typed for the D1S80 locus. We found 20 different alleles; their length ranges between 371 and 739 bp (17 - 37 repeat units of 16 bp). The allele frequencies are shown in table 1. The most common alleles are the allele 24 with a frequency of about 33% and the allele 18 with a frequency of 26%. 61 different genotypes could be observed. The most frequent genotypes are 18/24 (19%), 24/24 (10%) and 18/18 (8%). The power of discrimination is 0.95. For all 5 PCR-systems the Hardy-Weinberg equilibrium was tested; the genotype frequencies did not deviate from a Hardy Weinberg equilibrium.

4. Discussion

For the PCR-Systems HLA DQα, D1S80 and HUMTHO1 the distributions of allele frequencies in our Swiss population sample are similiar to those observed in other Caucasians, e.g. in Germany, in England and in the Netherlands % [3, 6, 8, 9]. The more complex STR-systems SE33 and D21S11 are difficult to compare with other population studies because there is no standardisation of allelic ladder markers and nomenclature, but this does not exclude the application of this powerful marker systems in individual laboratories.

The automated analysis of the STR-systems by use of fluorescence detection and internal lane standards demonstrates many advantages. There are no typing problems because of electrophoretic migration variability and bandshifting. One can simultaneously analyze at least 3 STR-systems . The precision of measurements is one base for allele lengths of less than 300 bp. The automated analysis by the Genescanner provides higher accuracy, reproducibilty and sensitivity than traditional techniques. The 5 investigated polymorphic systems are reliable and sensitive marker systems that are well suited for use in forensic casework.

Acknowledgments:The authors thank R. Fatzer and E. Garbely for excellent technical assistance.

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Swiss Population Data For 3 STR-Systems (SE33, HUMTHO1, D21S11), HLA DQα And D1S80

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1. Introduction

The application of any new genetic marker system in forensic casework requires a sufficiently large data base of the relevant population. This study presents a data base of the 5 PCR-systems SE33, HUMTHO1, D21S11, HLA DQα and D1S80 for a Swiss population sample. Allele and genotype frequencies were determined of at least 195 unrelated individuals.

2. Methods

a) STR-Systems SE33, HUMTHO1 and D21S11

Primer sequences: SE33 [5]: 5'->3' AAT CTG GGC GAC AAG AGT GA; ACA TCT CCC CTA CCG CTA TA . HUMTHO1 [2]: 5'->3' GTG GGC TGA AAA GCT CCC GAT TAT; GTG ATT CCC ATTGGC CTG TTC CTC. D21S11 [7]: 5'->3' GTG AGT CAA TTC CCC AAG; GTT GTA TTA GTC AAT GTT CTC C.

PCR reaction mixture: 10 ng template DNA, 5 µl 10x PCR buffer (GeneAMP, Perkin Elmer Cetus), 8 µl 1.25 mM dNTP's, 1 µl 12.5 µM of each primer (one primer labeled with a fluorescent dye), 1.5 U AmpliTaq DNA Polymerase (Perkin Elmer Cetus), 4µl 0.2% BSA, sterile H_2O was added to a final volume of 50 µl; the reaction mixture was overlayed with a drop of oil.

Amplification conditions (Thermocycler: Biometra Triothermoblock):
SE33 and HUMTHO1: 94°C - 45 sec., 60°C - 30 sec., 72°C - 30 sec; 30 cycles.
D21S11: 94°C - 45 sec., 55°C - 30 sec., 72°C - 30 sec.; 30 cycles.
SE33 and HUMTHO1 were coamplified.

Analysis and detection: The PCR-products were analyzed and detected on an ABI 373A automated DNA sequencer (ABI GeneScan 672). The electrophoresis was carried out on 6 % denaturing polyacrylamide gels according to the ABI protocol (running conditions: 1600 V, 24mA, 8hs). The PCR-products of the 3 STR-systems are labeled with different fluorescent dyes (blue, green and yellow), therefore they were analyzed together on the same gel. An internal lane standard coelectrophoreses with the PCR-products in each lane using a color (red) different from the STR-samples. The standard is used to build up a calibration curve for each gel lane. The lengths of the STR-alleles are automatically calculated from the calibration curve.

Nomenclature: The alleles of SE33 and HUMTHO1 were designated as suggested in the collaborative STR-exercise of EDNAP [2]. For D21S11 we chose the following designation: we named the most common allele as allele 10 and numbered the next shorter or longer alleles according to repeat sizes of 4 bp. In all 3 STR-systems we designated intermediate alleles with decimals, e.g. allel 10.2 is two bp longer than allele 10.

Allelic ladder: For each STR-system we constructed our own allelic ladder from alleles observed in the examined population sample. DNA from different individuals were amplified and their PCR-products were mixed to an allelic cocktail. 0.5 - 1 µl of the cocktail were loaded in a gel track.

b) HLA DQ α: Amplification and typing reactions were performed according to the Cetus Protocol (HLA DQα Forensic DNA Amplification & Typing Kit).

c) D1S80: Amplification was carried out by following the recommended protocol of the D1S80 Forensic DNA Amplification Reagent Set (Perkin Elmer Cetus). The PCR-products were separated on 6% polyacrylamide gels with piperazine diacrylamide as cross-linker [1]. The bands were visualized by silver staining. The alleles were determined by side-to-side comparison with the allelic ladder provided in the reagent kit from Cetus.

3. Results

SE33: For SE33 a total of 33 different alleles could be observed in a sample of 195 individuals; their frequencies are shown in table 1. The allele frequencies range between 0.3% and 10%. Allele 6 and allele 16 are the most common alleles with frequencies of 10% and 9.5% respectively. We could distinguish between 130

HWE. In fact, Brenner and Morris, who first suggested the "allele binning" strategy for DNA data analysis, have indicated that using this approach Hardy-Weinberg tests are fraught with artifactual pitfalls, and they would view with skepticism claims that a population is or is not in HWE (7). In contrary, the approach of pooling rare alleles can not only decrease ΣEj caused by the absence of a large fraction of genotypes, but also maintains the characteristics of the distribution of allele frequencies in a sample. With this approach, HWE is defined on a feasible pooling allele level for a relatively small sample. For example, at COL2A1 locus all alleles with a frequency of less than 0.01 were pooled into one group, and on this level the Chinese sample is in HWE ($n=120$, $P>0.05$). This means that each allele with a frequency of more than 0.01 has been subjected to the test for HWE as an independent allele, and its frequency in the sample is the unbiased estimate of the population. Since rare alleles have been examined for HWE as one group, the frequency of this group is the unbiased estimate of all rare alleles in the population, and the frequency of each rare allele observed in the sample is the upper limit of its estimate in the population. Therefore, these data can be directly applied to calculate the relative propabilities dealing with individual identification and paternity analysis.

The analyses here indicate that, even though the genetic variation at VWA and COL2A1 loci is extensive, the chi-square test can be applied to assess the goodness of fit of the distribution of genotypes from their HWE. This method can also be employed to test HWE for other similar AMPFLP systems. Although some of the indirect approaches can also detect any gross divergence of genotype frequencies from their Hardy-Weinberg expectations, they are less sensitive for testing HWE. Some of the modified methods like binning alleles should be avoided because with these approaches the sample is not virtually examined for HWE.

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Table 2. Tests for HWE of genotype frequencies at the VWA locus

Test	n = 25		n = 50		n = 121	
	Observed	Expected	Observed	Expected	Observed	Expected
1. Number of						
heterozygotes	20	19.47	38	39.92	97	95.69
homozygotes	5	5.53	12	10.08	24	25.31
	P>0.05		P>0.05		P>0.05	
2. Number of distinct						
heterozygote genotypes	10	8.58	13	11.99	16	15.20
homozygote genotypes	2	2.75	5	3.92	5	4.67
	P>0.05		P>0.05		P>0.05	
3. Binning alleles						
3-allele model	x^2 =1.02		x^2 =4.16		x^2 =1.33	
	df=3		df=3		df=3	
	P>0.05		P>0.05		P>0.05	
4. Modified x^2						
	ΣEj=2.56		ΣEj=1.75		ΣEj=3.50	
	x^2 =11.44		x^2 =10.41		x^2 =16.18	
	df=7		df=12		df=14	
	P>0.05		P>0.05		P>0.05	

Compared with the above three tests, test 4 possesses some different features. Firstly, it is a contrasting of every observed genotype frequency with its respective expectation, and hence it can detect deviations of each specific genotype frequency from its HWE prediction. Secondly, it is noted that in a relatively smaller sample there is always a deficit between the total of observed and expected genotypes. This deficit is essentially equal to the totality of chi-square between the not observed genotypes and their respective expectations ($\Sigma((Oj-Ej)^2/Ej) = \Sigma Ej = N-\Sigma Ei = x^2$, df = 1). This feature is very useful for testing HWE with the chi-square test, because a relatively smaller sample size can be allowed and it is not necessary to observe all genotypes. Thirdly, it is ΣEj that offers a reference standard to judge whether or not the observed deviation from HWE is due to the absence of a large fraction of genotypes in a sample. Since ΣEj is defined with one degree of freedom, if Σj is smaller than the critical value with one degree of freedom, it will be assumed that the absence of some genotypes in a sample has only little influence on a test for HWE. For example, at the VWA locus, equation (1) can be directly applied to test HWE, if the sample size is large (n=121). In contrary, if ΣEj is larger than the critical value with one degree of freedom, the absence of a large fraction of genotypes will be considered as the main cause of deviation from HWE, and hence pooling relatively rare alleles to cancel this effect may be necessary for assessing the goodness of fit of the distribution of genotypes from their HWE in a relatively small sample.

It should be emphasized that the approach of pooling rare alleles (test 4) is different from that of binning alleles (test 3). With the approach of binning alleles, alleles with similar electrophoretic mobility or molecular weight are classified as one group and all alleles in a sample are divided into a few classes according to their electrophoretic mobility or molecular weight. Unfortunately, this classification is not significant for testing HWE, because there is no relationship between the electrophoretic mobility or molecular weight of a allele and its frequency in a sample, and for testing HWE only the allele frequency is important. Furthermore, binning alleles will change the characteristics of the distribution of the allele frequencies in a sample, and because of binning most of the independent alleles are not subjected to the test for HWE. Since new alleles and genotypes produced by binning differ from alleles and genotypes observed in a sample, with this approach the sample is not virtually examined for

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with a higher frequency, it is suggested to pool relatively rare alleles into one group. Afterwards a further Hardy-Weinberg test with equation (1) or a normal chi-square test can be carried out. If now the deviation from HWE is not significant any more, it can be concluded that for this VNTR locus on a particular pooling allele level the population sample is in HWE, and that the deviation from HWE observed for the first test is due to relatively small sample size.

RESULTS AND DISCUSSION

The results of the different tests for HWE are shown in Tables 1 and 2. In order to compare the efficiency of the different statistical tests, subsamples within these Chinese were sampled in random and tested for HWE (n=25 and n=50, tables 1,2). If the sample size was very small (n=25), no test revealed that there was a significant deviation from HWE in both VWA and COL2A1 loci. This is easily understood because with a smaller sample size the probability of a type II error (ß) is very large for any statistical test. The power of test (1-ß) becomes so low that any conclusion, that the sample is in HWE, is not reliable. With increasing sample size (n=50 and n=120), by test 4 a significant deviation from HWE was observed at the COL2A1 locus. Since HWE is an equilibrium of the distribution of alleles in the population among the various genotypes, a significant deviation from HWE should be observed, if many of the genotypes are not present in the sample. But tests 1, 2 and 3 failed to reveal this deviation from HWE at the COL2A1 locus (P>0.05), because they do not contrast the observed with the expected frequencies of all possible genotypes in a sample. Therefore, they are less sensitive approaches for testing HWE.

Table 1. Tests for HWE of genotype frequencies at the COL2A1 locus

	n = 25		n = 50		n = 120	
Test	Observed	Expected	Observed	Expected	Observed	Expected
1. Number of heterozygotes	20	17.37	36	36.45	90	84.32
homozygotes	5	7.63	14	13.55	30	35.68
	P>0.05		P>0.05		P>0.05	
2. Number of distinct heterozygote genotypes	6	5.52	14	12.51	19	16.71
homozygote genotypes	3	2.53	3	2.85	4	3.34
	P>0.05		P>0.05		P>0.05	
3. Binning alleles 3-allele model	x^2 =3.37 df=3 P>0.05		x^2 = 2.01 df=3 P>0.05		x^2 =1.93 df=3 P>0.05	
4. Modified x^2 1). ΣEj	ΣEj=1.16 x^2 =6.11 df=5 P>0.05		ΣEj=6.01 x^2 =67.44 df=7 P<0.05		ΣEj=7.78 x^2 =76.85 df=10 P<0.05	
2). Pooling rare alleles	-		ΣEj=2.15 x^2 =10.82 df=9 P>0.05		ΣEj=0 x^2 =7.95 df=10 P>0.05	

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COMPARISON OF DIFFERENT TESTS FOR DEVIATION FROM HARDY-WEINBERG EQUILIBRIUM OF AMPFLP POPULATION DATA

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INTRODUCTION

Since its introduction in 1985, VNTR polymorphism analysis has rapidly become a routine technique for forensic biology and population genetics. But so far there are still some controversies surrounding VNTR polymorphisms, one of them being whether they violate the assumptions of Hardy-Weinberg equilibrium (HWE). Concerning biostatistical tests the only significant difference between VNTR loci analysed by AMP-FLP and traditional serological markers is that there are more alleles at a particular VNTR locus, so that not all genotypes can be observed in a relatively small sample. Therefore, some indirect methods have been suggested to test HWE for these VNTR loci (1-4), but the efficiency of them was not evaluated. Our purpose here is to compare the efficiency of different statistical tests and to suggest a more sensitive new modification.

MATERIALS AND METHODS

EDTA-blood samples were collected from 121 unrelated Chinese individuals in Chengdu, Sichuan province, PR China. The amplification of VWA and COL2A1 loci and the electrophoretical analysis of PCR products were carried out as previously described (5,6).
The different tests for HWE include 1) observed versus expected number of heterozygotes (1-3), 2) observed versus expected number of distinct heterzygotes (1,2), 3) binning alleles (4). In addition, we suggest a modified statistical approach to test HWE (test 4).

$$\chi^2 = \sum_{j=1}^{h} \frac{(Oj-Ej)^2}{Ej} + \sum_{i=1}^{g} \frac{(Oi-Ei)^2}{Ei} = \sum_{j=1}^{h} Ej + \sum_{i=1}^{g} \frac{(Oi-Ei)^2}{Ei} \qquad (1)$$

$$(j=1,2,\ldots,h;\ i=1,2,\ldots,g;\ k=g+h)$$

where Oi and Ei are the number of the i th observed and expected genotype, respectively, while Oj and Ej are the number of the j th observed and expected genotype, respectively, which is not virtually observed in a population sample. k is the number of genotypes at a this locus. If m is the number of alleles, the degree of freedom (df) of the approximate chi-square distribution to test HWE can be obtained by

$$df = (k-h+1) - m = (g+1) - m \qquad (2)$$

ΣEj can be obtained by $\Sigma Ej = N - \Sigma Ei$ (3)

where N is the number of individuals in a sample. If a significant deviation from HWE was observed using equation (1) with ΣEj greater than the critical value with one degree of freedom, a relatively small sample size may be one of causes of deviation from HWE and further tests need to be carried out. Because the sampling error of the allele frequency is much larger in relatively rare alleles with a lower frequency than in relatively common alleles

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Since correct genotyping is permitted and the distribution of the genotypes is not continuous, the conventional formula of the Hardy-Weinberg rule can be applied to assess the goodness of fit of the distribution of genotypes for VWA, HUMTH01 and FES, respectively. No evidence of deviation from the Hardy-Weinberg equilibrium was observed in the Chinese population ($P>0.05$).

Population studies at the locus HUMTH01 have been reported by several groups (1,8-10). Because of the problems associated with the use of different electrophoretical systems and different allele ladders it is still difficult to compare population data generated in different laboratories. Adjusting the allele distributions to the previously not correctly identified allele 10-1 (allele 9.3) as shown by Nellemann et al (1993)(8), we conclude that our allele frequencies are similar to those reported by Edwards et al (1992)(1) for Asians, with allele 9 being the most frequent allele. More effort should be put into further clarification of allele frequency distributions for various Asian nationalities. Caucasian data from Europe and the US (1,8-10) and data for Greenland Eskimos (8) and US-Blacks (1) differ from the Chinese population. A more detailed comparison of our Chinese population data with German population data for HUMTH01, VWA and FES generated in our laboratory is in preparation.

In conclusion, using high resolution electrophoresis we obtained correct VWA, HUMTH01 and FES typing results that correspond to the allele identifications of the CRSE using automated fluorescent detection. Since the genotype data are in conformance with the predictions of Hardy-Weinberg equilibrium, the observed allele frequencies can be used to calculate the relative probabilities dealing with individual identification and paternity analysis. Therefore, the information obtained in this study provides a basis for the forensic application of VWA, HUMTH01 and FES in Chinese populations.

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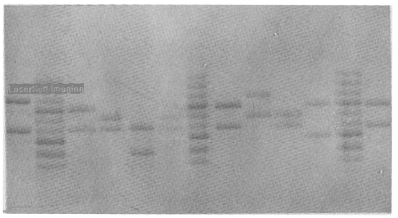

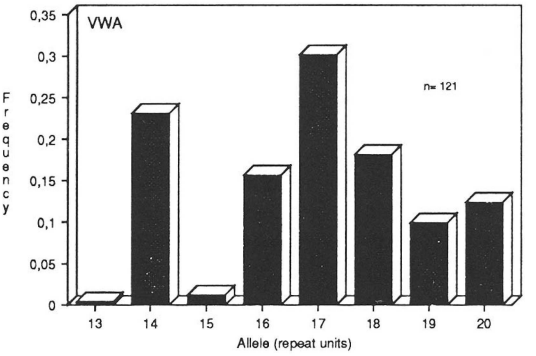

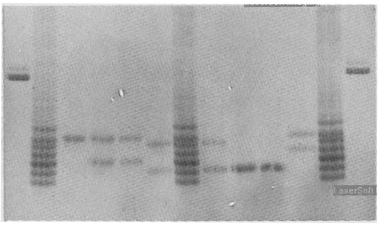

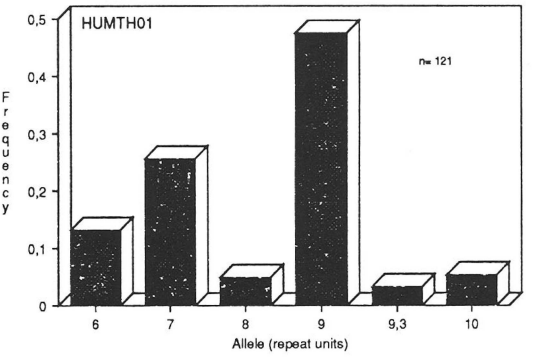

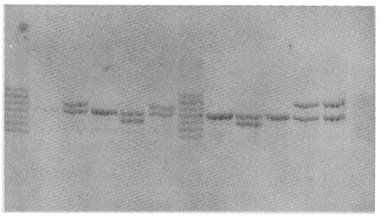

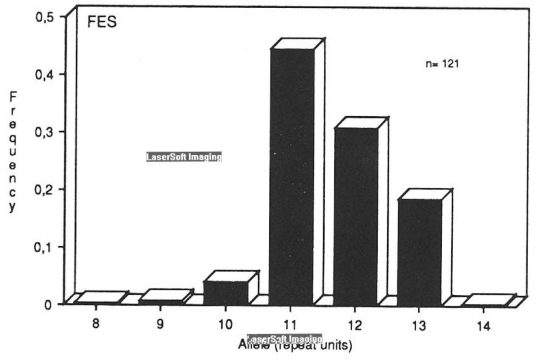

RESULTS AND DISCUSSION

Figures 1, 2 and 3 show the electrophoretic separation and the allele frequencies of VWA, HUMTH01 and FES, respectively. A total of 8 alleles for VWA, 6 alleles for HUMTH01 and 7 alleles for FES was observed in this Chinese population sample. K562 genotypes are VWA (16/16), HUMTH01 (9.3/9.3) and FES (9M/12), respectively.

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POPULATION GENETICS OF THREE STR POLYMORPHISMS IN A CHINESE POPULATION

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INTRODUCTION

Short tandem repeats (STRs) belong to a type of DNA polymorphisms with core sequences of a few base pairs in length. They are analysed using the polymerase chain reaction (PCR) and show variable allele lengths from 100 to 500 bp. The PCR products have to be separated by polyacrylamide gel electrophoresis with a resolution of one base pair (1). STRs offer three obvious advantages for forensic purposes. Firstly, the allele classifications can be based upon the number of tandem repeats relative to an allele ladder, so that correct typing is permitted. Secondly, samples containing very small amounts of DNA can be analysed. Thirdly, the amplification of alleles is also possible with highly degraded DNA. Therefore, we analysed 3 STR systems (VWA, HUMTH01 and FES)(2-4) to obtain preliminary allele frequency data for a Chinese population.

MATERIALS AND METHODS

EDTA-blood samples were collected from 121 unrelated Chinese individuals in Chengdu, Sichuan province, PR China. DNA was extracted from EDTA-blood by the Chelex method(5). The amplification was carried out with the following primers:

VWA (Kimpton et al.1992)(2)	5'-CCC TAG TGG ATG ATA AGA ATA ATC
	5'-GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG
HUMTH01 (CRSE)	5'-GTG GGC TGA AAA GCT CCC GAT TAT
	5'-GTG ATT CCC ATT GGC CTG TTC CTC
FES (CRSE)	5'-GGG ATT TCC CTA TGG ATT GG
	5'-GCG AAA GAA TGA GAC TAC AT

Each amplification sample contained 2-40 ng human genomic DNA, 1xTaq buffer (Promega), 1.5mM MgCl2, 150µM of each nucleotide, 1U Taq polymerase (Promega) and 0.25 µM of each primer. A total of 28 cycles was carried out in a Thermocycler (Biozym) with denaturation for 1 min at 94°C, annealing for 1 min at 60°C (for FES at 54°C), and extension for 2 min at 72°C.

Electrophoretical separation of the PCR products was performed in polyacrylamide gels (750µm thick, horizontal) with following conditions:

VWA	7.2% T, 3% C, 24 mM leading buffer.
HUMTH01	7.2% T, 3% C, 31 mM leading buffer.
FES	7.0% T, 3% C, 24 mM leading buffer.

A discontinuous buffer system was employed, Tris-Sulfate, pH 9.0 as leading buffer and 280mM Tris-Borate, pH 9.0 as tracking buffer. The separation distance was 20 cm and electrophoresis was stopped when the bromophenol blue front had reached the anodal end of the gel. The gels were silverstained according to Allen et al.(6).

Allele determination was carried out by comparison with the allele ladders of VWA, HUMTH01 and FES that have been constructed and evaluated by the Central Research and Support Establishment (CRSE) of the Forensic Science Service, UK.

The test for Hardy-Weinberg equilibrium was carried out using chi square test. The expected heterozygosity was calculated according to the equation $h=(1-\Sigma Xi^2)(n/n-1)$, where h=expected heterozygosity, Xi=allele frequencies and n=the number of alleles(7).

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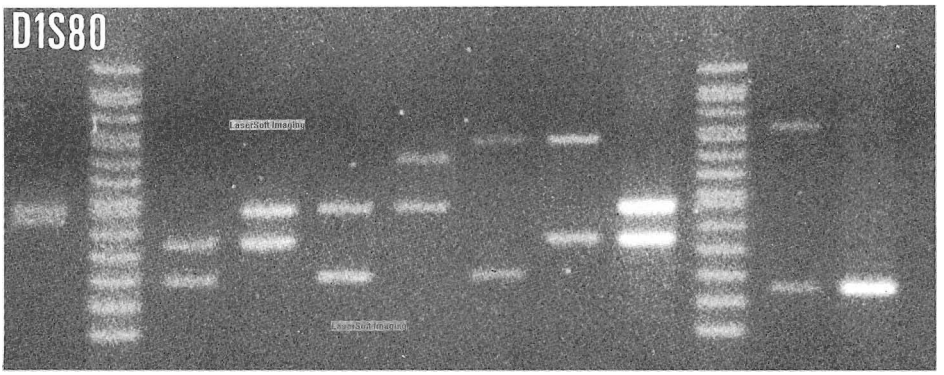

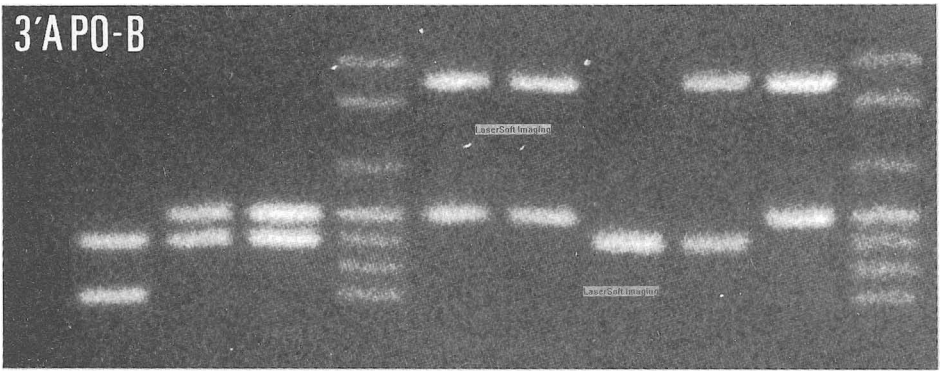

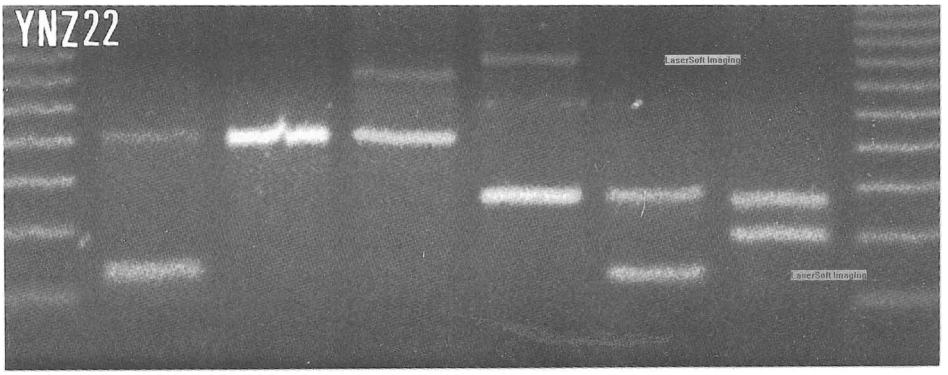

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	D1S80	3'ApoB	YNZ22
Heterocigosity index (HI):	78.24%	77.70%	81.93%
Power of discrimination (PD):	94.03%	92.36%	95.92%
"A priori" Chance Exclusion (CE):	63.67%	58.86%	69.77%
Essen-Möller value (EM):	9.33	9.47	9.35

D1S80 was examined in 105 paternity testing cases. Of these, 75 cases of practically proved paternity (W≥ 99.73) using conventional markers were confirmed, supporting the assumed autosomal codominant type of inheritance. In the other 30 cases of known exclusion, there are 21 incompatibilities, meaning an exclusion of 70%

3'ApoB was examined in 17 paternity testing cases. Of these, 16 cases of practically proved paternity (W≥ 99.73) using conventional markers were confirmed.

3'Apo B	D1S80		YNZ22
29 = 0	14 = 0	29 = 0.057	1 = 0.065
31 = 0.109	15 = 0.003	30 = 0.008	2 = 0.213
33 = 0.036	16 = 0.001	31 = 0.006	3 = 0.090
35 = 0.198	17 = 0.004	32 = 0.008	4 = 0.271
37 = 0.395	18 = 0.227	33 = 0.003	5 = 0.055
39 = 0.020	19 = 0.001	34 = 0.004	6 = 0.042
41 = 0.008	20 = 0.022	35 = 0.001	7 = 0.016
43 = 0	21 = 0.026	36 = 0.001	8 = 0.035
45 = 0.020	22 = 0.038	37 = 0.003	9 = 0.116
47 = 0.085	23 = 0.017	38 = 0.003	10 = 0.068
49 = 0.097	24 = 0.363	39 = 0	11 = 0.010
51 = 0.024	25 = 0.048	40 = 0.001	12 = 0.013
53 = 0.004	26 = 0.025	R* = 0.006	13 = 0.003
55 = 0	27 = 0.012		14 = 0
57 = 0.004	28 = 0.063		15 = 0.003

Table 1. Allele frequencies.

CONCLUSIONS

The high degree of variability of each PCR polymorphism makes these markers extremely useful in forensic haemogenetics.

In paternity testing the accumulated "a priori" chance exclusion of the three loci is 95.48 %, and the accumulated Essen-Möller value is 8.27

From the point of view of population genetics it would be advisable to extend the study of this polymorphisms to other population groups.

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STUDY OF THREE MINISATELLITES (D1S80, YNZ22, 3'ApoB) PERFORMED BY P.C.R. IN THE POPULATION OF CATALONIA (NORTH-EAST SPAIN).

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The polymerase chain reaction (PCR) method has been applied to amplify three minisatellites: D1S80 (pMCT118), 3'ApoB, and YNZ22 (D17S5), variable number of tandem repeats (VNTRs) on a random population sample from Catalonia.

Loci studied:	D1S80 (n=386)	3'ApoB (n=126)	YNZ22 (n=177)
Allele sizes (bp):	±300-1000	±500-1000	±170-1200

METHODS:

DNA was extracted using phenol-chloroform-isoamyl alcohol or Chelex TM 100.

PCR amplification of D1S80 locus was achieved by the method of Budowle et al. (1991), using the primers described by Kasai et al. (1990). The methods of Boerwinkle E. et al. (1989), and Batanian et al. (1990), with slight modifications, were used for the amplification of 3'ApoB, and YNZ22 loci respectively.

Detection of the different alleles, from PCR amplified products, was carried out by ethidium bromide agarose gel electrophoresis. We tried too, horizontal polyacrilamide gel electrophoresis, but, giving no much better resolution, we used routinately agarose gels.

RESULTS:

Alleles Observed:

D1S80: 26 alleles (25 common and one rare variant) and 82 genotypes.
3'ApoB: 15 alleles (11 common and 4 rare variant) and 35 genotypes.
YNZ22: 14 alleles and 45 genotypes.

Allele frequencies are shown in table 1. The distribution of the genotypes were in Hardy-Weimberg equilibrium for the three loci.

D1S80: Chi square = 16.469;	d.f. = 9;	0.10 > P > 0.05
3'ApoB: Chi square = 5.9368;	d.f. = 5;	0.50 > P > 0.30
YNZ22: Chi square = 10.6869;	d.f. = 6;	0.10 > P > 0.05

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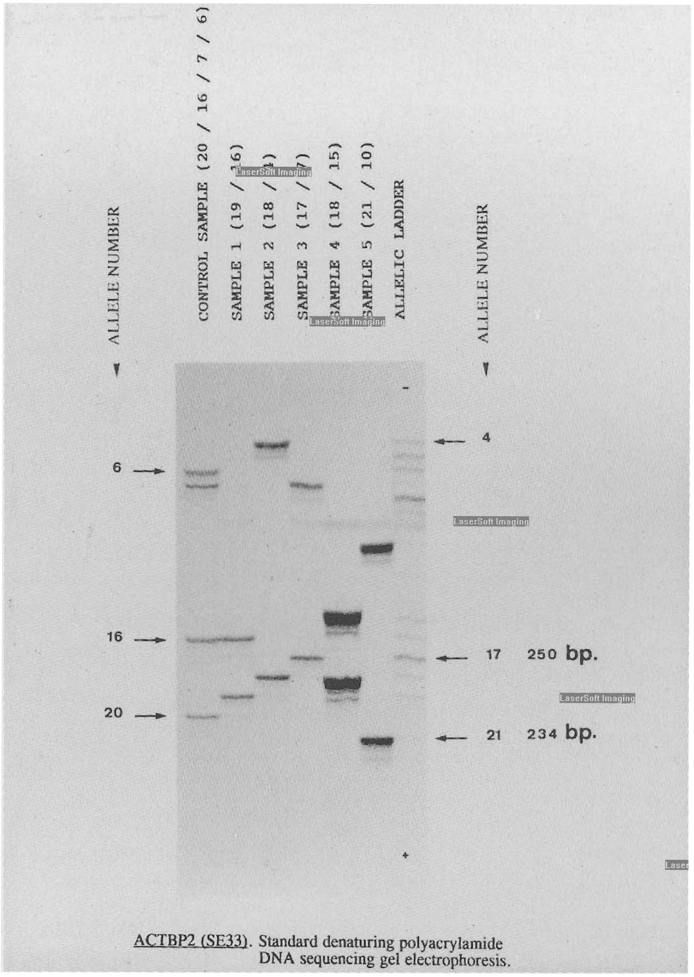

ACTBP2 (SE33). Standard denaturing polyacrylamide DNA sequencing gel electrophoresis.

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RESULTS AND DISCUSSION

Table 1 shows the genotype and the allele frequencies obtained. The study demostrated the presence of 25 alleles, 21 of which are identical to the ones observed by Polymeropoulos M.H., et al. (1992). The additional 4 alleles correspond to: R* (326bp) under the allele 1; 8' (286bp) betwen A8 and A9; 22 (230bp) and 23 (226bp). No alleles of size 322 were found.

There is no significant difference between the observed and expected numbers of heterozygous and homozygous individuals in the sample, indicating no departure from Hardy-Weinberg equilibrium ($0.10 > p < 0.05$) of allele frequencies at the VNTR site (Chakraborty et al. 1991).

Heterocigosity (HI) observed= 92.61%
Power of discrimination (PD)= 99.33%
"A priori" Chance Exclusion (CE)= 88.08%
Essen-Möller value (EM)= 9.01

We have observed an homogeneous distribution of allele frequencies, similar to the one described by Wiegand P. et. al., but we have not established any comparison because the allelic nomenclature is different.

Allele	Freq.	Allele	Freq.
R*	0.003	13	0.018
1	0.003	14	0.044
2	0.010	15	0.054
3	0.013	16	0.060
4	0.027	17	0.080
5	0.064	18	0.094
6	0.084	19	0.070
7	0.074	20	0.040
8	0.064	21	0.040
8'	0.048	22	0.003
9	0.030	23	0.007
10	0.020		
11	0.027		
12	0.023		

Table 1. Allele frequencies.

CONCLUSIONS

The high degree of variability of the ACTBP2 polymorphism makes it extremely useful in forensic haemogenetics.

From the point of view of population genetics it would be advisable to extend the study of this polymorphism to other population groups.

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SUITABILITY OF THE ACTBP2 (SE33) STR-POLYMORPHISM FOR LEGAL MEDICINE INVESTIGATION IN THE POPULATION OF CATALONIA (NORTH-EAST SPAIN).

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INTRODUCTION

The human genome contain DNA segments with variable number of short tandemly repeated sequences (STR). The copy number of such core sequences reveals a highly polymorphism larger than that detected by classical serologic and biochemical genetic markers.

Here we describe the genotype and allele frequency distribution at one locus on chromosome 6, the human beta actin pseudogene H-beta-Ac-psi-2 (ACTBP2), the polymorphic $(AAAG)_n$ repeat begins at base pair 176. The polymorphism is typed using the polymerase chain reaction (PCR). Given that this system has been studied only in few populations the objective of this paper is to present data on the frequencies of ACTBP2 alleles and on the distribution of different genotypes in a population sample from Catalonia (Spain).

MATERIAL AND METHODS

The nomenclature system adopted is based in the one recently proposed by Polymeropoulos et al. (1992).

Samples from 149 unrelated individuals were collected. The study was carried out with blood donors samples from the "Hospital Clínic" Blood Bank, and with samples (blood and hairs) of unrelated paternity cases and university employees.

DNA was extracted using phenol-chloroform-isoamyl alcohol (Auxbel et. al.) or Chelex TM 100 (Walsh et al.).

PCR amplification of ACTBP2 was accomplished by the method and primers described by Polymeropoulos M.H. et al. with slight modifications. PCR reactions were carried out in a 25 μl volume containing 10 to 50 ng of genomic DNA template, 2.5 pmol each oligonucleotide, 200 μM each dGTP, dCTP, and dTTP, 2.5 μM α^{35}S-dATP at 500 Ci/mmol., 50 mM KCl, 10 mM Tris (pH8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.5 units of Taq polymerase. Samples were processed through 30 temperature cycles consisting of 1 min.24 seg. at 94°C, 2 min. at 57°C, and 2 min. at 72°C. The last elongation step was lengthened to 10 min. The PCR products were resolved using denaturing polyacrilamide-urea gel (40x20x0.04 cm.) electrophoresis, along with a ladder of a cocktail sample. Gels were then fixed, dried, and processed for autoradiography.

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We have introduced the HLA-DQA1 polymorphism study in 51 paternity testing cases. The 54,5% of exclusions obtained, corresponding to 12 incompatibilities in 22 cases of non-paternity, is in good agreement with the theoretical a priori chance exclusion value (C.E. = 0.58).

DISCUSSION

The studied sample of the Portuguese population is composed of individuals whose genetic background is from several geographical zones of Portugal - North, Center and South, and also Madeira and Açores Islands, with higher incidence (57% of individuals) from the South.
When compared to other Caucasian data, our allele frequencies show some differences in the distribution of HLA-DQA1.1 and HLA-DQA1.3 alleles, which are more frequent in our population than in Barcelona (3), Italian (4) or US Caucasian populations(5).
Our study also shows that data on HLA-DQA2 and HLA-DQA3 allele frequencies, in the Portuguese population, are very similar to the Italian ones.
We can infere that HLA-DQA1 polymorphism exibits differences among several ethnic groups.
Due to the power of exclusion of 0.58 and the power of discrimination of 0.92 we can assign the HLA-DQA1 system as a good genetic marker in the Portuguese population both for paternity investigation and for individual identification in forensic analysis.

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Table 1. Distribution of HLA-DQA1 Genotypes and Allele Frequencies in the Portuguese Population

Genotype	Observed		Expected	
	n	%	n	%
1.1-1.1	3	2.83	4.57	4.31
1.1-1.2	4	3.77	4.98	4.70
1.1-1.3	4	3.77	4.98	4.70
1.1-2	7	6.61	7.47	7.05
1.1-3	3	2.83	3.94	3.72
1.1-4	20	18.87	13.49	12.73
1.2-1.2	2	1.89	1.36	1.28
1.2-1.3	1	0.94	2.72	2.56
1.2-2	5	4.72	4.08	3.85
1.2-3	2	1.89	2.15	2.03
1.2-4	8	7.55	7.36	6.94
1.3-1.3	0	0.00	1.36	1.28
1.3-2	6	5.66	4.08	3.85
1.3-3	4	3.77	2.15	2.03
1.3-4	9	8.49	7.36	6.94
2-2	3	2.83	3.06	2.88
2-3	4	3.77	3.23	3.04
2-4	8	7.55	11.04	10.41
3-3	1	0.94	0.85	0.80
3-4	4	3.77	5.83	5.50
4-4	8	7.55	9.06	9.40
TOTAL	106	100.00	106.02	100.00

HLA DQA1.1= 0.2075
HLA DQA1.2= 0.1132
HLA DQA1.3= 0.1132
HLA DQA 2 = 0.1698
HLA DQA 3 = 0.0896
HLA DQA 4 = 0.3066

χ^2=12.21
df= 15
$0.50 < p < 0.70$
CE= 0.58

In the Portuguese population the HLA-DQA1.1-4 genotype is the most frequent (18.87%), followed by HLA-DQA1.2-4, DQA1.3-4, DQA2-4 and DQA4-4 genotypes (7.55%-8.49%). In our sample, we have not observed any HLA-DQA1.3-1.3 genotype.

The allele frequency analysis reveals that the most common HLA-DQA1 alleles in the Portuguese population are: HLA-DQA4 allele and HLA-DQA1.1 allele with frequencies, respectively, of 0.3066 and 0.2075.

In our sample, we have obtained, a power of discrimination of 0.9210 and an allelic diversity of 0.8042, which is in good agreement with other populations studied at the some locus (5).

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HLA-DQA1 POLYMORPHISM IN A PORTUGUESE POPULATION

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INTRODUCTION

The polymerase chain reaction (PCR) is a powerful and sensitive techique which offers new tools for forensic analysis both in identification of stains and in paternity investigation.
By use of PCR amplification and reserve dot-blot procedure the polymorphism at the HLA-DQA1 locus revealed six major allelic types - DQA1.1, DQA1.2, DQA1.3, DQA2, DQA3 and DQA4 (1).
We present data on HLA-DQA1 allele frequencies and distribution of the different genotypes at this locus in a sample of a Portuguese population, mainly from the Southern part of the country.

MATERIAL AND METHODS

Blood samples were obtained from 106 unrelated individuals collected for paternity cases presented to the Institute of Legal Medicine in Lisbon.
The individuals of the investigated samples can be divided according to their genetic background into five geographical zones of Portugal: 57% from the South, 10% from the Center, 16% from the North, 11% from Madeira and 6% from Açores Islands.
DNA was extracted from bloodstains with Sigma chelating resin, using the method described by Barros et al. (2).
Amplification by PCR was performed during 32 cycles on approximatly 0.1µg of human genomic DNA using a DNA thermal cycler (denaturation at 94ºC for 1 min, annealing at 60ºC for 30s and extension at 72ºC for 30s).
The HLA-DQA1 alleles were typed by the reverse dot-blot procedure as described in the Amplitype HLA-DQ Alpha Forensic DNA Amplification and typing Kit (Perkin Elmer).

RESULTS

The distribution of HLA-DQA1 genotypes and allele frequencies for the Portuguese population studied are shown in Table 1. The distribution of genotypes indicate that there is no deviation from Hardy-Weinberg equilibrium, as shown by the χ^2 test (χ^2= 12.21; d.f. = 15; $0.50 < p < 0.70$).

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Phenotypes	Ancona		Parma		Pavia		Pisa		Pooled	
	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp
6	9	8.14	17	15.45	8	7.94	6	8.64	40	39.29
6-7	10	10.77	14	11.71	12	10.35	11	13.20	47	46.79
6-8	10	10.54	11	10.41	11	10.81	15	10.32	47	42.91
6-9	12	12.93	13	15.62	12	12.19	15	10.56	52	51.44
6-10	17	17.00	22	25.38	17	19.55	17	18.96	73	80.91
6-Others	1	0.48	1	0.98	1	0.23	2	1.68	5	3.36
7	3	3.57	1	2.22	2	3.38	4	5.04	10	13.93
7-8	7	6.97	4	3.95	9	7.05	8	7.88	28	25.55
7-9	9	8.56	6	5.92	6	7.95	4	8.07	25	30.63
7-10	13	11.25	10	9.62	14	12.75	22	14.48	59	48.17
7-Others	0	0.32	0	0.37	0	0.15	2	1.28	2	2.00
8	4	3.41	1	1.75	4	3.68	2	3.08	11	11.72
8-9	8	8.37	2	5.26	7	8.31	6	6.31	23	28.09
8-10	11	11.00	12	8.55	12	13.32	10	11.32	45	44.18
8-Others	0	0.31	1	0.33	0	0.15	0	1.00	1	1.84
9	6	5.13	7	3.95	5	4.68	2	3.23	20	16.84
9-10	12	13.50	12	12.82	18	15.02	13	11.59	55	52.97
9-Others	1	0.38	1	0.49	0	0.17	2	1.03	4	2.20
10	9	8.88	11	10.42	12	12.04	8	10.40	40	41.65
10-Others	0	0.50	0	0.80	0	0.28	1	1.84	1	3.46
Others	0	0.01	0	0.02	0	0.00	0	0.08	0	0.07
Total	142	142.00	146	146.00	150	150.00	150	150.00	588	588.00
χ^2		3.875		11.477		6.610		16.130		12.186
df = 15		P>.99		P>.70		P>.95		P>.30		P>.50

Tab.1: HUMTH01 phenotype frequencies in the sub-samples and in the pooled sample

Allele	Ancona	Parma	Pavia	Pisa	Pooled
6	.2394	.3253	.2300	.2400	.2585±.0128
7	.1584	.1233	.1500	.1833	.1539±.0105
8	.1549	.1096	.1567	.1433	.1412±.0102
9	.1901	.1644	.1767	.1467	.1692±.0109
10	.2500	.2671	.2833	.2633	.2662±.0129
Others	.0070	.0103	.0033	.0233	.0111±.0030

Tab.2: HUMTH01 allele frequencies in the sub-samples and in the pooled sample

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forensic practice. A simple comparison of these frequencies with those obtained by Edwards *et al* (1992) and Wiegand *et al* (1992) showed no apparent differences (fig. 3).
This study confirms that the HUMTH01 polymorphism is particularly suitable for forensic medicine applications, because of its information content. The exclusion chance is 0.583, the discrimination index is 0.923 and the expected heterozigosity is 0.790.

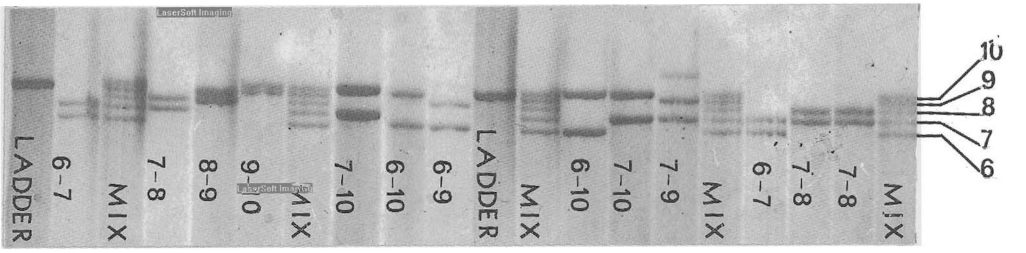

Figure 1: HUMTH01 phenotypes observed by PAA electrophoresis and silver staining

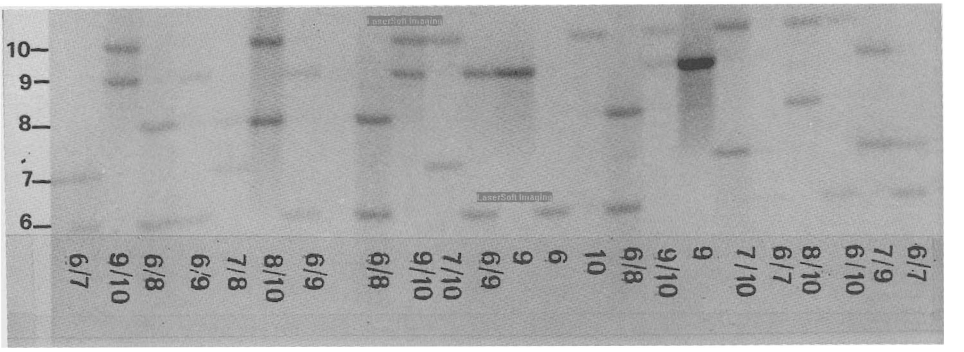

Figure 2: HUMTH01 phenotypes observed by denaturing sequencing gel

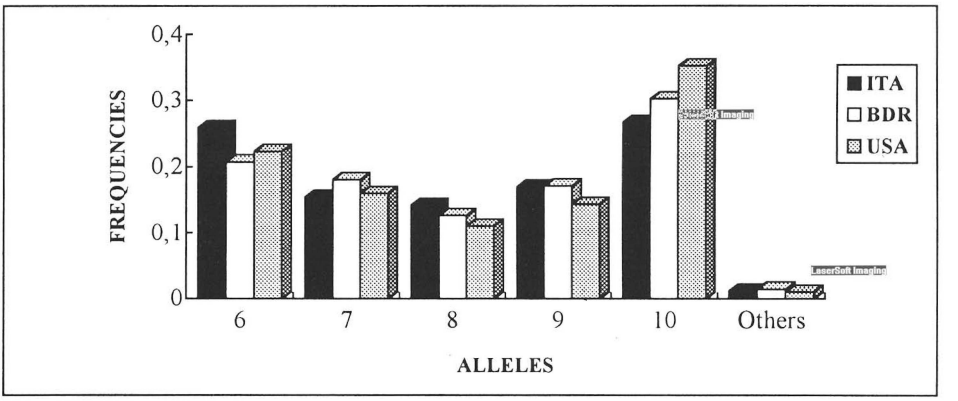

Figure 3: Comparison of our data (ITA) with data from Wiegand (BDR) and from Edwards (USA)

THE DISTRIBUTION OF HUMTH01 POLYMORPHISM IN NORTHERN AND CENTRAL ITALY

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INTRODUCTION

HUMTH01 polymorphism, first described by Edwards *et al* (1991), is due to a variation in the length of a tetrameric short tandem repeat on locus 11p15.5. Seven alleles were identified in the range 183-207 bp (Edwards *et al* 1992).
The aim of this study is to give a contribution to the knowledge of the allelic frequency distribution of this locus in Northern and Central Italy. Population samples were obtained from four Regions: Lombardy, Emilia, Tuscany and Marches. This study was made possible by the collaboration of the Legal Medicine Institutes of four Universities: Pavia (Lombardy), Parma (Emilia), Pisa (Tuscany) and Ancona (Marches).

MATERIALS AND METHODS

The population sample consisted of 588 healthy unrelated subjects: 150 resident in Pavia (45.10 N / 9.10 E), 146 resident in Parma (44.48 N / 10.20 E), 142 resident in Ancona (43.38 N / 13.30 E) and 150 all born in Pisa (43.43 N /10.23 E).
DNA typing was carried out in the laboratories of Ancona, Pisa and Parma according to the method described by Brinkmann *et al* (1992) with minor modifications. In the laboratory of Pavia, amplification products were radiolabeled by dATP^{35}S and fractionated on denaturating sequencing gels (6% PAA, 7M urea).
Alleles were assigned by means of home-made ladders consisting of human alleles (fig 1); the fragment sizes were measured on denaturating sequencing gel by comparison with a DNA sequence ladder derived from known sequences (fig 2).
An adequate number of samples were exchanged between the laboratories to test the reproducibility of the typing results.
The subsamples belonging to the four regions were tested for heterogeneity using G^2 statistics as described by Piazza *et al* (1989). They did not differ significantly from each other and were thus pooled to form a single population for further analysis.

RESULTS AND DISCUSSION

Table 1 shows the observed and expected frequencies in the four sub-samples; the Hardy-Weinberg equilibrium was satisfied. The 5 common alleles found were designated according to Edwards *et al* (1992). Rare alleles ($p < .025$) were pooled.
The heterogeneity test for allele frequency was not significant ($G^2 = 15.25$, df = 12, $P > .20$); the allelic frequencies in the sub-samples and in the pooled sample are shown in table 2 together with the standard error. These values are representative of the Northern and Central Italy, and may be used in

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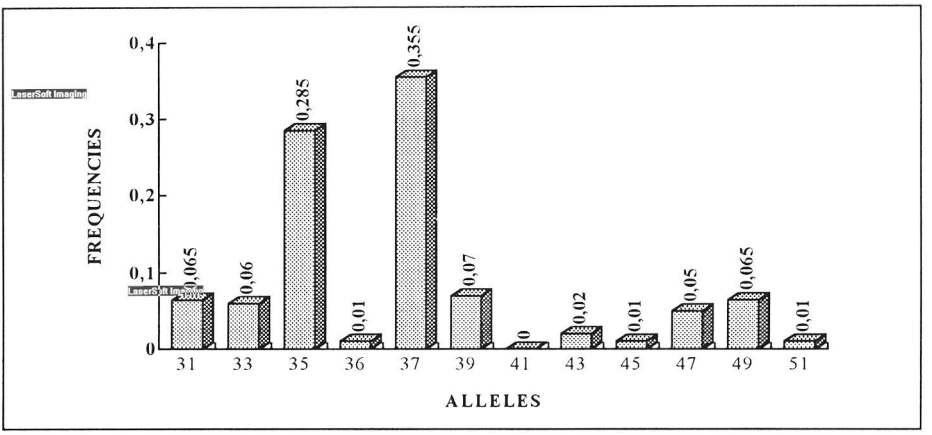

Figure 2: bimodal distribution of APO B allele frequencies

confirms its usefulness in forensic medicine. However, further studies, using larger sample sizes, would needed to provide more accurate estimation of rare allele frequencies.
Although some authors prefer typing by denaturing polyacrylamide gel electrophoresis, we found that agarose gel electrophoresis provided a clear identification of DNA fragments differing by only one repeat unit (14-16 bp).

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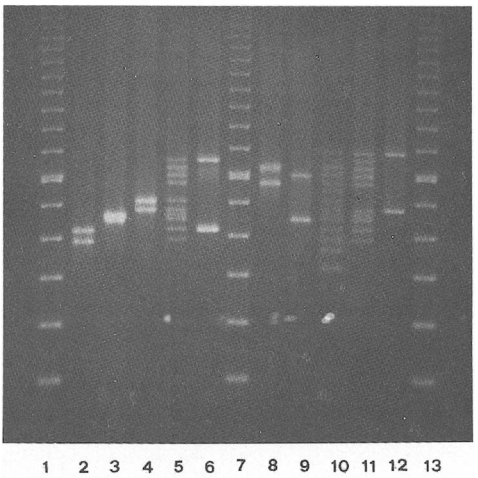
Type	Observed	Expected	Allele frequencies
31-33	1	0.85	APOB*31 = 0.065
31-35	5	3.77	
31-36	1	0.13	APOB*33 = 0.060
31-37	6	4.49	
33-33	1	0.42	APOB*35 = 0.285
33-35	3	3.77	
33-37	3	4.49	APOB*36 = 0.010
33-49	3	0.85	
35-35	10	8.41	APOB*37 = 0.355
35-36	1	0.58	
35-37	17	20.01	APOB*39 = 0.070
35-39	4	4.06	
35-43	1	1.16	APOB*41 = 0.000
35-45	2	0.57	
35-47	1	2.90	APOB*43 = 0.020
35-49	2	3.71	
35-51	1	0.58	APOB*45 = 0.010
37-37	12	11.90	
37-39	9	4.83	APOB*47 = 0.050
37-43	2	1.38	
37-47	6	3.45	APOB*49 = 0.065
37-49	3	4.49	
37-51	1	0.69	APOB*51 = 0.010
39-49	1	0.91	
43-47	1	0.20	
47-47	1	0.25	
49-49	2	0.42	
Total	100	89.45	

Table 1: phenotypes distribution and allele frequencies in the population sample from Tuscany



Lanes

1,7,13= 100 bp ladder
2= 31-33
3= 35-36
4= 37-39
5= allelic ladder (31-33-35-36-37-39-43-45-47-49)
6= 33-49
8= 43-47
9= 35-45
10= allelic ladder (25-29-31-33-35-36-37-39-41-43-45-47-49-51)
11= allelic ladder (31-33-35-36-37-43-45-47-49-51)
12= 37-51

Figure 1: APO B phenotypes

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STUDY OF THE APO B POLYMORPHISM IN TUSCANY (ITALY)

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INTRODUCTION

The APO B VNTR locus is located next to the 3' end of the apolipoprotein B gene and consists of a series of AT rich tandem repeats, each 14-16 bp long (for a review about the genetic relationship between the 3'-VNTR and diallelic apolipoprotein B gene polymorphism, see Renges *et al*, 1992; for a recent proposal of a molecular model, see Desmarais *et al* 1993). Its polymorphism was described by Boerwinkle *et al* (1989) and by Ludwig *et al* (1989) using the PCR technique. Since then, the APO B has become a genetic marker quite frequently used in forensic practice.
In this study we report the distribution of the APO B polymorphism in a population sample from Tuscany.

MATERIALS AND METHODS

The study was conducted on a population sample of 100 unrelated healthy individuals born in Tuscany (Italy).
DNA extraction was performed by the phenol-isoamyl alchool method following the protocol developed at the FBI Academy, Quantico (Budowle *et al*, 1990).
Genomic DNA samples were amplified according to the method described by Boerwinkle *et al* (1989) with minor modifications. The electrophoretical separation of the amplified fragments was carried out in agarose gel (2% v/w). A home-made allelic ladder was used for classifying phenotypes (fig 1).
Hardy Weinberg equilibrium test was conducted by using the proportion of heterozygous individuals, as well by using a four allele model as suggested by Skowasch *et al* (1992). The allele pools were: group 1 = APOB*31-33, group 2 = APOB*35, group 3 = APOB*36-37, group 4 = APOB*39-51.
In order to test for the heterogeneity between population samples, the G^2 statistics was used, as described by Piazza *et al* (1989); in this case also alleles were pooled in four groups.

RESULTS AND DISCUSSION

In table 1, the distribution of observed and expected APO B phenotypes and of allele frequencies is shown. Eleven different alleles (ranging in size from $\cong$ 600 bp to $\cong$ 900 bp) and twenty seven phenotypes were identified. The allelic designation follows the nomenclature originally suggested by Boerwinkle *et al* (1989).
No significant deviation from Hardy-Weinberg equilibrium could be found in our population sample, both by heterozygosity test (observed 74, expected 77.3, χ^2 = .605, df =1; P = .30-.50) and by four allele model (χ^2 = 4.28, df 6, P = .70-.50).
The allele frequency distribution appears to be bimodal, as in nearly all described Caucasian and not Caucasian populations [see: Schnee-Griese *et al* (1991), Deka *et al* (1992), Renges *et al* (1992), Desmarais *et al* (1993), März *et al* (1993), Hixson *et al* (1993)]. One mode is around APOB*35 and 39 alleles, and a less frequent mode is observed at APOB*47 and 49 alleles (see fig 2).
The heterogeneity test between our gene frequencies and those previously reported by Giorgetti *et al* (1991), d'Aloja *et al* (1992) and Novelli *et al* (1992) for Continental Italy was at the boundary (G^2 = 19.853, df = 9, P $\cong$ 0.02). The favourable phenotype frequency distribution of the APO B system

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Table 2: Allele frequency distribution of the different HLA class II loci in the Belgian population

DQA1	Freq.	DQB1	Freq.	DPB1	Freq.	DRB1	Freq.
0101	0.154	0201	0.200	0101	0.084	0101	0.092
0102	0.204	0302	0.083	0201	0.155	0102	0.014
0103	0.092	0501	0.132	0202	0.003	0103	0.012
0201	0.131	0502	0.027	0301	0.084	0301	0.119
0301	0.128	0604	0.049	0401	0.413	0400	0.036
0401	0.016	0605	0.017	0402	0.109	0401	0.068
0501	0.273	1301	0.198	0501	0.027	0402	0.008
0601	0.002	1303	0.051	0601	0.003	0403	0.002
		1402	0.012	0901	0.005	0407	0.002
		1503	0.019	1001	0.030	0700	0.137
		1601	0.012	1101	0.015	0801	0.012
		1602	0.119	1301	0.017	0802	0.004
		1603	0.081	1401	0.005	0803	0.006
				1501	0.007	0804	0.002
				1601	0.005	0901	0.006
				1701	0.012	1001	0.004
				1901	0.020	1101	0.113
				2001	0.003	1102	0.008
				2301	0.003	1103	0.004
						1201	0.018
						1202	0.002
						1301	0.137
						1303	0.006
						1305	0.002
						1401	0.020
						1404	0.002
						1500	0.141
						1601	0.023

DQA1*0101 contains the alleles DQA1*0101 and DQA1*0104. Alleles DQA1*0301 and DQA1*0302 can not be discriminated and are typed as DQA1*0301. DRB1*1500 contain the alleles DRB1*1501 and DRB1*1503. Allele DRB1*1401 includes DRB1*1407. Allele DRB1*1101 includes the alleles DRB1*1104 and DRB1*1105. Allele DRB1*1305 includes allele DRB1*1306. Allele DRB1*0400 contains the alleles DRB1*0404, DRB1*0405, DRB1*0408 and DRB1*0410. Alleles DRB1*0701 and DRB1*0702 can not be discriminated and are typed as DRB1*0700. DRB1*1600 contain the alleles DRB1*1601 and DRB1*1602.

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Table 1: Summary of the characteristics for the different HLA Class II loci determined in this study.

Locus	Observed no. of alleles[1]	Heterozygosity (%)[2]	Power of discrimination	Power of paternity exclusion
DQA1	8 (218)	82.0 ± 0.7	0.942	0.639
DQB1	13 (205)	87.1 ± 0.6	0.970	0.739
DPB1	19 (202)	77.8 ± 1.6	0.930	0.604
DRB1	28 (251)	90.1 ± 0.4	0.981	0.796

[1] In parenthesis are the number of individuals
[2] Expected heterozygosity and the standard error calculated according to Nei (1978)

A comparison with other Caucasian population frequencies for the DQA1 locus (Helmuth et al., 1990; Sajantila et al., 1991; Kloosterman et al., 1993; Allen et al., 1993) showed that there was no significant difference for the different statistics (heterozygosity, power of exclusion and power of discrimination) with our population and only slight differences are seen between individual alleles. However, the DQA1 system developed by us, discriminates between three alleles which are typed as one allele in the AmpliType HLA-DQα Kit (Perkin-Elmer/Cetus) which was used for determination of the population frequencies in the other populations. As a consequence, with the commercial kit, a false inclusion could be obtained if the second allele (0401, 0501 or 0601) in the genotype would be different between the reference sample (e.g. 0101/0401) and the evidence sample (e.g. 0101/0501).
The reversed dot blot method, as applied in this study, has been extensively used for HLA association studies (Insulin Dependent Diabetes Melitis, rheumatoid arthritis..), corneatransplants and paternity determinations. Currently, these methods are evaluated for application in forensic cases. The most polymorphic locus (DRB1) can be used without any problem of interpretation for forensic samples which are known to originate from one person. In sexual assault cases, however, mixtures of vaginal cells from the victim and semen from the assailant can not always be completely separated by differential lysis and therefore mixed DNA samples are obtained. Due to the complexity of the typing - a pattern which is a combination of several SSO's - problems of interpretation could be foreseen. Preliminary experiments with the DPB1 and DRB1 system indicate that in those samples the DRB1 system is favourable over the DPB1 system. Moreover, a combination of the two systems could increase the informativity in those cases where both victim and suspect do have the same genotype for one system.

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DISTRIBUTION OF HLA CLASS II GENES IN A CAUCASIAN POPULATION AS DETERMINED BY PCR AND REVERSED-DOT-BLOT TYPING

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INTRODUCTION

The human leucocyte antigens (HLA) loci are extremely polymorphic and are, therefore, of interest for identity testing and paternity determinations. The advent of the polymerase chain reaction (PCR) has made it possible to determine in more detail the different alleles at the nucleotide level. Analysis of the PCR products can be performed either by sequence analysis or by hybridization with sequence-specific-oligonucleotides (SSO's). In order to identify the existing alleles, a large panel of SSO's has to be used which makes hybridisation with individual SSO's a tremendous task. The development of the reversed-dot-blot technique, however, has made it possible to identify each allele in a minimum of time by hybridization of the amplified product to a membrane on which all the different SSO's bound are bound (Saiki et al., 1989).
We have developed a reversed-dot-blot assay (Buyse et al., 1993) for all the polymorphic HLA Class II genes (DQA1, DQB1, DPB1 and DRB1) and have applied this method for the determination of the allele and genotype frequency in the Belgian population.

MATERIALS AND METHODS

A panel of 200 DNA samples was obtained from random blood donors while additional DNA samples were from blood samples received for paternity determinations. All individuals were Caucasians of Belgian descent.
The polymorphic second exon of the HLA Class II genes was amplified by PCR and analyzed by hybridisation to sequence-specific-oligonucleotides bound to membranes as described by Buyse et al. (1993). Detection of the positive spots was done either by chemiluminescence (AMPPD, Tropix, Bedford, MA) or by a colorimetric assay (Inno-Lipa, Innogenetics, Gent, Belgium).

RESULTS AND DISCUSSION

In this study, we determined the allele frequency for all HLA class II loci in a random sample of the Belgian population. The method applied, PCR in combination with reversed-dot-blot hybridization, is able to identify 89.2% of the defined alleles from the latest HLA workshop (1992). Only for 5.4% of the total number of possible genotypes, it is not always possible to define exactly the genotype because of the complex pattern generated by the different SSO's which could give identical patterns for different genotypes.
The number of alleles that were observed in the population sample ranged between 8 for DQA1 and 28 for DRB1 (Table 1). The lowest heterozygosity was seen for DPB1 (79.7%) although it had the second largest number of alleles among the HLA class II loci. The reason can be found in the presence of one dominant allele (0401: 41.3%) in the population while for the other loci the maximum allele frequency is not more than 27.3% (DQA1: 0501) with the majority of the alleles below 20% (Table 2).
The observed genotype distributions were tested for Hardy-Weinberg (HW) equilibrium by two different methods: (1) excess of homozygosity was determined by comparing the number of observed and expected homozygotes (Wahlund, 1928) and (2) an exact test of HW proportion for multiple alleles based on Monte Carlo methods (Guo and Thompson, 1992). Both tests could not show any departure from Hardy-Weinberg equilibrium in the genotype distributions for all the HLA class II loci in the Belgian population sample.

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chromosomes). Table 3 reports tests for independence of genotypic frequencies (5) at the above loci: no significant departure from the hypothesis of random associations between TPO and 3'APOB VNTR markers was found thus suggesting that these markers can be used together in fingerprinting analyses.

Two-locus homozygosity/heterozygosity

TPO		APOB Hom.	Het.
Hom.	(obs.)	4	13
	(exp.)	3.25	11.07
Het.	(obs.)	41	123
	(exp.)	37.82	128.85

X^2 with 1 d.f.= 1.04 (P>0.25)

Variance of heterozygous loci

	(obs.)	(exp.)
Mean	1.66±0.04	1.69±0.04
Variance	0.27	0.25

Tab.3

Both polymorphisms resulted to be very informative in all the tested groups, with PIC values (8) ranging from 0.78 (APOB) to 0.90 (TPO).

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with peaks at 37 and 49 repeats (each repeat is 15bp long (3)). Again, some alleles showed different frequencies between groups (see arrows).

ALLELES	ALBANIANS	GREEKS	CALABRIANS	SARDINIANS
21	**5** (0-10)	0	0	**6** (0-12)
23	0	**6** (0-12)	0	0
→ 26	0	0	**8** (2-14)	0
29	0	**6** (0-12)	0	0
31	**115** (92-139)	**72** (53-92)	**134** (112-157)	**78** (58-98)
33	**66** (48-84)	**67** (48-85)	**59** (44-74)	**78** (58-98)
34	0	0	0	**6** (0-12)
35	**242** (210-273)	**222** (191-253)	**244** (216-272)	**267** (234-300)
37	**418** (381-454)	**394** (358-431)	**366** (334-397)	**383** (347-420)
39	**33** (20-46)	**56** (38-73)	**34** (22-45)	**56** (38-73)
40	0	0	**4** (0-8)	0
41	**11** (3-19)	**11** (3-19)	**4** (0-8)	**11** (3-19)
43	**5** (0-10)	0	**4** (0-8)	**6** (0-12)
45	**5** (0-10)	**11** (3-19)	**13** (5-20)	**6** (0-12)
46	0	0	0	**6** (0-12)
47	**38** (24-53)	**61** (43-79)	**42** (29-55)	**28** (16-40)
48	0	0	0	**6** (0-12)
→ 49	**33** (20-46)	**72** (53-92)	**63** (47-79)	**44** (29-60)
50	0	0	0	**6** (0-12)
→ 51	**27** (15-40)	**22** (11-33)	**21** (12-30)	**6** (0-12)
→ 53	0	0	0	**11** (3-19)
55	0	0	**4** (0-8)	0

Tab. 1b

Three test statistics (5) were used to verify whether TPO and APOB genotypic distributions conformed to HWE predictions: i) the observed and expected number of homozygotes and heterozygotes; ii) the observed and expected numbers of distinct homozygous and heterozygous genotypes; iii) the likelihood ratio G (6) computed according to ref.5. In each sample, and for both polymorphisms, the observed genotypic numbers fitted with those expected at HWE.

The heterogeneity between groups, with respect both to TPO and 3'APOB allele frequencies, was assessed by using the G test: preliminarly the test was applied to all the populations, showing that heterogeneity was significant only with respect to the TPO gene. Then pairwise comparisons between TPO allelic distributions were performed thus obtaing the results summarized in Tab. 2:

	ALB	GR	CA	SA
ALB	--	NS	S	S
GR		--	NS	S
CA			--	S
SA				--

Tab. 2

As for the majority of the known polymorphisms, the Sardinian sample was different from each other; the Calabrian sample, which was distinguishable from the Albanian one, did not show significant heterogeneity when compared with the Greek sample. This is in line with the hypothesis that the Greek colonization, which occured in South Italy in the VIII century b.C., played a role in the constitution of the Southern Italian gene pool (7).

Since both TPO and APOB loci lie on the short arm of chromosome 2, possible preferential allele associations were investigated by using pooled data from Greeks and Calabrians (362

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POPULATION GENETICS OF VNTR MARKERS (TPO AND 3'APOB LOCI) IN THE MEDITERRANEAN AREA (ALBANIA, GREECE AND ITALY)

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The use of hypervariable markers in population genetic studies indicated that different ethnic groups can show distinguishing features of allele frequencies, even though the range of DNA fragment size is almost the same (1). It is not clear, however, how much efficient are these markers in distinguishing populations of the same major ethnic group.

By using PCR, electrophoresis and ethidium bromide staining, VNTR markers at Thyroid Peroxidase (TPO) and 3'Apolipoprotein B (3'APOB) loci (2,3) were analyzed in a total of 364 DNA samples: 91 from Tirana (Albania); 83 from Athens (Greece); 98 and 92 from Calabria and Sardinia (Italy), respectively. The allele frequencies (X1000), evaluated by gene counting, and the respective 95% confidence intervals are reported in Tables 1a (TPO) and 1b (3'APOB).

ALLELES	ALBANIANS	GREEKS	CALABRIANS	SARDINIANS
→ 4	**10** (3-17)	0	0	0
5	**5** (0-10)	0	0	**5** (0-10)
→ 6	**5** (0-10)	**12** (4-21)	**14** (6-23)	**22** (11-32)
→ 7	**56** (40-73)	**54** (37-72)	**48** (33-63)	**5** (0-10)
8	**5** (0-10)	**18** (8-28)	**5** (0-10)	**16** (7-25)
→ 9	**87** (67-107)	**36** (22-51)	**53** (37-68)	**16** (7-25)
→ 10	**10** (3-17)	**12** (4-21)	**5** (0-10)	**32** (19-45)
→ 11	**71** (53-90)	**42** (27-58)	**34** (21-46)	**22** (11-32)
12	**71** (53-90)	**72** (52-92)	**77** (58-95)	**108** (85-130)
→ 13	**61** (44-78)	**60** (42-79)	**53** (37-68)	**118** (95-142)
→ 14	**77** (58-96)	**133** (106-159)	**96** (76-117)	**48** (33-64)
→ 15	**260** (229-292)	**193** (162-223)	**159** (133-184)	**204** (175-234)
16	**97** (76-118)	**102** (79-126)	**115** (93-138)	**134** (109-159)
→ 17	**5** (0-10)	**42** (27-58)	**58** (42-74)	**81** (61-101)
→ 18	**41** (27-55)	**54** (37-72)	**38** (25-52)	**11** (3-18)
→ 19	**15** (7-24)	**36** (22-51)	**67** (50-85)	**22** (11-32)
20	**26** (14-37)	**30** (17-43)	**34** (21-46)	**22** (11-32)
21	**31** (18-43)	**24** (12-36)	**48** (33-63)	**32** (19-45)
→ 22	**5** (0-10)	0	**19** (10-29)	**27** (15-39)
23	**26** (14-37)	**36** (22-51)	**24** (13-35)	**27** (15-39)
24	**10** (3-17)	**12** (4-21)	**14** (6-23)	**16** (7-25)
25	**10** (3-17)	**6** (0-12)	**29** (17-40)	**22** (11-32)
26	**5** (0-10)	0	0	**11** (3-18)
27	0	0	**5** (0-10)	0
28	0	**6** (0-12)	**5** (0-10)	0
29	**5** (0-10)	**6** (0-12)	0	0
31	**5** (0-10)	0	0	0
→ 34	0	**12** (4-21)	0	0

Tab. 1a

As to TPO, a total of 28 alleles were identified (Tab. 1a) which varied in size from 4 to 34 repeats (each repeat is 50bp long (4)). All groups shared a unimodal distribution with peak at 15 repeats; some alleles, however, showed significantly different frequencies between groups (see arrows).

As to 3'APOB, a total of 22 alleles were identified (Tab. 1b) varying in size from 21 to 55 repeats. The bimodal distribution of allele frequencies already found in other populations was observed,

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Table 1
Distribution of observed and expected HUMTH01 and MIT-MH26 phenotypes.

MITMH26

Type	Observed	Expected	Chi square
1-1	1	0.82	0.04
3-3	7	5.40	0.47
4-4	18	16.71	0.10
6-6	12	9.60	0.60
7-7	1	1.45	0.14
1-3	5	4.20	0.15
1-4	6	7.39	0.26
1-6	4	5.60	0.46
1-7	4	2.18	1.52
3-4	16	19.00	0.47
3-6	16	14.40	0.18
3-7	3	5.60	1.21
4-6	23	25.33	0.21
4-7	14	9.85	1.75
6-7	5	7.47	0.81
sum	135		

HUMTH01

Type	Observed	Expected	Chi square
6-6	10	9.76	0.01
7-7	5	3.66	0.49
8-8	1	2.56	0.95
9-9	14	10.00	1.60
10-10	15	8.57	4.81
11-11	0	0.01	0.01
6-7	7	11.95	2.05
6-8	11	10.00	0.10
6-9	22	19.76	0.25
6-10	19	18.29	0.03
6-11	1	0.49	0.54
7-8	8	6.13	0.57
7-9	14	12.10	0.30
7-10	10	11.20	0.13
7-11	0	0.30	0.30
8-9	10	10.13	0.00
8-10	9	9.38	0.02
8-11	1	0.25	2.25
9-10	7	18.52	7.17
9-11	0	0.49	0.49
10-11	0	0.46	0.46
sum	164		

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classified with the help of home made admixtures of different individuals. Individual alleles apparently originated from iteration (6 to 11 repeat for HUMTH01, 1 to 7 repeats for MIT-MH26) of a four base pair-long repeat unit. Interestingly, in MIT-MH26 distribution some alleles were missing in the virtually complete ladder of sizes (fragments with 2 and 5 tandem repeats). HUMTH01 distribution showed a complete scale of alleles (from 6 to 11 repeat units), but an additional allele with incomplete repeat unit (three base pair unit instead of the expected four) was present in a significant pool of individuals (the so called 10-1 allele). A high average heterozygosity was evident in both systems (HUMTH01:.75; MIT- MH26:.72). Unlike the two systems just dealt, HUMACTBP2 was a more complex polymorphism to study. It encompassed a larger DNA region in which the occurrence of incomplete repeat units had its natural counterpart in a wider number of alleles. Additionally, alleles with apparently the same size were supposed to underlie internal sequence differences by slightly irregular migration of the relevant bands. Owing to these peculiarity, a correct definition of the entire HUMACTBP2 pattern of alleles was only - and partially - achieved by the use of the automated sequencer. Also, setting a current allelic ladder containing the most frequent alleles was a proportionally difficult task. Due to these difficulties, a tentative distribution of observed and expected genotypes is achieved by PAGE and silver staining procedures. An A.L.F. typing of all genotypes so far detected is presently in progress. The average heterozigosity of the system amounted to .92 and no mutation were observed in family segregation. We presently believe that HUMACTBP2 system will be useful in a fewer forensic instances than those envisioned for other STRs.

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ALLELE FREQUENCIES OF THREE STRs IN AN ITALIAN POPULATION SAMPLE

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Several short tandem repeat (STR) (1) polymorphisms have been recently described which are potentially useful in procedures of criminal identification and in paternity tests. STRs consist of short DNA stretches arranged in 2-6 base pair long tandem repeats. They are abundant in the human genome and generally are highly polymorphic. Because of their small size (generally 300 bp) STRs are more likely to be investigated from degraded DNA samples. Tetranucleotide-repeat microsatellites are of primary interest, because they are more easily genotyped.
Preliminar to their use in forensics, an archive of data to adopt as reference should be made available. In conformity with this guideline, we promoted a study aimed at obtaining the allele distribution of some recently described STRs. We here report frequencies of HUMTH01 (11p15.5-p15) (2), MIT-MH26 (D7S460) (3), HUMACTBP2 (chr 5 or 6) (4) in our reference population (Central and Southern Italy).

MATERIALS AND METHODS

Genomic DNA was extracted from whole blood samples, obtained by venipuncture from at least 100 unrelated Italian donors. Standard PCR protocols were adopted to amplify HUMTH01, MITMH26 and HUMACTBP2 loci, with some modifications apported whenever fluorescent primers use was required. Amplified products were separated in polyacrylamide gels (native or denaturing) followed either by silver staining or by analysis with an automated apparatus relying on argon fluorescence (A.L.F., Pharmacia).
Both the non-denaturing gels and the automatic apparatus (6% T and 7 M urea) were able to simultaneously separate the allelic patterns of the three loci. However, with the native horizontal polyacrilamide gels, different acrilamide sieves had to be used, according to the size of alleles (8% T for HumACTBP2, 7.5% T for HumTH01 and MIT-MH26, 5% C common to all the systems). For HumACTBP2, an accurate typing was achieved only by automated laser fluorescence.

RESULTS AND DISCUSSION

In Table 1, the distribution of observed and expected HUMTH01 and MIT-MH26 genotypes is shown. Fragments were identified and

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heterozigosity rate (0,80) and the allelic diversity (0,837±0,0344). On the basis of the allelic frequencies found the (P_D) was calculated as 0,902, and the overall results obtained in the present study show that 3'-ApoB locus is a reliable tool in identification and paternity analysis.

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Fig.1. 3'-ApoB alleles after agarose gel electrophoresis and ethidium bromide staining. From left to right: pGem, 49-53, 31-39,33-35,35-43,123 bp, 36-37, 35-37, 33-39, 47-49, pGem.

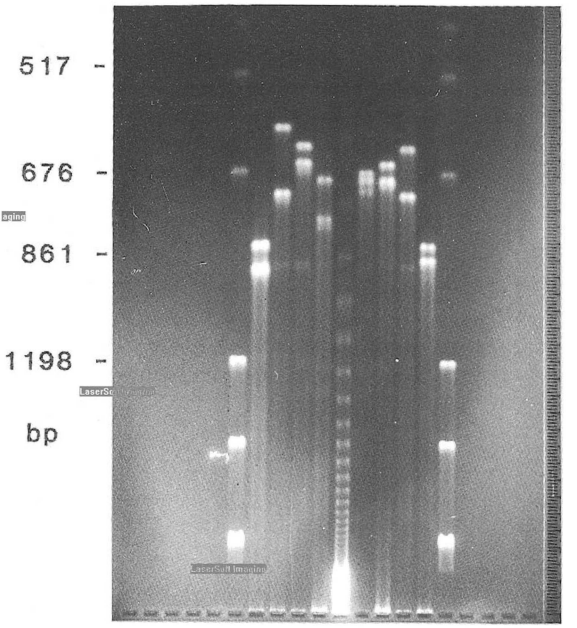
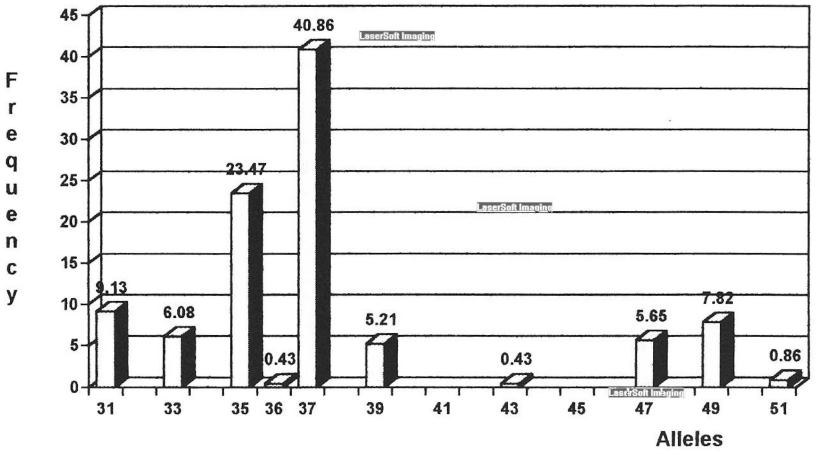


Fig.2. Frequency distribution of 3'ApoB VNTR alleles



Allelic frequency data were found to be similar to those observed in other population studies, both in Italy and in Europe. Allele 36 seems to be a rare variant; a previous observation (Schnee-Griese and Teifel-Greding, 1991) of a similar allele in a larger population sample (n=340) suggested that this mutational event was unlikely to be recent.

The χ^2 test (χ^2=6,9; 0,7<P<0,8; 9 df) showed no significant deviation from Hardy-Weinberg equilibrium. Further evidence of good agreement with Hardy-Weinberg equilibrium was the similarity between the

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Polymorphism of the 3'-ApoB VNTR system in a population sample from the province of Parma.

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INTRODUCTION

DNA polymorphism (Wyman and White, 1980; Jeffreys et al., 1985) analysis has been introduced into routine casework in many forensic haemogenetics laboratories. PCR amplification (Saiki et al., 1985) of target DNA has been shown to be especially useful in typing small or partially degraded specimens.
AMP-FLP's (3'-ApoB, YNZ22, D1S80, Col2A1) (Boerwinkle et al., 1989; Ludwig et al., 1989; Kasai et al., 1990; Horn et al., 1989; Wu et al., 1990) are among the most reliable polymorphisms studied so far, as Mendelian inheritance and balanced allelic distribution have been established in many population studies.
3'-ApoB polymorphism lies on the short arm of chromosome 2 at the 3' end of the Apolipoprotein B gene and consists of tandemly repeated, 15-16 bp long, AT rich sequences (Knott et al., 1986).
A high number of alleles has been observed in previous population studies; a maximum number of 14 alleles has been discovered in sample of 318 individuals from northern Europe (Ludwig et al., op cit), whilst other population studies have shown a number of alleles ranging from 11 to 13 (Giorgetti et al., 1991; Rand et. al., 1992, d'Aloja et al., 1992).
The 3'-ApoB polymorphic system has been analysed on a population sample from the province of Parma (Northern Italy) to obtain database information necessary for practical applications to criminal and paternity caseworks.

MATERIALS AND METHODS

115 blood samples were obtained from healthy blood donors living in the province of Parma and stored at -80 °C until use. DNA was extracted following the protocol developed at the FBI Academy, Quantico (Budowle and Baechtel, 1990). Amplification was carried out in a thermal cycler PTC 100-60 (MJR, Watertown, MA USA) for 29 cycles, at the following conditions: denaturation at 94 °C 1 min, annealing at 58 °C 1 min, extension at 58 °C 5 min, followed by a final extension time of 7 minutes.
The primers used were those proposed by Boerwinkle et al. (op cit): 5'-ATGGAAACGGAGAAATTATG-3', 5'-CCTTCTCACTTGGCAAATAC-3'.
The amplification mix contained 30 ng DNA, 2U Taq DNA-polymerase (Promega, Madison, WI USA), 5 μl buffer (500mM KCl, 100mM Tris-HCl, 15mM MgCl, 1% Triton X-100), 1 mM each primer, 200mM each dNTP up to a total volume of 50 μl.
10 μl of amplified product were separated by electrophoresis in 2,5% agarose gel (NuSieve 3:1 Agarose, FMC, Rockland, ME USA) in 1X TAE buffer at 70V for 16h. After ethidium-bromide staining, bands were identified by comparison with molecular size standards (123 bp ladder, pGem, ϕX174), applying the local reciprocal method of Elder and Southern (1983).
Alleles were pooled into four groups (Brenner and Morris, 1990; Rand et al., op cit) in order to obtain suitable numbers for χ^2 calculation under Hardy-Weinberg law; the heterozigosity rate, allelic diversity, and discriminating power (P_D)were also calculated.

RESULTS AND DISCUSSION

Ten alleles were observed in the population sample studied, ranging in size from about 600 to 900 bp (Fig.1) and differing from each other mostly by multiples of a repeat unit of about 30 bp. A further allele was observed, which differed from contiguous alleles (35 and 37) only by 15 bp (half of the standard repeat unit). The frequency distribution of alleles is shown in Figure 2.

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FIGURE 1

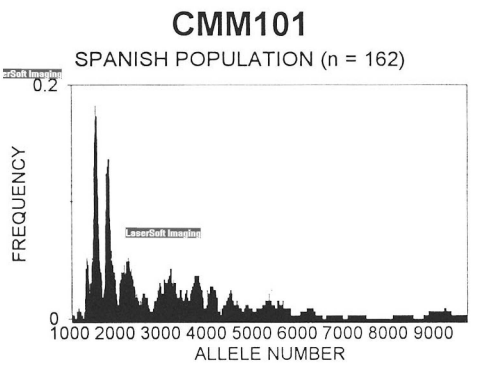


FIGURE 2

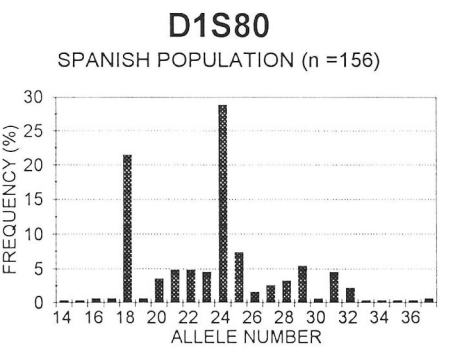


FIGURE 3

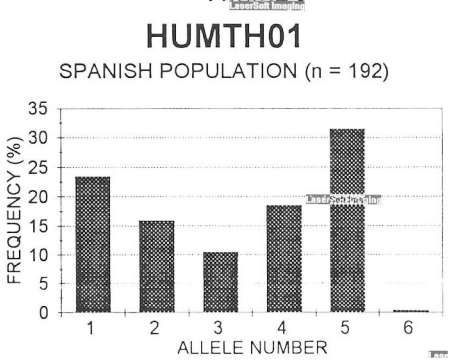


FIGURE 4

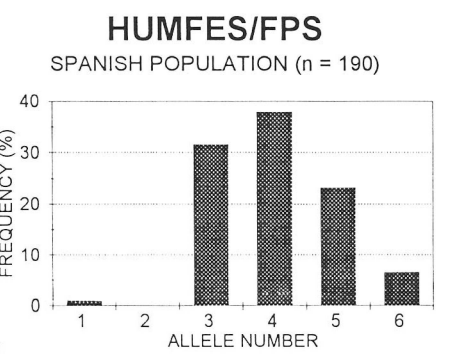
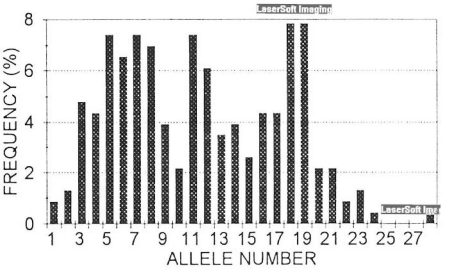


FIGURE 5



RESULTS

Figures 1 and 2 shows the allele frequency distribution for the D14S13 and D1S80 loci. The allele frequency distribution in the spanish population is similar to other already published for white-caucasian individuals. A total of 24 different D1S80 alleles were observed in 156 individuals. Two alleles (#18 and 24) were found to be relatively common in the Spanish sample having frequencies of 0.175 and 0.305, respectively. Similarly, for STR's locus HUMTHO1 (Fig 3), there were 6 alleles observed in 190 individuals with 4 alleles occuring at a frequency greater than 0.100. We have found a mutation in the allele number 5 of the HUMTH01 locus produced by the addition of a single nucleotide. There were 6 alleles for HUMFES locus (Fig 4) with 3 of them relatively common. Allele number 2 was not found in any case for this locus. HUMFES and HUMTH01 loci have a similar allele distribution to other caucasian population groups. ACTBP2 locus (Fig 5) shows a variation in the alleles mobility depending the electrophoretic conditions, as it has been reported for other AT-rich VNTR (Sullivan et al., 1992). This fact can represent a potential problem for the interlaboratory data comparison and protocol standardization.

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ALLELIC FREQUENCY IN THE POPULATION OF SPAIN USING THREE HYPERVARIABLE LOCI

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INTRODUCTION

Tandemly repeated sequences represent a rich source of highly polymorphic markers useful in genetic linkage analysis, genome mapping and personal identification. The interpretation of the results from the analysis of these genetic markers, utilized in forensic and paternity testing, requires the existence of a database for the appropriate population.

Studies to determine the allele frequencies of single locus have been carried out for some world populations. The substructure of populations and their influence in forensic cases has generated a great deal of controversy (Lewontin and Hartl, 1991; Chakraborty and Kidd, 1991). We have constructed spanish population databases for the SLPs, YNH24, TBQ7, MS31 and MS43 (Valverde et al, 1993). In order to complete the spanish databases, we have studied other polymorphic loci. In the present paper we report the databases for five different loci in the spanish population. One of these markers is the D14S13 locus, detected by the CMM101 probe (Nakamura et al., 1988a). The other four loci are detected by PCR amplification followed by electrophoretic separation. The four analyzed loci are: D1S80 (Nakamura et al., 1988b; Budowle, 1991), HUMTH01 (Polymeropoulos et al., 1991a), HUMFES/FPS (Polymeropoulos et al., 1991b) and ACTBP2 (Polymeropoulos, M.H. et al., 1992)

MATERIALS AND METHODS

SAMPLES: DNA was extracted from 190 unrelated volunteer donors, randomly selected from Spain. The high molecular weight DNA was extracted following the perchlorate protocol as described (Valverde et al., 1992; Díez et al., 1992).

D14S13 LOCUS: 5-10 μg of DNA were HaeIII digested, and the resultant fragments electrophoresed and blotted following the EDNAP protocol recomendations. The CMM101 probe was ^{32}P labelled by random priming.

LOCI DETECTED BY PCR (D1S80, HUMFES, HUMTH01, ACTBP2): All the amplification were performed in a final volume of 50 μl in a Perkin-Elmer thermocycler. The different cycle temperatures, primers sequences and electrophoresis conditions were as described (Budowle, 1991; Polymeropoulos et al., 1991a,b, 1992)

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Table 1. Statistical evaluations

	Mean excl. chance	DI	Heterozy.
vWA	0.61	0.93	0.82

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RESULTS AND DISCUSSION

The 7 common alleles, identified in the range 138 to 162 bp and classified according to the number of repeat sequences, were found. A new smaller allele, tentatively designated as vWA*5, was discovered. No significant differences between allele frequencies in the 2 Italian samples were found. The most frequent alleles were vWA*11, vWA*12, vWA*10, in line with the frequencies found in previous studies on Caucasians (Kimpton et al.1992; Brinkmann, pers. comm.1992). The frequency distribution of vWa system alleles is shown in figure 2. Observed and expected genotypes were compared assuming the Hardy-Weinberg law (χ^2= 22.166 0.75< P <0.90; 28 d.f.). The Italian sample was then statistically compared with previous studies on Caucasians (Kimpton et al.1992; Brinkmann, pers. comm.1992) and no deviation from homogeneity was found: χ^2=8.1436 P=0.9030 ± 0.0094; G statistic = 8.7207 P=0.8820 ± 0.0094. The results of the present study suggest that this STR system could be a powerful tool for forensic purposes-Table 1-.

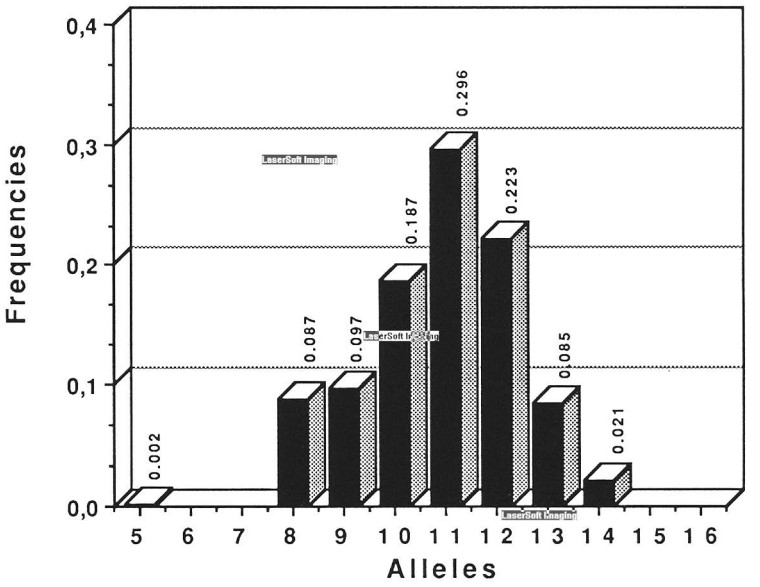

Fig. 2 Frequency distribution of vWa system alleles in an Italian population sample.

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ALLELE FREQUENCIES OF THE vWA STR LOCUS IN ITALY

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INTRODUCTION

The tetrameric (TCTA) tandem repeat system vWa, located in intron 40 of the vWF gene, was first described by Kimpton et al. (1992). The polymorphism of this locus has been analyzed in two population samples of North and Central Italy, by the polymerase chain reaction (Saiki et al. 1985). The aim of the present study was to set up a data base, to check whether allelic frequencies show inter-regional differences and to evaluate this system for forensic purposes.

MATERIALS AND METHODS

Sample preparation: DNA was extracted from blood samples of 211 unrelated healthy subjects according to the phenol-isoamyl alcohol method (Budowle and Baetchel 1990).
PCR reaction mix: 50 ng template DNA, 1 U Taq polymerase (Promega, USA), 2 µl buffer (Promega), 0.6 µM each primer, 100 µM each dNTP, made up to total volume of 25 µl, with the addition of 1-2 drops of oil.
Primer sequences (Kimpton et al. 1992)
vWA1= 5' CCCTAGTGGATGATAAGAATAATC3'
VWA2= 5' GGACAGATGATAAATACATAGGATGGATGG'3
Temperature cycling conditions:

denaturation	94°/1min
annealing	50°/1min
extension	72°/2min
cycles	30

Electrophoresis: denaturing polyacrylamide gel (6% T, 3% C, 7mM urea, thickness 750 µm), piperazine diacrylamide as crosslinker, 100mM formate; 20 cm. separation distance, 2% agarose plugs (Allen et al. 1989; Budowle et al. 1991); running time: 1000 V, 40 mA, 3 W, ramping every 10 min up to 15 W as suggested by Wiegand et al (1993).
The bands were visualised by silver staining (Budowle et al. 1991) and phenotyped by side-to-side comparison with an allelic ladder -Fig. 1-.

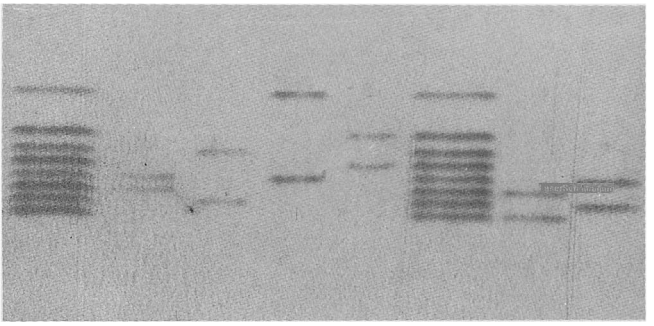

Fig. 1 Silver stained vWA system polyacrilamide gel of the amplified fragments. From left to right: allelic ladder is in lane 1,6. vWA phenotypes: lane 2: 11-12; lane 3: 9-13; lane 4: 5-11; lane 5: 8-10; lane 7: 12-14; lane 8: 11-13.

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differ significantly in the groups compared. In addition among donors from St.-Petersburg decrease in frequency of DQB-1B allele and increase in rate of DQB-X was significant as compared to the German population. The distribution of DQA alleles in donors from St.-Petersburg did not differ from that in the German population.

The date on high statistical significance of linkage between the DRB and the two DQ loci has been confirmed although, according to our findings, the incomplete association between DQB-3A and DQA-1B alleles was observed as compared to the German population.

Moreover, rare associations between DR and DQ loci (DRB-1/DQB-3B; DRB-11/DQB-1B; DRB-1/DQA-2; DRB-15/DQA-1C) were revealed.

In 17 (15.4%) individuals there are differencies between results obtained by HLA-DR DNA-RFLP and serological typing (mainly in definition of HLA-DR-3 and DR-6 specificities).

Thus the results obtained have confirmed the usefullnes of the RFLP method in typing class II HLA molecules; a new information about the distribution of alleles DRB,DQA and DQB loci in St.-Petersburg's group of donors is gathered and the differences in their distribution in the two populations are given.

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T a b l e. Distribution of DRB, DQA and DQB alleles determined by RFLP method in donors from St.-Petersburg.

DRB alleles	Number of alleles	DQA and DQB alleles	Number of alleles
DRB-1	23 (9.7)	DQA-1A	27 (11.4)
DRB-15	23 (9.7)	DQA-1B	43 (18.2)
DRB-16	9 (3.8)	DQA-1C	25 (10.6)
DRB-17-1	16 (6.8)	DQA-2	68 (28.8)
DRB-17-2	16 (6.8)	DQA-3	73 (30.9)
DRB-4	36 (15.3)		
DRB-11	24 (10.2)		
DRB-12	4 (1.7)	DQB-1A	43 (18.2)
DRB-13a1	12 (5.1)	DQB-1B	38 (16.1)
DRB-13a3	6 (2.5)	DQB-X	7 (3.0)
DRB-13a4	6 (2.5)	DQB-2A	33 (14.0)
DRB-13в	3 (1.3)	DQB-2B	16 (6.8)
DRB-14a	3 (1.3)	DQB-3A	45 (19.1)
DRB-14в	2 (0.8)	DQB-3B	54 (22.9)
DRB-7-1	4 (1.7)		
DRB-7-2/9	33 (13.9)		
DRB-8	12 (5.1)		
DRB-10	4 (1.7)		

Note: allele designated as DRB-7-2/9 corresponds to two alleles DRB-7-2 and DRB-9 which cannot be separated by the RFLP method; allele frequency in per cent is given in parentheses.

The distribution of HLA-DRB, - DQA, -DQB alleles in donors from St.-Petersburg was compared with data obtained in the German population [2,3]. The increase in frequency of DRB17-2 allele was found in donors from St.-Petersburg as compared to the German population (6.8% vs. 2.8%). Furthermore, in the group of healthy donors DRB16 frequency was also slightly increased(P =0.07). Frequencies of other alleles of DRB locus did not

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DISTRIBUTION OF HLA-DR AND -DQ ALLELES REVEALED BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS IN DONORS FROM ST.-PETERSBURG.

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As is known, class II HLA molecules are characterized by a wide allelic polymorphism which be defined by various immunologic methods. However, the immunologic approaches in the characterization of class II molecule polymorphism is associated with certain limitations put by both the errors of methods and impossibility to define all structural distinctive features of these molecules. In recent years, the successful studies on the allelic polymorphism of class II molecules became possible because of introduction of new approaches based on the investigations into HLA genes structure. Among them, the method based on the RFLP analysis of genes is the most reliable and accurate.

In the present work, the results of identification of HLA-DRB, DQA and DQB alleles in 118 healthy donors from St.-Petersburg as well the analyzed date on the association found out between different alleles of the above loci are presented.

The HLA-DRB and -DQ alleles were determined by restriction fragment length polymorphism (RFLP) analysis using DRB, DQB and DQA probe after TaqI digestion of DNA and Southern blotting. Identification of class II DRB, DQA and DQB alleles was made according by J. Bidwell [1]. The allele frequencies of DRB, DQB and DQA loci, linkage disequilibria between DR and DQ loci and haplotype rates were calculated. Also, allelic polymorphism within HLA-DR and DQ antigens were determined by microlymphocytoxicity method.

The RFLP analysis allowed to identify 18 HLA-DRB, 7 HLA-DQB and 5 HLA-DQA alleles. Distribution of DR and DQ alleles is presented in Table. It is to point out that among the donors examined, 18 (7.6%) were homozygous for DRB, 68 (28.8%) for DQA and 38 (16.4%) for DQB loci.

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Tab. 2: Phenotype frequencies of TC 11 (HUMTH01) of 268 unrelated individuals of Northern Germany. Heterozygosityindex is 76.7%. Distribution fits the Hardy-Weinberg equilibrium (χ^2-test, df: 10, $0.95 < P < 0.98$).

Phenotype	observed		expected
	number	%	%
6/6	13	4,9	4.8
6/7	20	7.5	7.0
6/8	10	3.7	4.5
6/9	12	4.5	7.5
6/10	49	18.3	15.2
7/7	7	2.6	2.6
7/8	11	4.1	3.3
7/9	15	5.6	5.5
7/10	26	9.7	11.1
8/8	4	1.5	1.0
8/9	11	4.1	3.5
8/10	15	5.6	7.1
9/9	10	3.7	2.9
9/10	34	12.7	11.9
10/10	31	11.6	12.0

In conclusion separation of TC 11-PCR products on nondenaturating polyacrylamide gels followed by ethidium bromide staining allows to resolve and detect the alleles of TC 11. Alleles 9.3 and 10 differ only by 1 basepair and could only be separated by denaturating sequencing gels.

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DNA samples were collected from 268 unrelated individuals, 105 three member families and two 7 member families from Northern Germany.

Results and Discussion

We could find five different TC 11 alleles. These were the alleles 6, 7, 8, 9 and 10. Table 1 shows the allele frequencies of altogether 536 analysed alleles. The allele with the highest frequency is allele 10 with about 35%. The allelic distribution is comparable to those found by Edwards et al.(2). Allele 8 has the lowest frequency with 10%. The heterozygosity index is 75.7%. With the nondenaturating gel used in our experiments we were not able to separate alleles 9.3 and 10. For this reason we typed both alleles as allele 10.

Tab. 1: Allele frequency of TC 11 (HUMTH01) in the German population. Alleles 9.3 and 10 could not be separated and are therefore typed as 10.

REPEAT	%
5	-
6	21.8
7	16.0
8	10.3
9	17.2
9.3 + 10	34.7
11	-

The distribution of observable phenotypes in a population sample of 268 unrelated individuals are shown in Fig.1 and Tab.2. The heterozygosity index is 75.7% The distribution of the observed phenotypes is not different from the expected distribution according to Hardy-Weinberg equilibrium (X^2-test, d.f.: 10, $0.95 < P < 0.98$). As expected it could be stated that the alleles associated randomly with each other at this locus and there is no detectable population heterogeneity.

Up to now we analysed 246 meioses in the family studies and we could not find any de novo mutation. For calculating mutation rates we need to analyse more families.

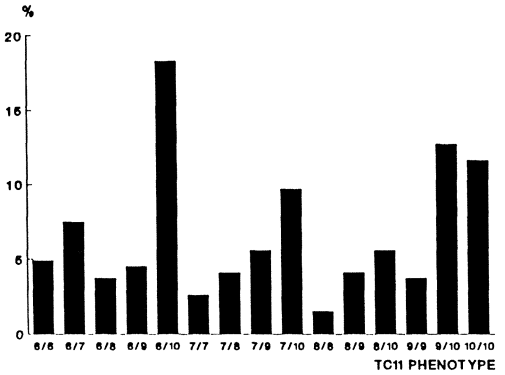

Fig. 1: Phenotype frequencies of TC 11 (HUMTH01) of 268 unrelated individuals of Northern Germany. Phenotypes are plotted against frequency in %.

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ANALYSIS OF THE SHORT TANDEM REPEAT POLYMORPHISM TC 11 (HUMTH01): ALLELE FREQUENCIES AND FAMILY STUDIES.

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Introduction

Short tandem repeat (STR) polymorphisms provide a potential tool to forensic casework. In order to use the system TC 11 (HUMTH01: human tyrosine hydroxylase intron 1 on chromosome 11 (1)) in forensic DNA analysis, detailed knowledge of allele frequencies is most important. In paternity cases it is also essential to know the mutation rate. For this reason we analysed unrelated individuals and families from Northern Germany.

Material and Methods

DNA was extracted from whole blood according to standard procedures. PCR was carried out with the primers (1):

TC 11/1: 5'-GTG GGC TGA AAA GCT CCC GAT TAT-3'
TC 11/2: 5'-ATT CAA AGG GTA TCT GGG CTC TGG-3'

The amplification conditions were: 50 mM KCl, 10 mM Tris HCl pH 8.3, 0.1% Triton X-100, 0.2 mM each dNTP, 0.5 μM each primer, 1 U Taq polymerase, 5-10 ng DNA. The reaction volume was 25 μl. The thermal cycler (Biometra TRIO-Thermoblock) was set to 80^{o}C for 5 min for hot start conditions and the reaction was started by adding primer TC 11/2 and Taq polymerase to the heated reaction mix (including DNA, TC 11/1 and $MgCl_2$). The reaction conditions were as followed:

94^{o}C - 1 min, 64^{o}C - 1 min, 72^{o}C - 2 min, for 30 cycles

8 μl of the amplification product were loaded on a 1 mm thick, 20 cm long polyacrylamidegel (6%T, 5%C) with bisacrylamide as crosslinker and seperated in 1x TBE for 3 h at constant 30 W (about 380-400V) until xylencyanol dye reached the anodal end of the gel. After electrophoresis the separated products were stained with ethidiumbromide (1 μg/ml) and visualized by means of UV light. The alleles were typed by comparing the products with an alleleic ladder which was constructed from alleles observed during this study.

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The heterozygosity (H) of each locus, the most common allele, the range of DNA fragments found for each locus, power of exclusion of the system, match probability, and the discriminant probablity, are all given in Table 2. The H in the Non-Basques for YNH24 and g3 are the lowest (83% and 85% respectively) perhaps due to the outbred nature of these populations. However the Basques with all four probes and the Non Basques for probes MS43 and MS1 gave the H values near or above 90%. The theoretical exclusion probability is lowest for probe YNH24 (Basques 82% and Non Basques 78%) while for all other probes the PE is above 90%. The cumulative PE is 99.99%. The discriminant probability for these loci ranges from 88-90% and similar values were also observed for the Non-Basque populations.

These results indicate that the distribution patterns of various VNTR loci are considerably different for both the Basque and Non-Basque populations, but all four loci are extremely useful in paternity and forensic testing.

Reference

Garber, B.A. and Morris, J.W. (1983) General equation for average power of exclusion for genetic systems of n codominant allele in one parent and no parent case of disputed parentage. In: Walker, R.H. (ed.) *Inclusion probabilities in parentage testing.* American Association Blood Bank. pp. 277-280.

Jones, D.A. (1972) Blood sample: probability of discrimination. *J.For.Sci.Soc.*, **12**, 355-7.

Table 1: Heterozygosity (H), Frequency (F) of most common allele, power of exclusion (PE), probability of match (PM) of four VNTR probes in Basque and Non-Basque populations

Probe	Population	H %	Range of fragments Kb	Common allele	% F	PE	PM
g3	Basques	89.0	1.5-18.5	6.1-6.4	8.4	0.9084	0.0354
	Non-Basques	85.0	1.5-16.1	2.8-3.0	5.7	0.9196	0.0270
MS1	Basques	96.8	1.7-1.9	5.6-5.9	8.6	0.9050	0.0400
	Non-Basques	98.5	1.3-1.9	4.1-4.4	6.5	0.9266	0.0298
MS43	Basques	87.9	3.0-14.3	7.8-8.0	6.0	0.9145	0.0382
	Non-Basques	89.0	3.0-15.4	8.4-8.6	8.4	0.9235	0.0285
YNH24	Basques	90.5	2.2-7.2	2.5-2.7	13.0	0.8241	0.0860
	Non-Basques	82.9	1.9-5.4	2.7-2.9	21.3	0.7765	0.1114

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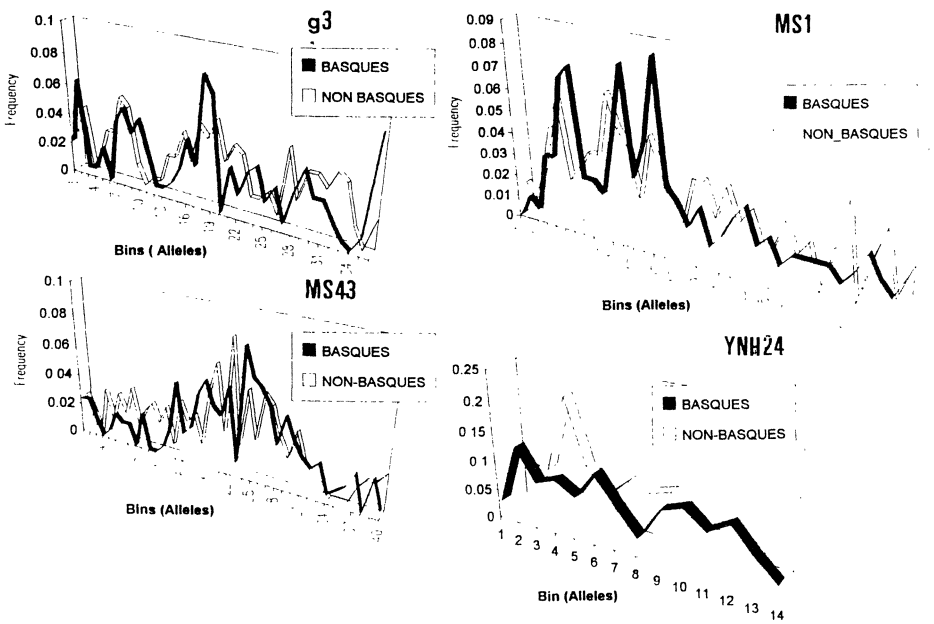
respectively. The frequencies of these bins were used to calculate the theoretical power of exclusion, probability of match, and the discriminate probability of each loci using the methods of Garber and Morris (1983) and Jones (1972) respectively.

Results and Discussion

The distribution of the allele frequencies detected by the single locus probes g3, MS1, MS43 and YNH24 are given in Figure 1 (a-d). For g3 probe there were three major peaks in Basques at bins 2 (1.6-1.8kb), 18 (6.1-6.4kb), and 19 (6.4-6.7kb), and a greater number of large DNA fragments were found in Basques (>12Kb). In Non Basques several alleles showed similar values. For the MS1 probe, the Basques showed three alleles of high values (bin 7, 3.2-3.5Kb; bin 12, 4.7-5.0Kb; and bin 15, 5.6-5.9Kb) while in the Non Basque population none of the alleles showed such a high frequency and the highest frequency was found for bin 10, 4.1-4.4Kb. The distribution of the MS43 alleles gave a considerable overlap between the Basque and the Non-Basque populations. Both populations showed a greater number of the fragments between 8.5 and 9.1Kb. The YNH24 probe showed considerable variation between these two populations. The Basques show the highest frequency of allele with low molecular weight DNA fragments (Bin 2, 2.5-2.7Kb; f=13%) while the highest frequency of the allele in Non-Basques was 21% for Bin 4, 2.7-2.9Kb.

Figure 1: Distribution pattern of allele frequencies for g3, MS1, MS43 and YNH probes in Basques and Non Basques



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ALLELIC HETEROGENEITY OF SINGLE LOCUS PROBES IN THE POPULATIONS OF THE BASQUE COUNTRY, SPAIN: APPLICATION IN PATERNITY TESTING

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Both for genetic and anthropological studies, the Basque population is one of the most interesting in Europe. Anthropologically, the Basques are generally regarded as the relict population, the last representative of an early people who were thought to occupy much of western Europe, whose descendents are now found only in the Northern Pyrenes Over the long span of their micro evolution, the Basques have maintained their unique language, structure, and they are distinct from other western Europeans in a number of cultural and social features. Genetically the Basques have maintained the purity of their gene pool. In spite of the successive waves of invasions from the Alpine and Mediterranean races, the Basques have resisted to assimilate any foreign gene pool in the past. However in their recent history there is a greater mixing with the local Non Basque population. Genetic differentiation, increased endogamy and only the recent mixing with Non-Basques suggest that the present day populations of the Basque Country (Basques and Non Basques) represents different genetic subgroups. The present investigation aims to understand the magnitude of the differences of polymorphisms at four variable number of tandem repeat loci among two main subgroups of the Basque country. The effect of the variation in allele frequencies on paternity testing is also evaluated.

Material and Methods

In Spain the Basques live in the provinces of Vizcaya, Guiptazcoa, Alava and Navara. In our recent field investigation in the region of Vizcaya and Guiptazcoa, 10ml EDTA blood samples were collected from individuals with both the grandparents of Basque ancestry. The Non Basque (Spanish) blood samples were also collected from the same region.

Blood samples were frozen and transported to Newcastle where DNA extraction was carried out. Four VNTR loci were studied, D1S7, D7S22 and D12S11 using g3, MS1 and MS43 probes from Cellmark Diagnostic, and the D2S44 locus was studied using the probe YNH24 from Promega. The DNA samples were digested with Hinf I and analysed by standard technique used for RFLP analysis.

The molecular weight of the DNA fragments was estimated using the molmatch program supplied by UVP. The sizes of separated DNA fragments formed 36 classes of fixed bins for D7S22 and D1S7 loci, 40 and 14 bins for D12S11 and D2S44 loci

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RESULTS AND DISCUSION

The allele sizes and the allele frequency distributions were determined for 5 VNTR loci: D1S7, D7S21, D12S11, D5S43 and D2S44.

The resolution limit and measurement reproducibility ranges of our electrophoretic system was estimated by determining the size variation of the genomic control (K562) run in duplicated on every gel (Table 1).

	MS1		MS31		MS43A		MS8		YNH24	
	HMWF	LMWF	HMWF	LMWF	HMWF	LMWF	HMWF	LMWF	HMWF	LMWF
N	106	106	99	102	120	73	73	75	37	36
MEAN	4835	4482	7858	7003	13587	5290	5550	4789	4028	2906
MAXIMUM	4912	4527	7997	7095	13930	5367	5634	4845	4070	2929
MINIMUM	4759	4432	7694	6852	13323	5239	5465	4716	3978	2867
SD	29	23	60	46	120	27	33	27	21	15
MVI	2.19%	1.10%	3.82%	3.48%	4.47%	2.42%	3.05%	2.70%	2.27%	2.12%

Table 1. Resolution limit and measurement reproducibility. HMWF=High Molecular Weight Fragment; LMWF=Low Molecular Weight Fragment; N= number of genomic controls examined; MVI=Maximal Variation Inter gel.

The allele frequency distributions of five VNTR loci in a population sample of individuals living in Madrid and individuals from different areas of Spain are shown in Fig 1A and 1B respectively. As can be seen the allele frequency profiles are very similar in both populations, and no differences were observed when the data of these populations were combined (Fig. 1C).

The observed heterozygosity, range of band size and the most common fragment size range of the five loci analyzed are shown in the following table :

	MS1	MS31	MS43a	MS8	YNH24
Heterozygosity (%)	94.6	92.6	90	77.5	91
Allele size range (Kb)	0.9 - >20	1.7 - 14.2	2.8 - 14.6	1.7 - 17	1.4 - 10.6
Common size range	4.5 - 4.6	7.1 - 7.2	8- 8.1	6.7 - 6.8	3 - 3.1
(Kb) - [Frequency]	[2.4 %]	[4.2 %]	[3.8 %]	[20.2 %]	[8.9 %]

On the other hand, the allele frequency distributions determined in Spain are in agreement with other data previously reported from Caucasian populations, where loci and restriction enzyme are in common [10-14].

ACKNOWLEDGEMENTS. We are grateful to I. Jimenez and MJ. Cirugeda for excellent technical assistance .

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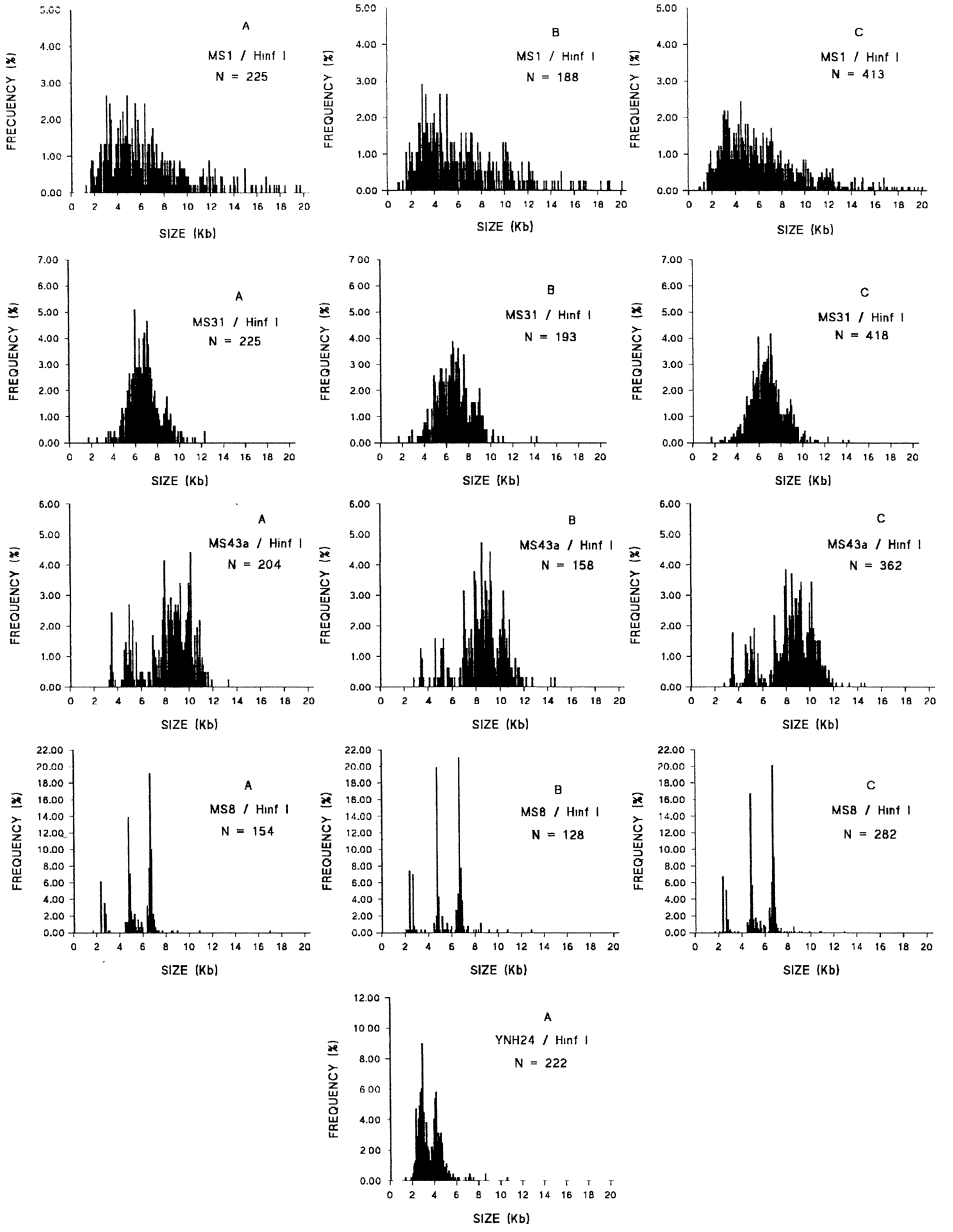

Fig 1. **DISTRIBUTION OF MS1, MS31, MS43a, MS8 AND YNH24 ALLELES.**
A. Unrelated individuals resident in Madrid B. Unrelated spanish involved in criminal cases C. Cobined data (A + B)

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ALLELE FRECUENCY DISTRIBUTIONS OF FIVE VNTR LOCI (D1S7, D7S21, D12S11, D5S43 AND D2S44) IN SPAIN.

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INTRODUCTION

Since the discovery of a hypervariable locus [1] numerous such loci have been found [2-4]. Of particular value and interest are the RFLP-analysis of hypervariable VNTR regions with the single locus probe hybridization technique. These markers have great potencial for individual identification in forensic analysis and paternity testing [5-7]. However, before they can be adopted for routine use in these applications, the allele frecuency distributions must be determinated [8].
Here, we report the allele frecuency distributions for the loci D1S7, D7S21, D12S11, D5S43 and D2S44 in the spanish population as determined by using oligonucleotide conjugated with alkaline phosphatase single locus probes (MS1, MS31, MS43a, MS8 and YNH24).

MATERIALS AND METHODS

Samples

The following population samples were analyzed:
a) EDTA blood samples from about 200 unrelated individuals living in Madrid (Central Spain).
b) EDTA blood samples from about 200 unrelated individuals spanish involved in criminal cases from different areas of Spain.

DNA profiling procedure

Genomic DNA was extracted by the standard phenol/chloroform protocol [7]. DNA was restricted with Hinf I. Separation of the DNA fragments was performed by agarose gel electrophoresis in TBE buffer according to the EDNAP group protocol. Gels contained a control DNA size marker (Hind III/lambda), three DNA control ladders (BRL) and two genomic controls (K562). Gels were depurinated, denatured and Southern blotted onto nylon membranes. Membranes were hybridised with the probes MS1, MS31, MS43a, MS8 (Cellmark Diagnostic) and YNH24 (Promega Corporation) according to the Non-Isotopic Chemiluminiscent Enhancement protocol (Cellmark Diagnostic). Autoradiography was carried out for 16h-3days.

Image analysis

All autoradiographs were analysed by means of the Bioimage equipment (Millipore) using the method Elder and Southern [9] for band size calculation with reference to the control DNA ladder at three differing positions on the autoradiographs.

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TABLE 4. Comparison of Various Studies in Genetic Marker Polymorphism for Turkish populations

	Sunni Turks n=1125	Turkish group in Germany (Hummel,1980) n=274	Hemşinli Turks (this study) n=88
Allele	**Allele Frequency**	**Allele Frequency**	**Allele Frequency**
*AK1*1*	0.96	0.965	1.0000
*AK1*2*	0.04	0.035	0.0000
*GLO*1*	0.43	not studied	0.4432
*GLO*2*	0.57		0.5568
*PGM1*1*	0.71	0.677	0.6111
*PGM1*1*	0.29	0.323	0.3889
*ESD*1*	0.904	not studied	0.9540
*ESD*2*	0.096		0.0454
*GC*1*	0.72	0.743	0.8160
*GC*2*	0.27	0.257	0.1839

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TABLE 3. Phenotypes and Allele Frequencies of AK1, GLO, PGM1, ESD, GC in Hemşinlis (Black Sea) (n=88)

Phenotype	Number		Allel Frequency	χ^2	p
	Observed	Expected			
AK1					
1-1	88	88	*AK1*1 = 1.000*		
2-1	0	0	*AK1*2 = 0.000*	-	-
2-2	0	0			
GLO					
1-1	22	17.28	*GLO*1* =0.4432	3.607	<0.02
2-1	34	43.43	*GLO*2* = 0.5568		
2-2	30	27.28			
PGM1					
1-1	34	32.85	*PGM1*1*= 0.6111	0.932	<0.05
2-1	38	41.82	*PGM1*1*= 0.3889		
2-2	16	13.31			
ESD					
1-1	81	80.09	*ESD*1* = 0.9540	44.616	<0.0005
2-1	4	7.71	*ESD*2* = 0.0459		
2-2	3	0.185			
GC					
1-1	113	109.47	*GC*1= 0.8160*		
2-1	78	84.98	*GC*2=0.18739*	5.193	<0.02
2-2	20	16.49			

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TABLE 2. Phenotypes and Allele Frequencies of AK1, GLO, PGM1, ESD, ACP1 & GC in Laz Turks(n= 211)

Phenotype	Number		Allel Frequency	χ^2	p
	Observed	Expected			
AK1					
1-1	166	168.37	*AK1*1 = 0.8933*	3.00	<0.05
2-1	45	40.18	*AK1*2 = 0.1066*		
2-2	0	2.39			
GLO					
1-1	52	43.98	*GLO*1* =0.4655	5.10	<0.02
2-1	85	100.99	*GLO*2* = 0.5344		
2-2	66	57.97			
PGM1					
1-1	20	52.25	*PGM1*1*= 0.500	79.62	<0.0005
2-1	169	104.5	*PGM1*1*= 0.500		
2-2	20	58.25			
ESD					
1-1	169	165.20	*ESD*1* = 0.9634	66.39	<0.0005
2-1	5	12.51	*ESD*2* = 0.0365		
2-2	4	0.23			
ACP1					
A-A	19	11.05			
B-B	128	120.04	*ACP1*A* = 0.2323		
C-C	-	-	*ACP1*B* = 0.7671	9.69	<0.02
A-B	57	72.86	*ACP1*C* = 0		
A-C	-	-			
B-C	-	-			
GC					
1-1	113	109.47	*GC*1= 0.7203*	1.433	<0.05
2-1	78	84.98	*GC*2=0.2796*		
2-2	20	16.49			

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TABLE 1. Phenotypes and Allele Frequencies of AK1, GLO, PGM1, ESD, ACP1, GC and TF in Sunni Turks (n= 1125)

Phenotype	Number		Allel Frequency	χ^2
	Observed	Expected		
AK1				
1-1	1028	1030.33	*AK1*1* = 0.96	0.22
2-1	97	92.58	*AK1*2* = 0.04	
2-2	0	0		
GLO				
1-1	200	208.01	*GLO*1* =0.43	0.72
2-1	565	551.47	*GLO*2* = 0.57	
2-2	360	365.51		
PGM1				
1-1	555	567.1	*PGM1*1*= 0.71	1.083
2-1	480	463.2	*PGM1*1*= 0.29	
2-2	90	94.2		
ESD				
1-1	935	931.61	*ESD*1* = 0.904	0.196
2-1	180	184.27	*ESD*2* = 0.096	
2-2	10	9.11		
ACP1				
A-A	104	108.11		
B-B	401	405.00	*ACP1*A* = 0.31	
C-C	8	9.11	*ACP1*B* = 0.60	1.636
A-B	426	418.50	*ACP1*C* = 0.09	
A-C	64	63.78		
B-C	122	121.50		
GC				
1-1	595	594.59	*GC*1=0.72*	0.255
2-1	442	446.58	*GC*2=0.27*	
2-2	88	83.84		
TF				
CC	1123	1102.61	*TF*C= 0.99*	3.534
CB	-	-	*TF*D= 0.01*	
CD	2	0.11		

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difference can be seen in a comparison between the values reported and our results. There is also a report for Turkish people in Iran by Amirshaki et.al. 1989, where differences were found . However this is not unexpected in the light of the genetic diversity of this population maintained through geographical, ecological, linguistic and cultural separation from the Anatolian people.

II. Laz Turks, form a considerably isolated group on the Black Sea region. The phenotypes and the allele frequencies of the red blood cell enzyme and protein systems studied appear in Table 2. The frequencies seem to be different compared to Sunni Turks ,our previous studies (Abacı et.al 1990, Atasoy et.al. 1990a,1990b, 1991,1993) and the group studied by Hummel et. al (1980). They are far from being in accordance with Hardy-Weinberg equilibrium, an expected consequence of the consanginous marriages that are predominant in the area. The present study will allow comparison of the Laz population with the rest of the world and especially with the different Turcic communities living in SW Caucasia. Possibly, investigation of different enzyme and protein systems among the above stated groups might supply interesting information in this regard.

3. Hemşinlis speak the Armenian language (Pastırma, 1970, Benninghaus, 1989), which may be an indication to relate them to todays Armenians, however there is no scientific, historical or sociological supporting evidence for it. . The phenotype distribution and gene frequencies obtained for Hemşinlis are presented in Table 3, wheras the comparison between the results found and the results of Sunni Turks of our previous studies (Abacı et.al 1990, Atasoy et.al. 1990a,1990b, 1991, 1992, Ateş et.al. 1990) and the group studied by Hummel et. al (1980) can be seen in Table 4. Altough the studied number seems to be a small one, there are some quite interesting results seen, like having a monomorphic AK system, and quite an elevated GC*1 frequency compared with the rest of the populations. Regarding the Hardy-Weinberg equilibrium, it is an expected situation to get a deviation, once the subjects were not genetically independent.. The frequencies of the genetic markers found should be compared with the frequencies of other Armenian groups to get a possible relatioship. Prior to such speculations, however, the results should be confirmed in a larger group of subjects.

We believe that the data reported here will also be a reliable tool for the quantitative evaluation of evolutionary divergence between populations and the evolutionary processess affecting each marker (Barbujani and Canella, 1987; Farabegoli and Barbujani, 1990).

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III. HEMŞINLI

Designation: Hemshin, Khemshin; Ger. Chemschinli; Tk. Hemşinli; Laz. Armeni
Self-designation: Homsetsi (Homsetsi) in east; Hemşinli in west.
Numbers: CENSUS : no indication. From village population 1975 ca. 24,000 Armenian-speaking, ca. 15-20,000 Turkish speaking.
Distribution: in two well-defined groups. 1. The western group, Baş Hemşin, live in Rize province in villages along Büyükdere, Ortaköy, Fırtına, Piskale and Abiviçe rivers, in compact valley settlements extending well up to the Pontic mountains. 2. The eastern group is found in Artvin province, on the river systems flowing through Hopa and Kemalpaşa, and apparently limited to the east by the Çoruh river.
Language: in the eastern group, Armenian, western dialect, and Turkish. In the western group, Turkish only, though Armenian place-names are retained. The Armenian spoken is in at least two dialects, and probably varies from village to village, with astonishing archaisms, changes in the sound system, and many Turkish loans (Dumezil 1964).
Religion: Sunni Islam: Hanefi, with some deviant practices: Bryer (1975) reports the recent survival of baptism in at leasts one area.
Group identity: inadequately recorded. It appears now to derive primarily from language in the eastern group, though this does not imply acceptance of Armenian origin. Some, at least , are unaware that they are speaking Armenian (Dumezil 1964). The western group, now thoroughly Turkicised, deny Armenian origin for themselves, but are still referred to as Armeni by the neighbouring Laz (Benninghaus, 1989). The Hemşinli were attracted to Islam from the XVth century (Bryer 1975), or converted later, ca. 1700 (Dumezil, 1964) Those who remained Christian left the region of Hemşin (Arm. Hamsen) for Trabzon, or went further west to Samsun or even Adapazarı: these later left for Armenia at the time of World War I(Benninghaus ,1989, Andrews, 1989).

MATERIAL AND METHODS

1. Sunni Turks:

The samples (n= 1125) were collected from healty Sunni Turk males(nm= 615) and females (nf = 510) between 18-70 years of age. Attention was paid to obtain the samples from people originating from diverse parts of the country. Each sample was classified as to sex and birth place. There is a high incidence of consanguineous (cousin preferred) marriage in the Anatolian population. Therefore extreme care was taken, to collect blood samples from unrelated individuals.

2. Laz Turks:

The blood samples were collected from the province of Artvin:
1) subprovince: Arhavi, district: Merkez, villages: a)Aşağı Hacılar (coordinates: 41.29-41.32) , b) Cumhuriyet (41.29-41.31) and c) Musazade (41. 29-41.33); 2) subprovince: Borçka, district: Merkez, villages: a)Demirciler (Mamanat) coordinates 41.23- 41.38, b) Düzköy (41.23-41.37); 3) subprovince: Borçka, district: Muratlı, village: Güreşen (Beglevan, Beylevan) (41.26-41.38); 4) subprovince: Hopa, district: Kemalpaşa, villages: a) Kemalpaşa (41.29-41.32), b) Dereiçi (41.28-41.31), c) Sarp (41.31-41.33); 5) subprovince: Hopa, district: Merkez, villages: a) Camlı köyü (Peronit, Perenit) (41.22-41.23), b) Sugüren (Kise) (41.23-41.26) from healthy males (nm= 112) and females (nf = 99) between 18-70 years of age. The ethnic denomination of the subjects was established by their mother tonque and based on this, the results from different areas were pooled .

3. Hemşinli

The blood samples were collected from the province of Artvin: subprovince: Hopa, district: Merkez, village: Pınarlı (coordinates: 41.23-41.37 from healthy males (nm= 60) and females (nf = 28) between 18-70 years of age. The ethnic denomination of the subjects was established by their mother tonque and their personal claim.

Whole blood was obtained in citrate (3.8g/dL) by venipuncture. Fresh packed erythrocytes were lysed in distilled water in a 1:2 ratio and stored at -20°C until analyzed. The analysis was done within ten days after collection and the samples were typed on cellulose acetate for AK1, GLO, PGM1, ESD, ACP1, GC and TF with the Sartorius Sartophor System and based primarily on methods described by B. Grunbaum (1981), with slight modifications. The gene frequency of each allele was calculated by gene counting . Chi square tests for heterogeneity and comparison between different comunities were carried out on the observed number of genes.

RESULTS AND DISCUSSION

This study which is part of a general investigation about genetic polymorphism on various ethnic groups living in Turkey, is concentrated on the Sunni Turks, Laz Turks, and Hemşinlis.

I. The phenotypes and the allele frequencies of the red blood cell enzyme systems studied in Sunni Turks appear in Table 1. Regarding the Hardy-Weinberg equilibrium, the frequencies provided a good fit to this statistical parameter. The present study will allow comparison of the Turkish Sunni population with the rest of the world. There is not much in the literature about the polymorphic systems for the Anatolian population except our previous studies and one reported by Hummel (1980) on Turkish people living in Germany. There is no information though, about the ethnic characteristics of the blood donors in these studies. Nevertheless, only a slight

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POPULATION STUDIES IN TURKEY

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INTRODUCTION

Significant genetic variation in populations can occur as a result of differential gene flow from neighboring populations. In a sociological study of people living in Turkey, 47 different ethnic groups are listed (Andrews, 1989). The present study aims to compare three of them -Sunni Turks, Laz Turks and Hemşinli- with each other.

Although many frequency studies of various genetic marker systems have been carried out on numerous populations throughout the world , there is no representative data for populations living in Turkey.

The only published studies are by Hummel et al.(1980) on a group of people from Turkey, working in Germany, which reports values on AK, PGM1, ACP1 and GC together with our previous studies (Abacı et.al.1990; Atasoy et.al.1990a; 1990b, 1991) where ethnic origin was not taken into consideration.

I. SUNNI TURKS

Designation: Turks; Ger. Türken; Self-designation: Türk (ler); Secondary self-designations: Yerli, Manav, Pallık (in parts of Artvin province), Dadaş (in Erzurum), Efe (in the Aegean) etc.
Numbers: CENSUS 1965: 28,289,680 persons speaking Turkish as mother tongue, but this includes persons speaking Turkic languages other than Turkish (no distinction being made), and those ethnic groups not listed by mother tongue in the Census. It also allows no distinctions for religious difference, and thus for example, includes Alevi Turks in very large numbers.Later census results give no classification by mother tongue.
Distribution: throughout Turkey, but in varying concentrations. In the provinces further east, such as Hakkari and Van, the available figures show the Turkish populations as almost entirely located in the towns and cities (provincial centres). Assimilation in the western and central areas is being accelerated by internal migration.
Language: Turkish (Türkçe) in some seven principal dialects. Turkish is a language of the southwestern, or Oğuz group (with Türkmen, Azeri, Gaşgayi etc.) in the Turkic branch of the Altaic family.
Religion: Sunni Islam: Hanefi. Some affiliation to dervish orders (Rufa'i, Mevlevi, Kadiri, Halveti, Bektaşi, Nakşbendi etc) still persists, with a tendency to endogamy within these groups, though this affects only a minority.
Group identity:
Most Turks are well aware of the smaller ethnic groups in the population, and can often say very precisely where these are to be found, thus defining themselves in contrast to these groups.

II. LAZ TURKS

Designation: Laz; Ger. Lasen (sing. Lase); Tk. Laz(lar); Georgian: C'an;
Self-designation: Lazi.
Numbers: CENSUS 1945 46,987 declared persons speaking Laz. CENSUS 1965 26,007 declared persons speaking Laz as mother tongue. 59,101 declared persons speaking Laz as second language. From village population 1975 ca. 90,000 in NE Anatolia, ca. 25,000 in W Anatolia. Feurstein 1983 : 250,000 Laz-speakers (sic).
Distribution: originally in extreme northeastern Anatolia, in the provinces of Rize (ca. 57,000 based on village populations in 1975) and Artvin (ca. 33,000), in villages, town and cities along the Pontic coast; there, in the northern mountain valleys, the proportion of the population speaking Laz ranges from ca. 50% in Pazar sub-province in the west, through ca. 80% in Ardeşen, ca. 45% (?) in Çamlıhemşin, ca. 80% in Fındıklı, ca. 100% in Arhavi, and ca. 45% in Hopa to ca. 5% (?) in Borçka (Andrews, 1989).
Language:
Laz (Lazuri nena), classed with Mingrelian in the Zan or Colchian branch of the Kartvelian or South Caucasian languages, and thus related to Georgian and Svan. Laz and Mingrelian are mutually comprehensible.
Religion: Sunni islam: Hanefi, to which the formerly Christian Laz were converted after about 1580 (Bryer, 1966).
Group identity: In general all the people living on the Black Sea cost anywhere east of Sinop or Samsun , or even as fas west as Zonguldak up to the Soviet border are called "Laz" in Turkey. In this wide concept or category of Laz are included those who really speak the Laz language, that is a language belonging to the southern (Kartvelian) branch of the Caucasian language group, together with the Georgians, Svans and Mingrelians.

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haplotype. This is compared with an expected PI calculated by counting the reciprocal matching allele fraction locus by locus, then using the product rule to predict **obs**. Approximately 50,000 haplotypes were sampled in this way. Grouping the data according to ranges of values of **exp**, **exp** and **obs** were compared by log-linear regressions. With **exp** < 2500 there was no discernable difference. Above that the number of observed multiple-locus matches was usually 0, so the statistical result depends on the convention chosen for the reciprocal of 0. Put otherwise, despite a conspicuous amount of built-in substructure, the product rule proves accurate to the limits of the available data.

Now, Lowentin claimed [3] that inter-ethnic differences are about 50% greater than inter-racial. Others have disputed this using different statistics or different data. However from our present point of view the dispute is splitting hairs -- within a factor of 2 one way or the other the magnitudes are the same [4]. The experiment with the mixed-race Virginians revealed no perceptible error in the product rule. Hence it is extremely unlikely that a mixed-ethnic population would give any different result.

Conclusions

A priori computations show that the product rule cannot fail significantly with any remotely believable amount of substructuring. An examination of live data confirms that with a plausible amount of substructing there is no discernable error in the product rule for product frequencies down to 1/2500. By computation on simple models it seems that product rule errors due to substructuring can only creep in gradually. Therefore, the conclusion that the product rule is a reasonable approximation can be extrapolated to even very much smaller products.

It is also worth noting in passing that the usual practice of computing a mixed-race frequency race by race is needlessly punctilious.

Acknowledgements

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A hypothetical population

Here's an example of the possible situation: At some mythical locus **A**, California population data shows 10 equally frequent alleles, A_1, A_2, ..., A_{10}. However, we find that F_{st}=2%, which in this case just means a 2% excess of homozygotes. Perhaps spurred on by this anomaly we analyze further and eventually hit on the winning idea of graphing the data according to political affiliation. California Democrats and Republicans are equinumerous and alike with respect to most alleles, but there is one allele, A_1, on the far left that is exclusive to Democrats (frequency=20% among Democratic alleles), and one far right allele, A_{10}, that is an invariable indicator of Republicanness (frequency=20% among Republican alleles). F_{st}=2% is a high figure compared to substructuring values suggested in the literature [e.g. 2].

To continue the thought experiment, imagine that there are several other similar loci, with alleles B_1, ..., B_{10} and C_1, ..., C_{10}, etc, where B_1, C_1, are purely Democratic, etc. Every allele has frequency 1/10 but 2-locus combinations ("haplotypes") like A_1B_1 have frequencies greater than 1/100 -- namely 1/50. It thus turns out that the chance that two sperm selected at random have the same haplotype is 1.04/100 -- a 4% error for the product rule.

It is interesting to consider the error across 3 or more loci. This example is easy to calculate, and with 3 loci the percentage error using the product rule is 3 times as great (the correct matching chance is 1.12/1000), with 4 loci about 6 times as great, and so on. The reason is easy to see: with three loci A, B, and C there is statistical dependence between triple the number of combinations: e.g. not just $A_1B_1C_x$ but also $A_1B_xC_1$ and $A_xB_1C_1$. Similar calculations with slightly more complicated models give the same sort of behavior, namely:

> To the extent that the product rule fails because of substructuring, as the number of factors increases the relative error grows, but rather slowly -- approximately as the square of the logarithm of the matching odds.

But this is a digression. The question remains as to whether the magnitude of substructuring built into the "California" model is relevant to amounts that one might be confronted with in the real world.

A real substructured population

To this end we consider the real DNA typing data from 3235 Virginians all typed in each of the 4 probes YNH24, TBQ7, EFD52, and CMM101 (D2S44, D10S28, D17S26, D14S13). The Virginians are about half Black and half Caucasian. Examining the Caucasian and Black data separately makes it clear that the Virginians are a substructured population. The Nei genetic distances between these races are 0.24, 0.12, 0.29, and 0.27 respectively for the four probes, compared to 0.4 for Republicans vs Democrats. F_{st} values for the mixed race population are slightly lower than in the California example.

A single test of the product rule is defined as follows. A person is selected, and a 2, 3, or 4 locus haplotype is selected from the person by choosing an allele at random from each of several loci. An observed PI (so called because of the relationship of this experiment to a paternity case) **obs** is then obtained by observing the reciprocal of the fraction of other people who are potential donors of approximately the same

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Evaluation of the Product Rule

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A couple of years ago a couple of population geneticists generated a stir with an opinion piece in Science [1]. This is somewhat remarkable considering that the article was nothing but speculation, extrapolation, and opinions. It contained no new data, no new ideas, and no science. Repercussions continue in American courts to this day.

Their fundamental concern was with the question of statistical independence of genetic traits. Traits A_1 at locus A and B_2 at locus B are statistically independent if A_1 occurs with the same probability in the presence of B_2 as in the absence. If independence holds, then the "product rule" holds: the proportion of e.g. sperm cells with the combination is simply the product of the proportions with the individual traits. Loosely speaking, we can say that the loci are independent if all their combinations of traits are independent. If the loci are not independent then of course the product rule fails, and on average (average over all combinations weighted by their chance of occurrence) always fails in the awkward direction of underestimating the probability of a combination.

Loci will obviously be dependent if they are linked on the same chromosome or if some issue of selectivity arises e.g. because some combinations are more or less hardy. These mechanisms are not of realistic concern when dealing with RFLP's on different chromosomes so sometimes Europeans and others living in countries more homogeneous than the United States falter at the post and fail to give Lewontin and Hartl's argument even what consideration it deserves. But the population geneticists are concerned about the possibility of selective breeding -- maybe people with trait A_1 preferentially seek partners with B_2. Of course they can't do it on purpose because B_2 is not expressed, but it could still happen. For example perhaps A_1 and B_2 are mostly prevalent among Blacks and most people mate according to skin color.

Selective breeding by race is not a problem because everybody knows (or at least assumes!) that statistics and calculations should be made separately race by race for this very reason and for other reasons. But might there not be some less obvious manifestations of "population substructuring" -- "ethnic substructuring" such as Poles mating with Poles, as Lewontin and Hartl suggest?

Perhaps. Then it is worth asking, how big would the effect be? It's not important that there may be some specific examples of combinations of traits that are non-independent between two loci. Specific examples are just anecdotes -- of course if they exist and you know what they are, then you should watch for them and make adjustments when they arise. But the real concern is the average impact of such combinations -- i.e. what is the expected error of the product rule?

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can be addressed by collecting data from a variety of different populations. The extensive polymorphism at the HLA class II loci make these loci very informative markers for analyzing allele and haplotype frequency data. The data discussed here indicate that, for the HLA class II loci, Native Americans show significantly less genetic diversity than other populations studied. The study of Asian populations suggests that the allele frequency differences between these groups are much greater than between different groups of European Caucasians. The analysis of DRB1-DQB1 haplotypes as well as DPB1 alleles suggests that some of these (e.g., DRB1*1602, DQB1*0301 for Native Americans or DRB1*0405, DQB1*0503 for Filipinos) may serve as population specific markers.

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other HLA class II loci, only a subset of the alleles found in other populations, were found in both North and South Amerindian groups. For this reason, genetic markers used for forensics inference, like the AmpliType HLA-DQα forensics test, are generally less informative (lower Power of Discrimination) in Amerindian populations than in other groups. Also, unlike the situation with other populations, distinguishing the subtypes of the DQα-4 type (4.1, 4.2, 4.3 or, more recently, *0501,*0401, *0601) can significantly increase the Power of Discrimination for this genetic marker in Amerindians. Future upgrades of the HLA-DQα reverse dot blot strip will contain additional probes for such subtyping.

As noted above, many DRB1 alleles are absent among Amerindians. A striking exception are the DR4 subtypes, where in Amerindians overall, several different DRB1 alleles were found. It is not clear whether many DR4 DRB1 alleles survived the putative population bottleneck that occurred during the migration from Asia or whether some of these DR4 subtypes were generated from the founding DR4 DRB1 alleles **after** colonization of the Americas. In the Cayapa Indians of Ecuador, a new DR8 allele, DRB1*08042, was identified (8). The finding of this new allele, presumably derived by point mutation from DRB1 *0802 within codon 86 (Gly-86 to Val-86) implicates the operation of selective forces favoring DRβ position 86 variants, because in Africa, DRB1*0802 gave rise to the same DRβ -86 variant (*08041) by another mechanism. Many DRB1 alleles that differ only at position 86 are found in many human populations and, recently, evidence indicating that the spectrum of peptides bound in the peptide-binding cleft of these molecules is different has been reported.

Although, like the other class II loci, only a few alleles at the DPB1 locus are found among most Amerindian groups, the allele frequency distributions can differ dramatically. For example, the frequency of DPB1*1401 is about 50% among the Cayapa Indians and is high among other Amerindian tribes in Ecuador and Colombia (8); this allele, however, is very rare (i.e., less than 3%) in most other Amerindian groups as well as all other human populations studied.

Some DRB1-DQB1 haplotypes appear to be unique to Native American populations; DRB1*1602, DQB1*0301 has been found in high frequency in all Amerindian groups studied and, to our knowledge, in no other groups. This marker, as well as others that are very frequent among Amerindians and absent or rare in other populations could serve as a population specific marker.

PACIFIC/ASIAN POPULATIONS

Recently, we have analyzed the class II polymorphism among two different Filipino populations as well as Indonesians, Malaysian, Papua New Guinea, and Hawaiian populations. One of the Filipino populations was sampled by using buccal swab samples collected at a high-school class reunion and a bowling alley (9). Several of these populations were characterized by a very high frequency of DR2 (and the haplotype associated DQA1*0102, or α1.2). However, the DR2 subtype at DRB1 was often different as was the DRB1-DQB1 haplotype in the different populations. Overall, these groups had a significantly different allele frequency distribution from previously reported Asian groups. In general, the differences between different Asian/Pacific groups was much greater than observed between different European Caucasian groups. This study also revealed several DRB1-DQB1 haplotypes that appeared to be unique to a given group. For example, DRB1*0405-DQB1*0503 was found only in Filipinos. DRB1*1502-DQB1*0502 was found at a very high frequency among Filipinos and was frequent among Indonesians but absent from the other populations studied.

SUMMARY/CONCLUSIONS

Forensics inference from genetic typing results is dependent on population data bases. Moreover, some of the issues associated with potential population substructure and assumptions of statistical independence (Hardy-Weinberg equilibrium and linkage equilibrium)

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Analysis by PCR/Oligonucleotide Typing of HLA Class II Alleles in a Variety of Human Populations

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INTRODUCTION

The analysis of allele frequency distributions in various human populations can provide valuable anthropologic genetics information as well as useful data for forensics inferences about identity. One of the more controversial issues in the area of forensic inference, has been the question of population substructure and how this might effect the calculations of statistical weight (i.e., likelihood of a "random match") associated with an inclusionary result. The issue of substructure within the so-called census populations and the possibility that different subpopulations can have significantly different allele frequencies can be addressed most directly by determining the allele frequencies in many different population groups.

In the case of the HLA loci, given their critical role in mediating specific immune responses, population data can also help in estimating the likelihood of finding a matched donor for organ and bone marrow transplants as well as in understanding HLA-associated disease susceptibility. In addition, the pattern of allele distribution in different populations can provide valuable information for understanding the selective forces that have shaped the generation and maintenance of the HLA polymorphisms.

The HLA class I and class II loci are thought to be the most polymorphic genes (coding sequences) in mammalian biology. We have developed HLA PCR/oligonucleotide probe typing systems, in both the conventional dot blot (immobilized PCR product and labeled oligonucleotide probes) and the more convenient reverse dot blot (immobilized oligonucleotide probes and labeled PCR product) formats (1-5). These HLA class II typing systems analyze polymorphism at the second exon of the HLA-DRB1, -DQB1, -DQA1 (available commercially as the AmpliType® HLA-DQα test), and DPB1 loci. The HLA-DQα test has been used extensively in forensic casework beginning in 1986 (6). Recently we have also developed a reverse dot blot HLA-A typing system for analyzing polymorphism within the second and third exons (7). Our HLA class II typing systems have been used to determine the distribution of class II alleles and haplotypes in a variety of different populations. Here we present the results of our population studies and discuss the implications of the observed allele distributions for forensic analysis, particularly for DQA1.

RESULTS/DISCUSSION

Native Americans

Using PCR/oligonucleotide probe typing, we have analyzed the distribution of HLA-DRB1, -DQA1, -DQB1, and -DPB1 alleles as well as DRB1-DQB1 haplotypes in a variety of North Amerindian and South Amerindian groups. These studies revealed a striking lack of genetic diversity among all of these groups; a relatively small number of alleles and haplotypes have been identified, consistent with a population bottleneck during the colonization of the Americas by migration from Asia across the Beringia land bridge. For example, at the HLA-DQA1 (previously, the DQα) locus, with very few exceptions that are probably attributable to admixture, only the alleles *0301 (previously, α-3) and the *0401 and *0501 (grouped in the AmpliType HLA-DQα forensics test as α-4) are found in the groups studied. Similarly, for the

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Table II shows the main results of our study. The between-case allele matching frequency of 1.46 per cent is far below the homozygote frequency of 5-10 per cent reported when traditional matching criteria are used (See e.g. Morton et al 1993). It illustrates that the present procedure exploits the hyperpolymorphism extensively.
The difference in allele matching frequency between between-case and within case female/male pairs is statistically significant. Kinship in paternity case matings (0.436%) is in accordance with kinship levels in Norway estimated by genealogic methods (Gedde-Dahl 1973).
The allele matching frequency in pairs of males involved in the same paternity case is 2.7 per cent, nearly twice that of between case pairs. This means that the kinship factor is of about the same size as is the allele frequency. Kinship is estimated at 1.3 per cent, a surprisingly high value. Data not shown here indicate that there is a 5-10 per cent proportion of closely related males between men involved in the same case.
The results demonstrate that bioassay of kinship by the procedure described is an effective tool to estimate kinship in pairwise relationships. Kinship levels in paternity case parties should be taken into account in paternity statistics. Studies in pairs of suspects in forensic casework might be of interest for forensic statistics as well.

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RESULTS AND DISCUSSION

Fig 1 illustrates the allele discrimination level obtained. It shows 5 pairs originally typed either as matches or near matches - within 1 mm apart. Pairs number 1 and 5 are matches, number 3 and 4 are non-matches about 0.5mm apart, while nomber 2 - after some hesitation - is typed as a match.

Fig 1

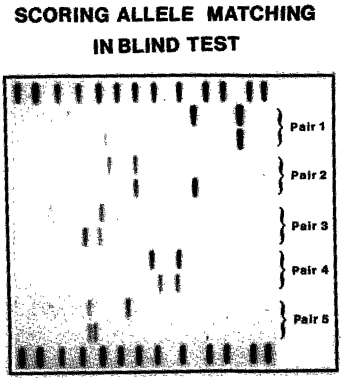

Table I TEST FOR TYPING BIAS

	ORIGINAL type distribution		BLIND TEST type distribution	
	match	near-match *	match	near-match *
between case pairs	50	50	51	49
within case pairs	50	50	52	48
mother/child pairs	100		98	2

*· less than 1mm migration distance, but not a perfect visual match

Since the original typing procedure did not allow blind typing, a blind test was performed which involved more than 10 per cent of all matches as well as a similar number of near-matches and obligate mother-child pair matches.
This test demonstrated that there was no systematic typing bias in the original typing, and that very few true matches were lost (Table I).

Table II MATCHING OF ALLELES IN A PATERNITY CASE MATERIAL
Comparison between 1052 between-case and 1588 within-case pairs of individuals, using 5 highly polymorphic VNTR probes

TYPE OF PAIR	Number of pairs	Allele compar.	Allele matches Number	Percent	KINSHIP %
Between-case	1052	21040	307	1.46	
Within-case					
female/male	1403	28060	530	1.89	0.436
male/male	185	3700	101	2.73	1.289

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Bioassay of kinship using VNTR alleles

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INTRODUCTION

Much attention has been given to forensic statistics. Focus has been on matching criteria, on binning procedures and likelihood ratios, and on VNTR genotype distribution in forensic populations (e.g. Lander 1989, Cohen 1990, Chakraborty 1991, Lewontin and Hartl 1991, Evett and Pinchin 1991, Nichols and Balding, 1991). Newton Morton deserves much credit for his valuable contributions in the field (see e.g. Morton 1992). Recently he and his colleagues demonstrated how probability calculations in forensic identification may be reliably performed if due care is given in particular to kinship (Morton et al 1993).

Kinship (F) is the probability that two alleles are identical by descent. In modern society, F in matings is ordinarily very small - and less than 1 per cent. Ordinarily allele frequencies are so high that estimates of kinship in matings would require extremely large materials. It struck us - however - that given a maximal electrophoretic exploitation of VNTR fragment length polymorphisms, allele frequencies might be brought down to a level which should allow estimates of kinship in such pairwise relationships.
The aim of our study was to establish a procedure for bioassay of kinship suitable for evaluating pairwise relationships like matings, alleged fathers, and suspects in criminal cases.

MATERIAL AND METHODS

The material we had at hand was 1403 consecutive paternity cases, all typed in the VNTR loci D2S44, D7S21, D7S22, D12S11, and D14S13.
We sorted three different kinds of pairs: 1052 between-case female/male pairs, representing the general paternity case population, 1403 within-case female/male pairs representing paternity case matings, and 185 within case male/male pairs representing the relationship between males involved in the same paternity case.
Only intragel comparisons between individuals not more than 2 lanes apart were included.
The matching criterium chosen was a perfect visual match - that is when an experienced typer decides that the alleles might well be identical. In our experience this matching criterium equals a sliding window of 0.2-0.3 mm.

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4 Probability of match

Denote by M the event that there is a one-allele match between two fragments and denote by $w_1 = \log y_1$ and $w_2 = \log y_2$, where y_1 and y_2 are the two observed fragment lengths. In such a situation the continuous version of Bayes Rule applies to find an estimator of the posterior probability of M given the evidences (y_1 and y_2):

$$\hat{p} = \hat{P}(M|_{y_1,y_2}) = \frac{\hat{g}(w_1, w_2|_M)P(M)}{\hat{g}(w_1, w_2|_M)P(M) + \hat{g}(w_1, w_2|_{\overline{M}})P(\overline{M})}.$$

where $P(M)$ and $P(\overline{M})$ are the *priori* probabilities and the rest of the terms in the ratio admit the following expression, when the gaussian kernel is used,

$$\hat{g}(w_1, w_2|_{\overline{M}}) = \hat{f}_h^W(w_1)\hat{f}_h^W(w_2) = \frac{1}{n^2h^2 2\pi}\left[\sum_{i=1}^{n}\exp(-(w_1 - W_i)^2/2h^2)\right]\left[\sum_{i=1}^{n}\exp(-(w_2 - W_i)^2/2h^2)\right]$$

$$\hat{g}(w_1, w_2|_M) = \frac{1}{n2\pi\hat{\sigma}\sqrt{2h^2 - \hat{\sigma}^2}}\sum_{i=1}^{n}\exp\left(\frac{-h^2(w_1 - w_2)^2 + 2\hat{\sigma}^2(W_i(w_1 + w_2) - w_1w_2 - W_i^2)}{2\hat{\sigma}^2(2h^2 - \hat{\sigma}^2)}\right)$$

5 Conclusions

a) The database used to implement the model show that the function g can clearly be assumed to be linear. Further more we can accept that $g(x) = x$.
b) The semiparametric model is flexible enough to admit a wide class of density functions for the true fragment length. However, the assumptions on the model imply that the conditional standard deviation of the observed length (measured by migration) is proportional to the actual fragment length.
c) The computational algorithm used is complex but may be run very fast in a PC by using sofisticated techniques (as the fast Fourier transform algorithm for density estimation).
d) The method outperforms the majority of the existing procedures, since it makes no parametric assumptions on the marginal distribution of the fragment sizes and does not discretize the continuous estimator of the migrated distance.
e) The calculations done above for the probability of single-allele match could be extended to the pairwise match just by formulating a new model with the same structure but incorporating some correlation between the error terms (ε) of both alleles in the pair.

6 References

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In the particular case of g being linear, the conditional mean and standard deviation of the observed fragment length grow linearly with the true fragment length. This was really an evidence in our practical experimental databases.

3 Estimation of the density function

The most natural graphical way of representing the ditribution of a continuous random variable is to plot its density function. In our case, we want to estimate f, the density of X. Unfortunately, we face some problems to do this:

1. No data for the variable of interest, X, are avaliable.
2. If we try to solve the previous point by using a sample of Y, we see in (1) that we need some information about g.
3. Even in the ideal case of g being known, we ignore the quantity σ -a measurement of the experimental error-, and we have to estimate it.

It is clear then that we have to carry out two estimations: estimation of σ^2 and functional estimation of g.

To do this, it is usefull to realize of the fact that, expression (3) implies:

$$Var(Y/m(X)) = e^{\sigma^2} - 1.$$

By using a nonparametric estimator, $\hat{m}$, of the regression function m (see for instance the kernel estimator of Nadaraya(1964) and Watson(1964)) and replacing the expectation by the empirical mean, we only need to deal with a preliminary sample of both variables X and Y (this means a control database for which the true fragment lengths are known) to estimate the standard deviation σ.

Once this step is performed, the function g is estimated, in view of (2), by rescaling the nonparametric regression estimator:

$$\hat{g}(x) = \frac{\hat{m}(x)}{e^{\hat{\sigma}^2/2}}.$$

With the observed sample of fragment lengths $(Y_1, Y_2, \ldots, Y_n)$, the kernel estimator (see Parzen (1962)) can be used to construct a nonparametric estimator of the density of W:

$$\hat{f}_h^W(x) = \frac{1}{nh} \sum_{i=1}^{n} K\left(\frac{x - W_i}{h}\right),$$

where $W_i = \log Y_i$, K is the kernel function and h is the bandwidth parameter.

Using this nonparametric density estimator and the convolution structure of W, we end up with a deconvolution density estimator of the density of the random variable Z. For the particular choice of gaussian kernel K, this deconvolution estimator coincides with the previous one but with a slightly small bandwidth: $\sqrt{h^2 - \hat{\sigma}^2}$.

Finally, a simple change of variable leads to the density estimator we wanted to find:

$$\hat{f}^X(x) = \hat{f}^W_{\sqrt{h^2-\hat{\sigma}^2}}(\log \hat{g}(x)) \frac{\hat{g}'(x)}{\hat{g}(x)}.$$

The important problem of choosing the smoothing parameter h was addressed, in practice, by using the smoothed cross-validation selector (see Hall, Marron and Park (1992)), while the computational matters were optimized by means of the fast Fourier transform algorithm for density estimation.

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SEMIPARAMETRIC DENSITY ESTIMATION WITH APPLICATIONS TO DNA PROFILING

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1 Introduction

The development of new methods for deeper statistical inference concerning VNTR systems in DNA profiling is the main purpose of this study. Most of the already existing methods treat the observed fragment size as a continuous variable (this is unavoidable since there is a continuous error in the measurement) but they discretize back the distribution of the fragment sizes (see for instance, Gill et al. (1991), Berry (1991) or Eriksen, Bertelsen and Svensmark (1992)).

The idea behind the study we present here is to handle the continuous distribution in a proper manner to answer the questions we are willing to (as estimation of the distribution of the true fragment size or probability of match, for instance). This is done without any parametric assumption, except on the part concerning the error, which is assumed to be normal. In other words, a semiparametric model for the probability density function is stated, estimated and finally used to find answers to questions like the ones given above.

2 The statistical model

Let us denote by X the variable of interest, namely the true number of Kbp of the fragment size. Of course, we can approximate this variable (which, in fact, is discrete) by a continuous version of it with probability density function denoted by f.

The most common approach to the problem of computing the true value of X, consists of running an electrophoretic experiment in which a migrated distance, W, is observed. In the basis of this observation, an approximation, Y, to the true value of X, is computed (typically $W = \log Y$, up to a certain constant).

If we denote by Z the *theoretical* migrated distance -this means the migrated distance that should be observed under ideal conditions and with no measurement errors-, we allow a general logarithmic-type relationship between X and Z:

$$Z = \log g(X),$$

where g is a link smooth function (without any parametric assumption on it) which enables to incorporate not only linear dependence between X and Y.

Taking into account all the relationships stated above, we end up with the following semiparametric model:

$$Y = g(X)e^{\varepsilon}, \tag{1}$$

where ε is a random error term, assumed to be normal with variance σ^2 and independent of the true fragment length.

Two important features of the model are that the conditional mean and variance of the observed fragment length, given the true fragment length, are linear and quadratic functions (respectively) of the link function $g(x)$:

$$m(x) = E\,(Y|_{X=x}) = g(x)e^{\sigma^2/2} \tag{2}$$

$$v(x) = Var\,(Y|_{X=x}) = m(x)^2(e^{\sigma^2} - 1). \tag{3}$$

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The results from the two populations appear very similar. The smaller size of the subpopulation databases (approximately 200 individuals for both cases) makes a less smooth distribution and some of the more defined low molecular weight alleles (see MS43A) appear to be relatively more common in the subpopulation. The results for the Afro Caribbean population are also very similar when general and subpopulations are compared to each other.

2). For both cases, estimates were made of the chance occurrence of the target crime profile. As can be seen in the table below, the differences that occur when the "correct" subpopulation database is used as opposed to the general database are relatively small. Examples of the values obtained when the F_{ST} coefficient is used to modify the general database results are given in the two right hand columns. These arbitrary levels of F_{ST} which are used in casework at the Metropolitan Police Laboratory can be seen to be extremely cautious. The true value of this parameter in urban populations is probably at least an order of magnitude less.

Comparison of calculations using the general and subpopulation databases:

	Subpopulation database	General database	1% F_{ST}	5% F_{ST}
Caucasian case	1 in 17,000,000	1 in 27,000,000	1 in 7,000,000	1 in 200,000
Afro Caribbean case	1 in 3,000,000,000	1 in 3,000,000,000	1 in 200,000,000	1 in 2,000,000

3). The "between person" comparisons method takes each profile in a database and compares it with every other. In each of our subpopulations this amounts to more than 20,000 comparisons. Likelihood ratios for each comparison are calculated and the results plotted graphically. It is important to emphasise that using four loci, none of the profiles matched any others within the databases. In order to detect any small effects of increased band sharing, the analysis was performed on each locus individually. Random samples of the same size as the subpopulations were selected from the general databases to act as controls.

For all the loci and populations examined the probability of obtaining a likelihood ratio of greater than 100 (medium strength evidence) was extremely low. There appear to be no detectable differences between the subpopulations and the general databases using this test.

Conclusion.

That human populations are substructured and do not interbreed randomly is self evident. The question that we as forensic scientists need to answer is whether this significantly affects the strength of the evidence we present.

The results from these two cases suggest that in British urban populations of Caucasian and Afro Caribbean people, the effects of subpopulations defined by place of residence and general appearance are insignificant. However in many forensic cases we are unable to accurately define the population from which the offender or innocent suspects originate, and it is for this reason that we at the Metropolitan Police Laboratory use a modification of the product rule. In all but the most unusual cases this is likely to be highly conservative.

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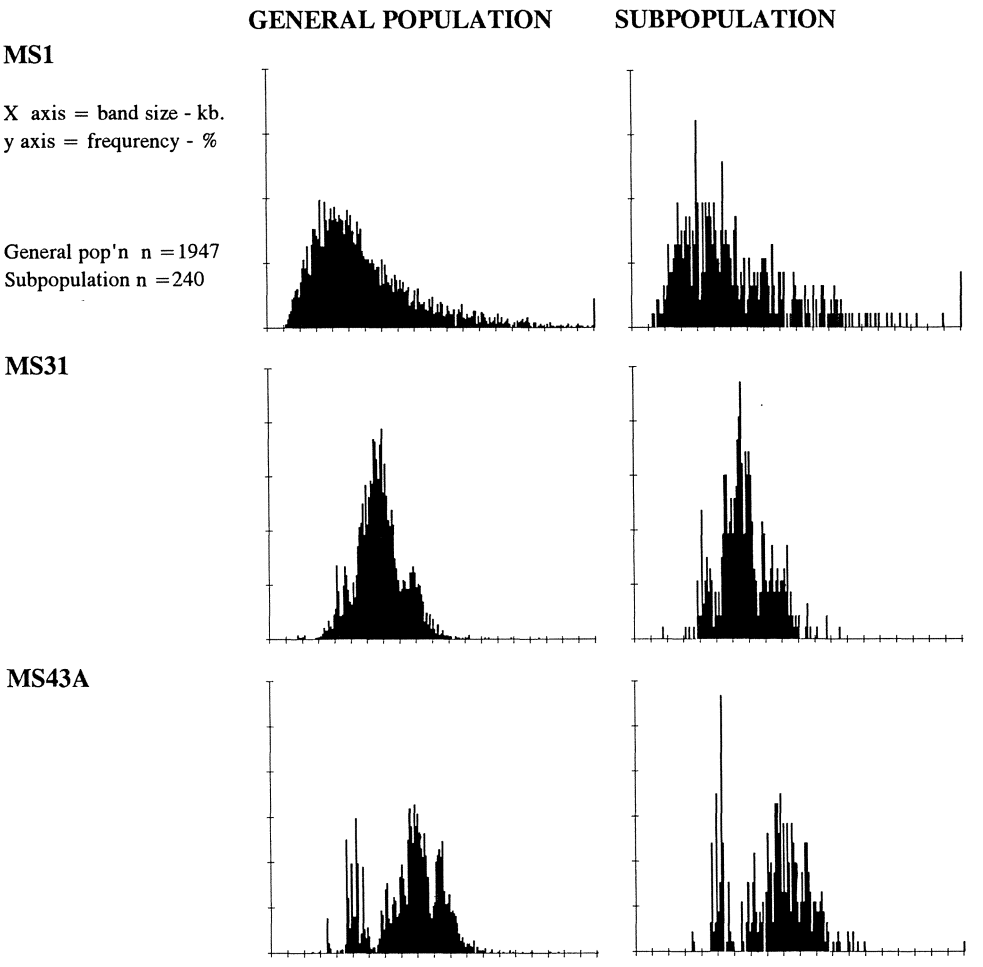
generate the allele distributions of the four loci used at this lab. These might then be considered the "correct" databases for the two cases. Comparisons were made with plots of the remaining individuals who form the broad general databases.

For the two cases, we have taken the target crime profiles and made estimates of the occurrence of the profile using the "correct" database for that subpopulation and compared this with the value obtained using the general database. We have also included examples of the frequency calculation using F_{ST} coefficients as a means of modifying the probabilities from the general database..[Nichols R.A. & Balding D.J.(1991) *Heredity,* **66**, 297-302]

Finally on the assumption that greater relatedness amongst a subpopulation might lead to an increased sharing of bands, we have used the "between person" comparison method [Evett I.W. Scranage J.K. & Pinchin R.(1992) *Journal of the Forensic Science Society*, Vol 32,(4),307-326]. to see if it is possible to detect any differences between the subpopulation and the general population.

Results.

1). Plots of the allele distributions at three of the loci are here shown for the Caucasian case.



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ESTIMATION OF GENETIC DIFFERENCE BETWEEN SUBPOPULATIONS USING DNA PROFILING DATA FROM CRIME INVESTIGATIONS.

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Introduction.

The evidential value of a DNA profile match is usually expressed to the court in terms of the estimated frequency of occurrence of that particular profile. ie. How many other people in the relevant population would be expected to have that profile. The chances of obtaining matching DNA profiles over the commonly used loci are extremely low in all populations that have been examined though estimating the degree of rarity has caused considerable debate.

At a meeting held earlier this year in London (The use of DNA Statistics in crime cases - MPFSL) it was suggested by Richard Nichols and David Balding of Queen Mary and Westfield College (London University) that police enquiries which lead to the arrest of the wrong suspect for a crime are more likely to lead to an individual from the same subpopulation group as the true offender. For example the innocent suspect may physically resemble the criminal or be a member of the same small and possibly inbred group living in that locality.

If there were to be a higher degree of kinship amongst the group containing the true offender, then the probability of finding another matching profile would be greater. Furthermore the distribution of alleles may be different and thus the figure for the chance occurrence of the profile which is calculated from general population data and uses the product rule could be unfair to the accused.

At the Metropolitan Police Laboratory we have collected DNA profiles in the course criminal investigations over the last four years. In two cases, which are the subject of this study, profiles have been obtained from large numbers of suspects which have then been shown not to match the "target" crime profile. These innocent suspects provide data to test whether the Nichols and Balding hypothesis has a significant effect in reality.

Methods.

Two cases were chosen where a DNA profile from the criminal had been prepared and the police had a description of the offender and knew in which area of London he was likely to live. In both cases a large number of suspects were apprehended and these were later shown to be innocent of the crime by DNA profiling. We have treated these individuals as a subpopulation selected by the police on the grounds of physical description and area in which they live.

Case 1:

This case consisted of a series if rapes committed by one individual in a residential area of South London. The assailant was described as Afro Caribbean and approximately 30 years old. Over 200 suspects from the local area were arrested and profiled before a match was found. Additional evidence confirmed the guilt of this individual.

Case 2:

This case consisted of a series of attacks on elderly women in another area of South London. In this instance the offender was Caucasian and again over 200 suspects from the local area have been profiled. No match has been found as yet.

For each case the DNA profiles of the "subpopulation" individuals were plotted to

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considered against the sum of all other phenotypes, was higher than expected (O=229, E=199, P=97%). This is even more significant, since the stratification of the population (or the heterogeneity of gene frequency) works in the opposite direction (i.e. towards an excess of homozygotes). We cannot distinguish, however, if this effect is due to an intrinsic methodological difficulty of the typing kit or it corresponds to a true genetic phenomenon. In any case, the effect is small and should be confirmed by further studies.

LIST OF PARTICIPATING LABORATORIES

Ist. Medicina Legale, Univ. **Ancona**; Ist. Medicina Legale, Univ. **Bari**; Ist. Medicina Legale, Univ. **Bologna**; Ist. Medicina Legale, Univ. **Brescia**; Ist. Medicina Legale, Univ. **Camerino**; Ist. Medicina Legale, Univ. **Ferrara**; Ist. Medicina Legale, Univ. **Genova**; Centro Genetica Medica, Immunopatologia, **Ozieri**; Ist. Medicina Legale, Univ. **Padova**; Ist. Medicina Legale, Univ. **Palermo**; Ist. Medicina Legale, Univ. **Parma**; Ist. Medicina Legale, Univ. **Pavia**; Dip. Biomedicina, sez. Medicina Legale, Univ. **Pisa**; Dip. Sanità Pubblica e Biostatistica, Univ. **Pisa**; Ist. Medicina Legale, Univ. **Roma** "Cattolica"; Ist. Medicina Legale, Univ. **Roma** "La Sapienza"; Ist. Genetica Medica, Univ. **Roma** "La Sapienza"; Servizio Polizia Scientifica, Polizia di Stato, **Roma**; Centro Nazionale Tresfusione Sangue, CRI, **Roma**; Dip. Biologia, Univ. **Trieste**; Ist. Medicina Legale, Univ. **Siena**; Ist. Medicina Legale, Univ. **Terni**; Ist. Medicina Legale, Univ. **Verona**.

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which, however, was due mainly to the Sardinian sample. After removing this sample the probability of heterogeneity was included between 90 and 95%.

4) Heterogeneity of the system reduced to 4 alleles

The typing method itself suggested to check the heterogeneity of the system after the frequencies of the alleles 1.1, 1.2 and 1.3 were combined in a single value. This analysis confirmed the peculiarity of the Sardinian allele frequencies. Two laboratories were now out of H.W. equilibrium: L9 (because of an excess of phenotypes 3 and 1/4) and L4 (with an excess of phenotypes 1/4).

5) Heterogeneity for the allele 4

Since the most represented phenotypes included the allele 4, it was meaningful to check the heterogeneity of this allele against all others (i.e. treating the system as a diallelic locus with three genotypes: 4/4, 4/- and -/-). None of the possible heterogeneity sources (phenotypic total, within-samples square law and between-samples gene frequency) was significant; however, there was a general slight tendency to deviate from H.W. equilibrium (P about 90%), towards an excess of heterozygotes.

6) Table of Italian (Sardinia excluded) allele frequencies

Allele	Gene count	Gene frequency
DQA1.1	496	0.167
DQA1.2	534	0.180
DQA1.3	181	0.061
DQA2	396	0.133
DQA3	232	0.078
DQA4	1133	0.381
TOTAL (1486 ind.)	**2972**	**1.000**

CONCLUSIONS

With the exception of one laboratory, which had mistyped a control sample and was very far from H.W. equilibrium (and for these reasons was excluded from the computation of the Italian allele frequencies), all other data were self-consistent (21 laboratories scattered on the entire national territory. The Sardinian sample was highly different from the other Italian samples. These were rather heterogeneous (P near 95%), probably reflecting a genetic differentiation within Italy, but not at the extent to invalidate the computation of the overall frequencies (see table above). The global sample was in H.W. equilibrium, so that the genotype frequencies may be estimated by the square law.

We should mention that there seems to be an excess of heterozygotes 1.1/4 (or 1/4 or -/4) in several laboratories. The frequency of the 1.1/4 phenotype in the global sample,

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AN ITALIAN COLLABORATIVE STUDY ON THE HLA-DQA1 LOCUS (GEFI's "GARDA 2" PROJECT)

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INTRODUCTION

In October 1991 the GEFI (Gruppo Ematologi Forensi Italiani) proposed a collaborative study on the use of AmpliType HLA-DQα Forensic DNA Amplification and Typing Kit (Parkin Elmer Cetus) for individual typing at the HLA-DQA1 locus (six alleles). The general recommendation for each participating group was to choose for the analysis a sample of at least 50 unrelated individuals, resident in the laboratory region. Five blind control samples from the Tenth International Histocompatibility Workshop were forwarded to each group. Twenty-three laboratories sent results in the deadline (see list below). We show here an analysis of the genetic heterogeneity between samples. A laboratory sent a selected subsample from its database and was excluded. The total number of considered individuals was 1623.

RESULTS

1) Heterogeneity of phenotype frequencies

About 2/3 of the expected values for the 21 phenotypes times 22 laboratories (= 462 cells) were lower than 2.0, making it impractical to test the equality of phenotypic proportions among laboratories. However, we did compute the χ^2s, in order to obtain a rough representation of the variation between samples. Two laboratories had a χ^2 value disproportionately higher than the mean; these were the Sardinian sample and a laboratory that had mistyped one control phenotype (L9).

2) Hardy-Weinberg equilibrium within laboratories

Owing to the small expected frequencies of the majority of phenotypes, we applied the method of Smith (*Ann.Hum.Genet.*: **50**, 163, 1986), which covers expectations even smaller than 0.5, to test each sample for accordance to the square law. The L9 sample was out of equilibrium (P>99.9%); a second laboratory had too high an accordance (P>99%). Another laboratory was above the level of 95% probability of deviation (but in a collection of about 20 independent random samples this should happen by chance just once).

3) Heterogeneity of gene frequency

The contingency table of the 6 alleles times 22 laboratories (grandtotal=3246 genes) was easily tested for heterogeneity of gene frequency, since no cell had an expected value less than 5.0. There was an overall high degree of heterogeneity (P>99.5%),

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determined from data bases compiled from mothers and tested men yields estimates of substructure one generation removed from the question at hand for disputed paternity (but not for disputed identity). Following the method of Morton, we have derived equations that yield estimates for $\alpha(F_{ST})$ from a determination of difference in matching of alleles between mother and tested man in each paternity case compared to matching between mother and random men:

$$PE_{M/AF}/PE_{M/RM} = \begin{matrix} 1\text{-}4\alpha \text{ (mother heterozygous)} \\ 1\text{-}2\alpha \text{ (mother homozygous)} \end{matrix}$$

For each paternity case, the tested man is considered to be a possible father of the mother, and the mean (motherless) exclusion probability ($PE_{M/AF}$) is compiled. This is compared to the same calculation treating each of the random individuals in the data base as a possible father of the mother ($PE_{M/RM}$). Excess sharing of alleles between mother and tested man would result in decreased probability of excluding the tested man as the father of the mother. This method has none of the limitations of Wahlund's method.

Estimates of α averaged from matching at 3 VNTR loci and calculated at three different values of delta are given in the table. N is the total number of mother-tested man comparisons made for each race. For the systems studied, our resolution is about $\delta = 2\%$ x MW. We use $\delta = 3\%$ x MW for casework; $\delta = 1\%$ x MW corresponds to a discrimination of less than one repeat unit. The mean values of α were computed for each race by averaging values obtained at loci D2S44(YNH24), D10S28(TBQ7) and D17S26(EFD52) [all restrictions HaeIII]. All estimates of α are close to 0, suggesting for each race that mothers and possible fathers are, for practical purposes, randomly paired. Similar results (based on fewer comparisons) were obtained for HLA and also for D1S47 [pL336, PstI].

Robustness of Estimates of α

δ	Caucasian	Hispanic	Black
1%	.0076	.0045	.0018
2%	.0016	-.0009	-.0010
3%	.0000	.0004	.0032
N	4751	3139	1823

Pairwise Independence of Loci

We tested for pairwise independence of phenotype matches among the five loci and three races. Overall, no departure from independence was found. Following the exposition of Morton, estimates of corrections needed in 4 DNA locus and 5 locus (4 DNA + HLA) phenotype matches were determined. For $\delta = 2\%$ x MW averaged over the three races the product rule would overstate the rarity of a four locus DNA phenotype match by a factor of 1.23, and for a five locus phenotype match (including HLA) by a factor of 1.32. Overstatement of PI values is estimated by the square root of these factors: the product rule would overstate 4 locus and 5 locus PI's by factors of 1.11 and 1.15 respectively.Use of even mildly conservative binning criteria would provide more than adequate compensation for both disputed identity and disputed paternity.

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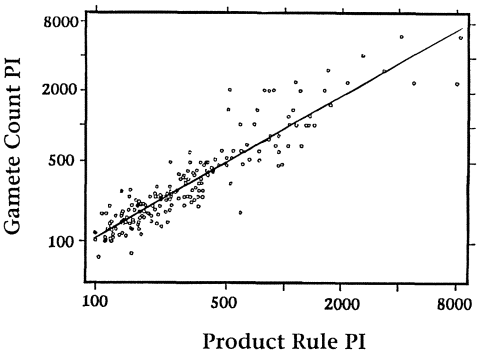

Fig 1 Correlation of gamete count and product rule PI. Data from 197 otherwise unselected cases for which product rule PI ≥ 100 for TBQ7 and EFD52. Gamete count PIs were obtained as described above from two locus data bases of size N=6146 (Caucasian), N=4148 (Hispanic) and N=2432 (Black). For one case with zero direct observation matches one match was assumed (Laplace's rule). 95% confidence intervals for slope and intercept of the regression line include 1 and 0, respectively.

Bioassay of Substructure of Paternity Populations

Critics have claimed that major US populations (Blacks, Caucasians, Hispanics) have substructure of sufficient magnitude to preclude use of intralocus (Hardy Weinberg) and interlocus product rules. It has been suggested that phenotype matching frequencies be compiled from data bases from the sub-population (ethnic group) of the suspect. Lacking such data bases, a ceiling principle could be adopted to allow for a "worst case" scenario. This view of population structure is equivalent to the affinal model "...the culprit is related to the suspect as closely as a spouse would be." [Morton, N.E. PNAS89(1992)2556]. For this model, Morton has shown that phenotype matching frequencies may be computed without ethnic specific data bases, provided that the inbreeding coefficient (α, F_{ST}), the standard estimate of population substructure, is available.

For disputed paternity, an analogous model is: the mother is related to the father as closely as a spouse would be. The actual ethnicity of the tested man is irrelevant for calculation of PI in VNTR systems - we know his phenotype and can accurately infer his genotype. What might be relevant is the ethnicity of other possible fathers, but since the relationship between mother and tested man reflects the relationship between mother and possible fathers, the issue of substructure (inbreeding) of the paternity population can be directly addressed from paternity casework. One result of inbreeding is increased homozygosity of offspring (Wahlund's rule). Estimation of inbreeding on this basis has two important limitations. First, at each autosomal locus the offspring is one of four possible results, so that Wahlund's method detects only 25% of similarities between mother and father. Second, the frequency of homozygosity expected on the basis of random mating and the frequency of silent alleles are critical to the calculations. For VNTR loci the former calculation depends on the level of discrimination of close alleles (delta), which can only be roughly estimated, and independent estimates of silent (null) alleles are difficult to come by for forensic (but not paternity) populations. One further limitation: excess homozygosity

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THE PATERNITY INDEX, POPULATION HETEROGENEITY, AND THE PRODUCT RULE

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Determination of PI's Across Genetic Loci Without Use of the Product Rule

Recent controversy regarding the validity of the product rule in forensic DNA identification cases raises the possibility of living in a "product rule free" world. We have examined methods to determine PI's across genetic loci without using the product rule. In analogy with HLA, collection of haplotypes from family studies would permit such calculations from tables, but fixed bins and very large numbers of haplotypes would be required. Following a suggestion of C. Brenner that a "product rule free" PI can be obtained directly from multilocus VNTR phenotype tables, we have devised a simple method that relies only on counting: i) Determine, from direct counting, the proportion of individuals in the multilocus phenotype data base that match at all loci tested. This yields the "exclusion probability" term of the PI [J. Morris. 3rd Intl Symp on Hum Ident 1992, Promega p. 177-190, 1993]. ii) For each of the randomly matching phenotypes, determine from direct counting the relative "fit" to the mother-child pair compared to the phenotype of the tested man. The average relative "fit" gives the "goodness of fit" term of the PI. iii) Multiplication of the two terms yields the directly observed "gamete count" PI. For example, suppose in a paternity case tested at 3 VNTR loci, the tested man, heterozygous at all loci, matches the single paternal allele at each locus, and that search of the three locus data base yields 43 of 6146 non-fathers who also match. The exclusion probability PI is 6146/43 = 143. Of the tested man's eight equiprobable gametes, only one contains the paternal marker at each locus, yielding 1/8 as the numerator of the "goodness of fit" term. If the 43 matching non-fathers consist of 38 who match as well as the tested man and 5 who are apparent homozygotes at one of the three loci, the denominator of the "goodness of fit" term would be 1/43 (38•1/8 + 5•2/8), yielding a "gamete count" PI of 6146/48 = 128.

This method requires that genotype be directly determined from phenotype, so it cannot be used for systems such as HLA or ABO. "Motherless" cases would require direct observation of multilocus phenotype frequencies to obtain the "gamete count" PI, which is not practical for VNTR systems. The ability to obtain multilocus PI's independent of the product rule yields a method for validation of the product rule for multilocus PI (Fig I).

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9. Population studies and biostatistics

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Discussion

Non-radioactive sequencing with digoxigenine marked primer was comparable to the radioactive technique. Advantages may be: 1. longer parts are readable in one application. 2. multiple use of sequencing gels. 3. easy waste disposal. As a disadvantage digoxigenine labeled sequencing is still a sensible technique, which may be improved in the future by incorporation of digoxigenin labeled nucleotides in the sequencing reaction.

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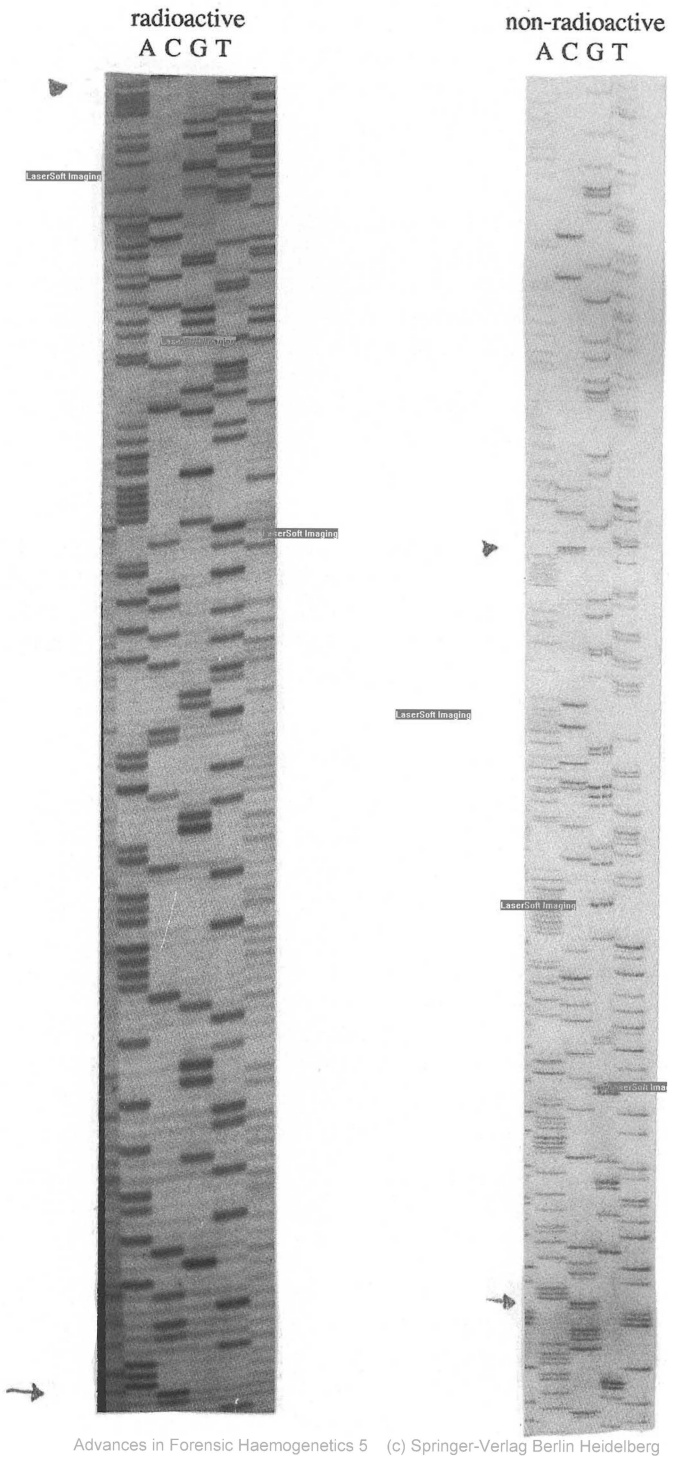
Figure 1: Comparison of radioactive and non-radioactive direct sequencing of exon 2 of the HPRT gene.
Arrows indicate the same region within the sequence.



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DIGOXIGENINE LABELED SEQUENCING OF THE HPRT GENE

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Protocols for radioactive sequencing of the Hypoxanthine-guanine-phosphoribosyltransferase(HPRT) gene have been reported by different authors. In contrast, only few described non-radioactive techniques using expensive sequencing automats. After having established a radioactive technique (modified according to Gibbs et al. 1990) we wanted to establish also a non-radioactive method.

Methods:

DNA extraction and PCR reactions

Genomic DNA was isolated from peripheral blood according to a non-organic method (Miller et al. 1988). The nine exons of the HPRT gene were amplified symmetrically in single PCR reactions (modified according to Gibbs et al. 1990) and amplified assymmetrically in second PCR reactions. The products of each PCR reaction were purified using glassmilk (Geneclean, Bio 101, La Jolly, USA).

Sequencing reactions

Sequencing reactions were performed according to the method of Sanger et al. (Sequenase 2.0 Kit, USB, Bad Homburg). In contrast to the radio-active method, were ^{35}S dATP was incorporated during labeling, digoxigenine-marked primer were used in the non-radioactive technique. Sequencing electrophoresis was carried out in a direct blotting electrophoresis apparatus (GATC, Konstanz) using a 4% polyacrylamide urea gel in TBE buffer. The sequencing products were blotted onto a nylon membrane which was transported past the lower end of the sequencing gel. The sequencing products were crosslinked to the membrane by UV light (transluminator, Pharmacia-LKB, Freiburg). Visualization was carried out with digoxigenine antibody conjugated alkaline phosphatase (DIG Nucleic Detection Kit, Boehringer Mannheim).

Results:

The results of sequencing reactions of exon 2 are shown in figure 1.
In the non-radioactive technique about 200 to 300 bp were readable, compared to 100 to 200 bp in the radioactive technique in one application on the gel. Sequencing gels could be run multiply in the direct blotting apparatus with comparable results. Sequencing reactions tended to become weaker within months, due to loss of digoxigenine on the primer.

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significant difference was observed between the number of observed and expected homozygous (8 vs. 6.0 ± 0.8) genotypes and heterozygous (49 vs. 49.5 ± 3.5) genotypes (1). However, when the allele frequencies were compared between females and males a significant difference was observed for one allele (no. 12; Fig. 2). Similarly, a significant difference was observed between males and females for two genotypes (3-11 and 11-12). The basis for these differences could be found by a segregation analyses and showed that there was a significant difference in the distribution of some alleles over the sex chromosomes: allele 12 was present on 72.7% of the X chromosomes while allele 11 was present on 31.2% of the X chromosomes. For allele 3 an equal representation was found on both sex chromosomes. Another explanation, heterogeneity of the population sample, could be rejected as there was no difference in allele or genotype distributions between females and males for several autosomal loci (D17S5, ApoB and IGHJ).

The DXYS17 locus is one of the most polymorphic VNTR loci typeable by PCR. The probability of identity between two non-related individuals is 0.048 and the probability for paternity exclusion is 0.673. In over 170 meioses, no evidence was found for any mutation and there were no alleles who failed to be amplified which would result in exclusion of maternity.

The amplification and analysis of the DXYS17 locus is very robust and has proven to be successful in over 100 forensic cases. The use of the A.L.F. DNA Sequencer for sizing of amplified VNTR allele is highly accurate and allows to compare two samples on two different gels (4). This is certainly an advantage in the analysis off forensic DNA samples where it is possible that some samples have to be compared with others typed several months before.

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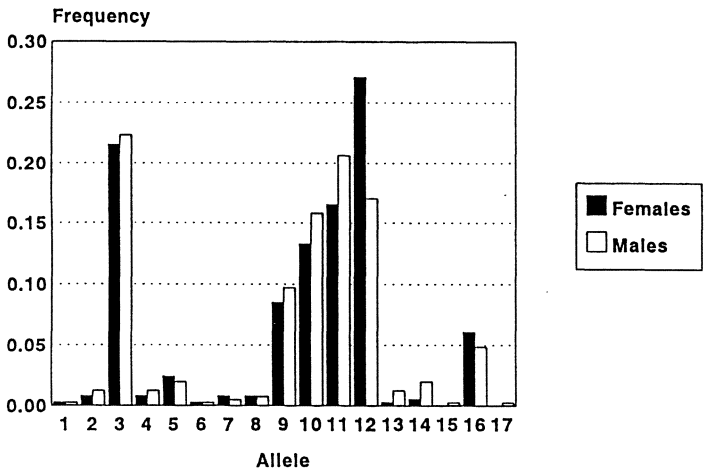

FIGURE 2: Distribution of DXYS17 alleles in the Belgian population classified to sex (188 females and 206 males). Alleles are ranked in descending order from the largest (lowest mobility) to the smallest (highest mobility).

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that these two alleles were of the same length but had a different internal structure. Sequence analyses proofed this hypothesis and showed that both alleles were almost identical at the 5' and the 3' end but with a different structure of the internal repeats. The DXYS17 consists of two different repeats: one of 28 bp and one of 33 bp. The 28-bp unit is similar to the 33-bp unit but shows a 5 bp deletion in the middle.

The observation that two non-identical VNTR alleles migrate with the same mobility on agarose gels or urea-PAGE gels and different on native PAGE gels has some important implications in forensics:

1. A genotype of the two alleles (9 and 10) would be identified as heterozygous on native PAGE gels and homozygous on agarose gels. This would lead to a false inclusion in case of two homozygotes (9-9 and 10-10) or a homozygote (9-9 or 10-10) and a heterozygote (9-10) when sizing of the amplified alleles would be done on agarose gels or denaturing PAGE gels.
2. The frequency of the two alleles is 9.1% (9) and 14.6% (10). A conservative frequency estimate (2p) for a homozygous typing is 47.4% while for a heterozygous typing a figure of 2.7% is obtained.

A population survey in 394 random Caucasians revealed in total 17 different alleles with a minimum frequency of 0.13% and a maximum frequency of 21.9%. The genotype distribution of the different alleles were in Hardy-Weinberg equilibrium as could be shown by different tests. The observed homozygosity (85.8% ± 1.8%) did not differ significantly from the expected (83.7% ± 0.5%). Also no

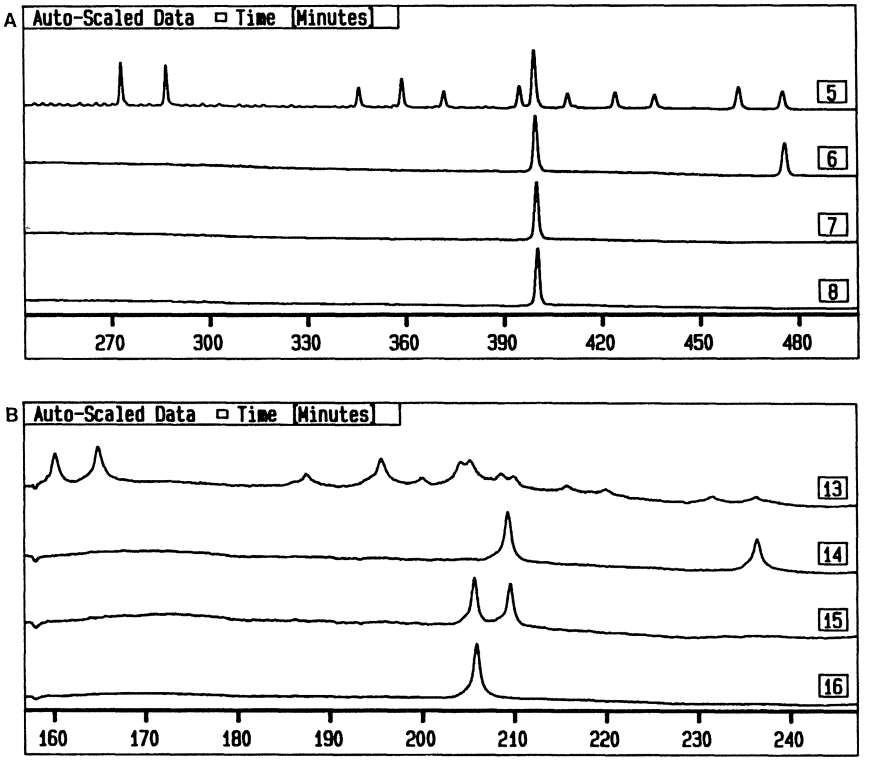

FIGURE 1: Analysis of the amplified DXYS17 alleles in a nuclear family (father: lanes 6 and 14; child: lanes 7 and 15; mother: lanes 8 and 16) on the A.L.F. DNA Sequencer in 6% Hydrolink Long Ranger gel with urea (A) and without urea (B). Lanes 5 and 13 represent an allelic marker with 13 of the 17 observed alleles in the Belgian population.

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HIGH RESOLUTION ANALYSIS BY PCR ON AN AUTOMATED DNA SEQUENCER OF INTERNAL VARIATION AT A PSEUDOAUTOSOMAL VNTR (DXYS17)

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INTRODUCTION

PCR-based DNA typing has become a valid alternative for the time-consuming and laborious typing of VNTR loci by Southern blot analysis. It has not only increased the sensitivity of DNA typing to levels of a few nanogram of DNA but it allows also to have a higher resolution for small size differences. Genotyping becomes possible which is in contrast to conventional Southern blot methods where the low resolution for small size differences, sometimes, lead to 'pseudo-homozygosity' (1).
At the present time, PCR analysis of only a few VNTR loci has become applied in identity testing and paternity determinations (e.g. ApoB, D17S5, COL2A1 ..). In order to increase the discriminatory power of PCR based DNA typing, we investigated the pseudoautosomal VNTR DXYS17 as a candidate marker in combination with a fluorescent approach for detection of the alleles on an automated DNA sequencer.

MATERIALS AND METHODS

DNA was extracted according to standard procedures from venous blood samples of 394 random Caucasian individuals of Belgian origin.
Amplifications were carried out in a Perkin-Elmer 9600 with 25 cycles consisting of 25 sec. at 94°C for denaturation, 25 sec. at 55.9°C for annealing and 2 min. 20 sec. at 68°C for extension. All reactions were done in a volume of 15 μl containing a modified Taq-buffer (2), 2 mM $MgCl_2$, 0.2 mM dNTP, 1 μM of each oligonucleotide (602-A 5'-ACTCACTAGCACATGCTGTG and 602-B 5'-GCGCAAAGAAATTCTGGTGA) and 250 ng of DNA.
10 μl of the amplified products was used for a quality control on 6% polyacrylamide (PAGE) gels. Positive amplifications were diluted 16-fold and 5 μl was loaded on a 6% Hydrolink Long Ranger gel and run on the A.L.F. DNA Sequencer. Analysis of the runs was done with the A.L.F. Fragment Manager Version 1.0.

RESULTS AND DISCUSSION

Conventionally, sizing of amplified VNTR alleles is performed either on high concentration composite-agarose gels or on PAGE gels with visualisation of the alleles by ethidium bromide. These gels can not be re-used and do not allow for standardization of the electrophoresis conditions other than the gel matrix. Therefore, we used an automated DNA sequencer to do a population survey for the alleles of the DXYS17 locus. The A.L.F. DNA sequencer was chosen for this purpose and this system showed to give highly reproducible runs: the maximum difference in mobility between two identical alleles on the same gel was 0.56% and between the transmitted alleles from parent to child was 0.09%. Moreover, fragments between 500 and 1100 bp could be separated in less than 4 hours and the gel could be reloaded with other samples. Preliminary experiments with shorter gel plates, where the well-to-laser distance was reduced from 24 cm to 12 cm, showed that the length of the run could be reduced to 1 hour with only a slight decrease in the difference in mobility between identical alleles (maximum 0.88%).
Sizing of the amplified fragments on the A.L.F. System was initially done in denaturing (urea) conditions. However, when comparing the resolution for separation of the different alleles with non-denaturing conditions, we observed that two different alleles migrated with the same speed in urea gels and with different mobility in native gels (Fig. 1A and B). This observation was reproducible and we hypothesized

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Thus, there are two antithetic versions in each exon , "A" or "B" in exon 12 and "1" or "2" in exon 14, and the resulting four combinations of them were shown to correspond to the four alleles (*F13A*1A, *1B, *2A, *2B*). When assumed that one of the four alleles was an ancestral monomorphic gene, the point mutations in exon 12 or 14 brought about two new alleles and the fourth one was likely to result from intragenic crossing over between the two of the pre-existing three alleles. Although we have not obtained direct evidence for crossing over event yet, relatively long intronic sequences intervening between the exons of the F13A gene seem to favor intragenic recombination.

Besides the four common alleles, several rare variant alleles have been reported thus far. A rare variant named as *F13A*4* was analyzed by SSCP and a putative exon harboring mutation was sequenced. Two samples of F13A 1B-4 showed different SSCP patterns, one in exon 11 and the other in exon 12. Careful IEF analysis revealed a very small difference in mobility between the F13A 4 bands of the two samples and the two *F13A*4* alleles were then tentatively renamed as *F13A*4I* and **4W* (fig.4A). Polymorphic bands were demonstrated by SSCP in exon 11 for *F13A*4I* and in exon 12 for *F13A*4W* as presented elsewhere in this volume by the same authors. A C to G transversion in the codon (CAA to GAA) encoding residue 468 and a G to A transition in the codon (CGG to CAG) for residue 540 were found to specify *F13A*4I* and **4W*, respectively. Amino acids inferred from the nucleotide sequences were Glu from Gln for **4I* and Gln from Arg for **4W*, being compatible with IEF separation pattern of both bands. Codons for residue 564 ("A"/"B") and for residues 650-651 ("1"/"2") are CTG564-GTT650-GAG651 for *F13A*4I* and CCG564-GTT650-GAG651 for *F13A*4W*. *F13A*4W* was previously identified as *F13A*4* by comparing the mobility with original *F13A*4* (Board and Coggan, 1981) on agarose gel electrophoresis (Suzuki et al. 1986). *F13A*4I* and **4W* were discriminated from each other only by comparing them side-by-side in "subtyping" gels. These findings therefore suggests that sequence data is finally required to discriminate variant alleles that have been defined only by electrophoretic procedures.

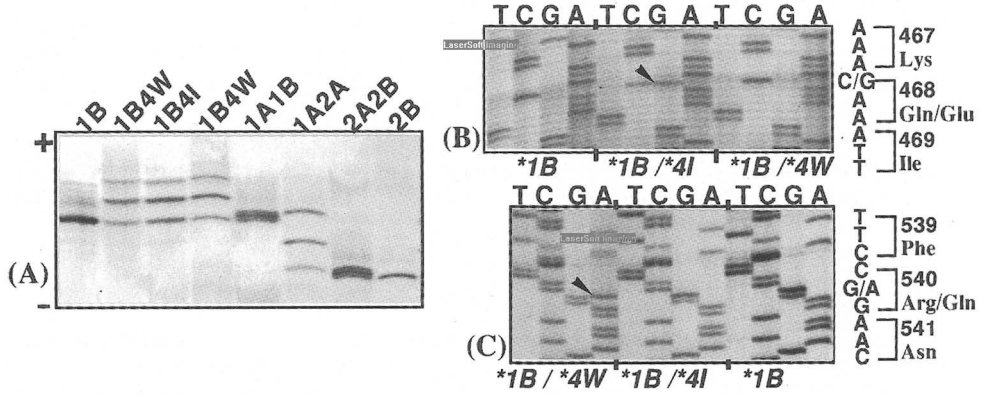

Fig.4. Separation of F13A*4I and *4W by AGIF (A). Sequences in the regions of the codon for the amino acid residue 468 in exon 11 (B) and of the codon for the amino acid residue 540 in exon 12 (C). Arrow heads indicate substitutinal bands.

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agarose gel electrophoresis to produce a single band under appropriate amplification conditions. Two exons were found to be responsible for the allelic differences. A C/T transition of the second nucleotide of the codon encoding the amino acid residue 564 in exon 12 was identified as a nucleotide difference between the *F13A*1A* and **1B* allele, and between the *F13A*2A* and **2B* allele (fig.3A). This transition leads to substitution of Leu564 in *F13A*1A* and **2A* for Pro564 in *F13A*1B* and **2B*, resulting in subtypic splits of the former diallelism (*F13A*1 and *2*) into the present four alleles. Exon 14 was found to harbor two changes at the first nucleotides of the contiguous codons encoding amino acid residues 650 and 651 (fig.3B). These two mutations in exon 14 were found to define the former diallelism. Nucleotide changes detected in this study and deduced amino acids were summarized in table 1.

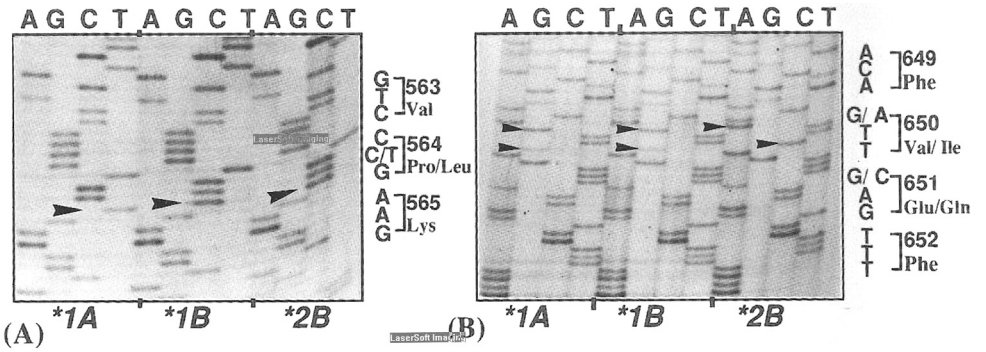

Fig.3. Sequences in the regions of the codon for the amino acid resideu 564 in exon 12 (A) and of the codons for the amino acid residues 650 and 651 in exon 14 (B). Arrow heads indicate substitutinal bands.

Table 1. Allele specific base changes in F13A polymorophism

alleles	exon12	exon14		abbreviation
	codon 564	codon 650	codon 651	
*F13A*1A*	C<u>T</u>G(Leu)	<u>G</u>TT(Val)	<u>G</u>AG(Glu)	T-GG
*F13A*1B*	C<u>C</u>G(Pro)	<u>G</u>TT(Val)	<u>G</u>AG(Glu)	C-GG
*F13A*2A*	C<u>T</u>G(Leu)	<u>A</u>TT(Ile)	<u>C</u>AG(Gln)	T-AC
*F13A*2B*	C<u>C</u>G(Pro)	<u>A</u>TT(Ile)	<u>C</u>AG(Gln)	C-AC

The Glu/Gln substitution at residue 651 inferred from the nucleotide change in exon 14 seemed to be consistent with the mobility difference between *F13A*1A/*1B* and *F13A*2A/*2B*. The substitution at residue 564 is a neutral one and thus undetectable as a mobility shift by usual zone electrophoresis. According to the hypothesis by Righetti and Gianazza (1980) based on the data showing IEF separation of hemoglobin variants with neutral mutations (Whitney III et al. 1979), increase in hydrophobicity of polypeptide chains in general leads to decrease in isoelectric point via loss of positive charge and vice versa. This hypothesis predicts that F13A 1B and 2B carrying proline at position 564 are likely to exhibit a little higher isoelectric points, i.e., more cathodal mobilities than their point mutation counterparts with leucine, F13A 1A and 2A since proline is more amenable to be exposed to water and thus less hydrophobic than leucine. The separation by IEF of each subtype of *F13A*1* and **2* was in agreement with this consideration.

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MOLECULAR BASIS FOR POLYMORPHISM OF THE "a" SUBUNIT OF COAGULATION FACTOR XIII

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INTRODUCTION

Two major alleles of the "a" subunit of coagulation factor XIII (F13A), *F13A*1* and **2* (Board 1979) were found to split into four alleles, *F13A*1A, *1B, *2A,* and **2B* (fig.1, Suzuki et al. 1988). We hypothesized on the basis of the differences in isoelectric points between the four allele products that two point mutations and one intragenic crossing over event might generate the four alleles (fig 2). In this study the 14 coding exons (Ichinose and Davie 1988) of the four F13A alleles were amplified by using polymerase chain reaction (PCR) and sequenced to reveal nucleotide differences between the alleles and to provide support for the hypothetic model, and nucleotide changes in two rare variant alleles were also presented.

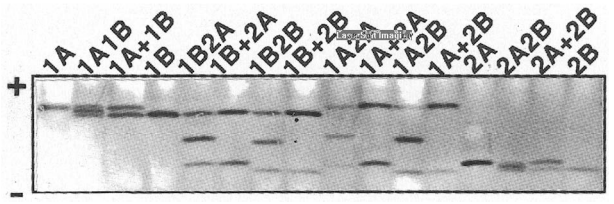

Fig.1. Separation of ten F13A phentypes deduced from four common alleles. Plasma samples were subjected to agarose gel (1%) isoelectric focusing with carrier ampholyte mixture of pH5-6 and 5-6.5 and 2M urea.

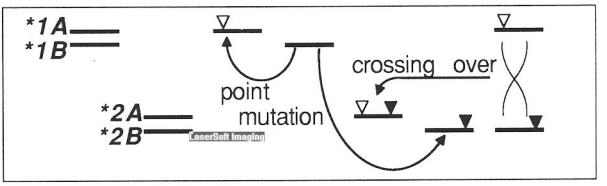

Fig.2. Hypothetic model for the genesis of F13A polymorphism. Although the most frequent *F13A*1B* allele is assumed here as an ancestral monomorphic gene, any other alleles are compatible as an ancestor with the hypothesis.

MATERIALS AND METHODS

Fourteen pairs of oligonucleotide primers, each of which corresponds to the flanking sequences of the 14 coding exons were synthesized according to the genomic sequence reported by Ichinose and Davie (1988). Single-stranded DNAs of sense and antisense sequences of each coding exon were prepared from double-stranded PCR products and then sequenced by dideoxy sequencing.

Single strand conformational polymorphism (SSCP) was analyzed by using polyacrylamide gel electrophoresis and silver stain.

RESULTS AND DISCUSSION

Genomic DNAs extracted from three homozygetes, 1A, 1B and 2B, and one heterozygote 1A2A were amplified by PCR and sequenced. PCR using each primer pair was demonstrated by

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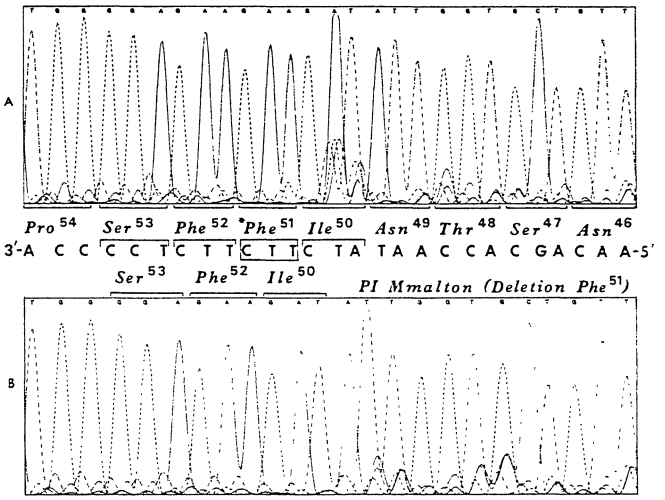

Fig. 3. Identification of the PI Mpalermo mutation in exon II by non-radioactive automated direct sequencing. (A) Normal sequence for the M1(Val213) allele concerning the amino acid 46-54 region. (B) Sequences of Mmalton and Mpalermo are identical in this region. A triple nucleotide deletion at the codon position for amino acid Phe51 characterizes the mutation.

In contrast to Mpalermo with a M1(Val213) background, the Mmalton allele derives from a M2 base allele (Curiel et al. 1989; Fraizer et al. 1989). To clarify the origin of both alleles we employed DNA haplotyping. While the Mmalton allele is associated with the haplotype 5'-SstI(-), 5'-AvaII(-), and 3'-AvaII(135), the Mpalermo allele was associated with the haplotype ++(135) in both families, indicating that the two alleles were generated in two independent mutational events. PI*Mnichinan, another deficiency allele of the M-family is identical to Mpalermo with the exception of a G → A transition at the codon 148 causing Gly → Arg substitution (Matsunaga et al. 1990).

We conclude that sequencing of the PCR amplified DNA is the most efficient technique for differentiation of very low producing alleles in the PI system.

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fluorescent oligonucleotide primers and T7 DNA polymerase (Sequenase 2.0 USB Co.) were used. Fluorescent labeled reaction products were analyzed with an Applied Biosystems 373 automated DNA sequencer.

RESULTS and DISCUSSION

The PI phenotypes were identified by IEF and immunoprinting (Fig. 2). Mother and child of family A. as well as both parents of family S. have shown slightly reduced α1AT band patterns. They were assumed to be heterozygous carriers of a deficient PI M variant which is located only a trace cathodal to the normal M2 subtype. Two individuals were found to be homozygous for this variant (lanes 7 and 8) which has been tentatively designated Mpalermo. Sequence analysis of this mutant demonstrated a 3bp deletion in exon II (TTC → Phe) which also occurs in the deficiency allele Mmalton (Fig. 3).

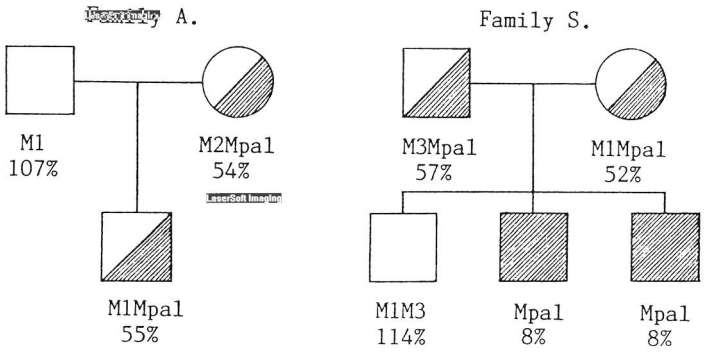

Fig. 1. Pedigree of two unrelated families with the deficiency allele PI*Mpalermo (Mpal). The phenotype is listed below each family member together with the serum α1AT level (% of normal value). Hatched symbols represent the rare Mpal variant.

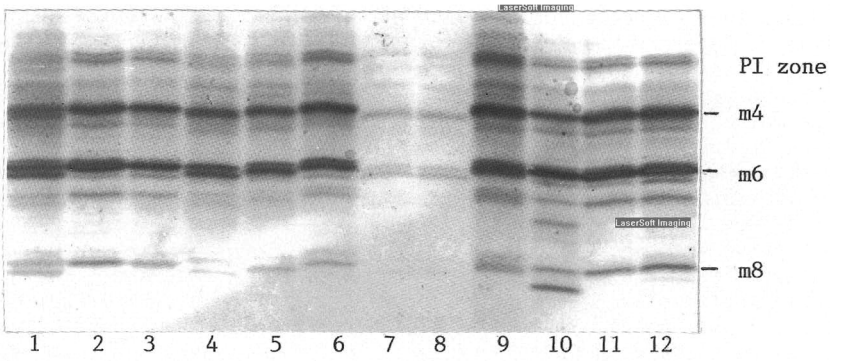

Fig. 2. Presentation of normal and deficient PI phenotypes as obtained by IEF on polyacrylamide gel (pH range of 4.2-4.9) followed by immunoprinting with a monospecific α1AT antiserum. Lane (1) M1M2; family A.: (2) father= M1, (3) child= M1Mpal, and (4) mother= M2Mpal; family S.: (5) father= M3Mpal, (6) mother= M1Mpal, (7) child 1= Mpal, (8) child 2= Mpal, and (9) child 3= M1M3; (10) M1Z; (11) M1, and (12) M1. Anode is at the top.

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PI*Mpalermo: A NEW ALPHA-1-ANTITRYPSIN DEFICIENCY ALLELE DETECTED BY DNA SEQUENCE ANALYSIS IN TWO FAMILIES

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INTRODUCTION

Alpha-1-antitrypsin (α1AT) is a member of the serine proteinase inhibitor (serpin) superfamily. The 52-kD glycoprotein is mainly synthesized in the liver and functions as the major plasma inhibitor of neutrophil elastase. α1AT deficiency is an inherited autosomal disorder associated with chronic obstructive lung disease and childhood liver disease (Crystal 1990). The highly polymorphic single-copy gene has been mapped to chromosome 14q32.1. Approximately 80 different allelic variations are known (Cox 1990). These alleles can be categorized into four groups: (1) normal, (2) deficient, (3) null, and (4) dysfunctional. The major clinical importance relates to the deficiency alleles (associated with reduced serum α1AT levels) and null alleles (no detectable α1AT in serum). According to their allelic frequencies PI*Z (0.01-0.02) and PI*S (0.02-0.04) are the most common deficient variants in European populations whereas all other abnormal PI variants are rare.

In the present study we characterize the gene and protein of the highly deficient α1AT allele, PI*Mpalermo.

MATERIALS and METHODS

1. Study of families

The allele PI*Mpalermo was evaluated in two unrelated families from the Mediterranian area (Fig. 1). Family A. (southern Italy) contained the index case, a one-year-old male heterozygote who was clinically treated for asthma. Quantitative determination of the patient's and her mother's α1AT serum levels by radial immunodiffusion have shown a reduction to nearly 50 %. According to the mother's birth place the new deficiency allele was named PI*Mpalermo. In an unrelated Turkish family two boys (three and four years of age) were found to be homozygous for Mpalermo. The only physical abnormality detected in these two children was a hyperreactive bronchial system. In none of the affected family members a liver disease is known.

2. Phenotyping and haplotyping

PI phenotypes in serum were classified by isoelectric focusing (IEF) on polyacrylamide gels using two different techniques (Weidinger 1992). Genomic DNA of the eight individuals examined was isolated from peripheral leukocytes of EDTA blood using standard procedures. DNA haplotyping was performed according to Meisen et al. 1988.

3. Amplification and direct sequencing

All coding exons (II-V) of the α1AT gene were amplified by polymerase chain reaction (PCR) using specific primers and genomic DNA as template (Faber et al. 1990). Sequencing was performed by a radioactive and an automated non-radioactive method (Faber et al. 1993). For automated DNA sequencing

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the prosimians. The bands of anthropomorphous apes are partly within the human range and partly outside (Fig.3). Again one can find the tendency of decreasing allele sizes associated with the distances in the evolutionary tree. For an overview of the family tree of primates see Washburn (1985).

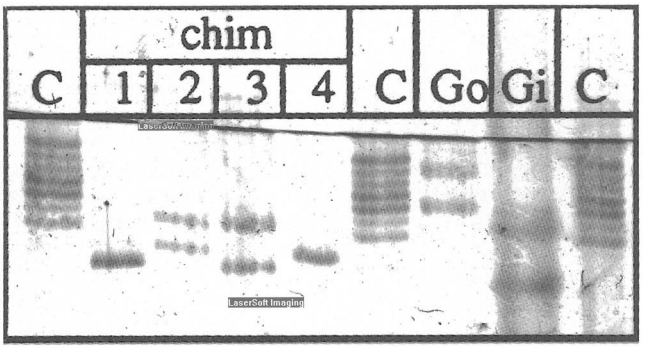

Fig.3 The HumVWA system in apes. Bands can be observed within and without the human range
C: Hum. allele cocktail
chim: Chimpanzee
Go: Gorilla; Gi: Gibbon

Codominant inheritance of STR alleles in a mendelian fashion could be observed in all species investigated so far (8 meioses in Chimpanzees, 3 in Gibbons, 6 in Crab-eating Macaques, 4 in Rhesus monkeys). This fact and the high degree of polymorphism found in most primate species demonstrates that PCR STR systems that have been established in forensic DNA profiling are also informative DNA markers for non-human primates.

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The electrophoretical separation of amplified HumTHO1 and HumD21S11 fragments was carried out in polyacrylamide gels (6% T, 3% C; thickness 750 µm) and denaturing polyacrylamide gels (6% T, 3% C, 10 M urea) for HumVWA fragments with piperazine diacrylamide as cross-linker (Budowle et al. 1991) using a discontinuous buffer system (Allen et al. 1989). The separation distance was 20 cm (15 cm for HumD21S11). Bands were visualized by silver staining according to Budowle et al. (1991).

Results and discussion

STR loci could be detected in a variety of non-human primates. In HumD21S11 a high degree of polymorphism could be observed as exemplified for Chimpanzees and Crab-eating Macaques (Fig.1).

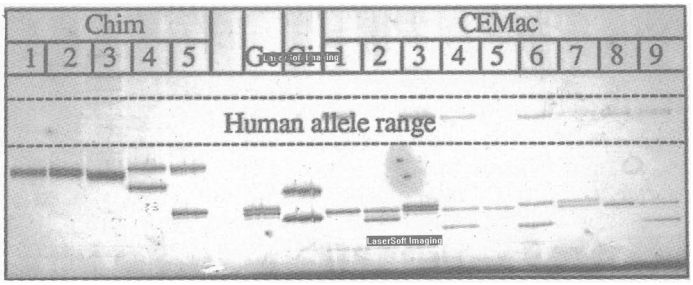

Fig.1: Polymorphism in D21S11. The range of human fragments is marked by broken lines.
Chim: Chimpanzee
Go: Gorilla
Gi: Gibbon
CEMac: Crab-eating Macaque

Concerning the allele sizes, it is evident that shorter alleles are more frequent in non-human primates. Sequence data indicate that this is due to less repeat units (data not shown). Phylogenetically older species such as Crab-eating Macaques show alleles relatively far outside the human range, while in Chimpanzees the fragments are intermediate in size and nearly reach the lower allele range of humans.

Fig.2: Polymorphic patterns in HumTH01. The fragments of the apes are located within the human range.
C: Human allele cocktail
chim: Chimpanzee
Go: Gorilla
Gib: Gibbon

Polymorphism in HumTH01 could only be detected in anthropomorphous apes (Fig.2). In this system the alleles of the apes are located within the human range and the alleles 5-7 are most common. Old world monkeys show no polymorphism, all species investigated so far are monomorphic having only one fragment with 3 repeats. The Capuzine monkey (a New world species) also shows allele 3 and in the less evolved Tamarine an even shorter allele could be found. Polymorphism in the HumVWA system seems to be phylogenetically older than in HumTHO1 and can already be observed at the level of

STR-Polymorphisms in non-human Primates

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Introduction

PCR STR systems have proven to be powerful tools in forensic DNA typing. Here we report on the STR systems HumD21S11, HumVWA and HumTH01 in which VNTR polymorphisms can also be detected in many non-human primates, and are therefore useful for DNA typing purposes such as paternity testing or choosing breeding partners in monkeys and apes. There are some structural differences compared to human alleles, which are associated with the evolutionary distances between the different primate groups. As only few data exist for some species these results should be regarded as preliminary.

Materials and methods

The primate species and numbers of individuals investigated were as follows:

Group	Anthr.Apes	Old World Monkeys	New World Monkeys	Prosimians
Spec	Chimpanzee (14)	Rhesus(23)	Capuzine(2)	Whitehead Lemur(1)
	Gorilla(4)	Crab-eating Macaque(11)	Tamarine(1)	Catta(1)
	Gibbon(5)	Green Monkey(1)		

DNA was isolated from single and pooled hair roots in 50 µl reaction volumes containing 5 µl 10X PCR buffer (Promega,USA), 10 µl proteinase K (2mg/ml), 3 µl 0.1M DTT.
The samples were incubated at 37°C overnight or at 56°C for 3 hours. Blood samples were collected on cotton fabric and extracted with Chelex 100 according to Wiegand et al.(1993). Aliquots of 1-10 µl were used for PCR.
PCR amplification was performed using published conditions and primers.
HumTH01 : (Edwards et al. 1992)
HumVWA : (Kimpton et al. 1992)
HumD21S11: (Sharma and Litt 1992) with the following modifications:
temperatures (29 cycles):
94°C - 15s
62°C - 30s
72°C - 75s

10x PCR-buffer and Tth-polymerase (ITC,FRG)

Thermocyclers: HumTH01 and D21S11: Triothermoblock (Biometra,FRG)
HumVWA: TC9600 (PerkinElmer/Cetus,USA)

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for different haematological disorders [13]. Since single aminoacid substitutions may have a profound effect on peptide binding and T-cell recognition [14] accurate molecular typing, including the use of the SSO DQB-3.5 probe we suggested in this study, should be mandatory, before BMT from unrelated donors is performed.

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co-amplification of the DQB2 locus. Next, an aliquot of amplified material was subjected to another amplification using 50 pmol of DQB130 and 0.5 pmol of DB86 (5'-CTG-CAG-GGT-CGT-GCG-GAG-CTC-CAA-CTG-3') ampli-primers and vice versa. The single strand DNA was purified in microconcentrator Centricon 100 (Amicon) and directly sequenced with the Sanger method on both strands. The sequence pattern of the novel allele, formally indistinguishable from a double crossing over, can most likely be explained by an unidirectional gene conversion event between the DQB1*0302 (recipient) and DQB1*0402 (donor) alleles. While both the 5' and 3' sequences of the DQB1*0305 allele belong to the DQB1*0302, those comprised between these two stretches are derived from the DQB1*0402 allele. In particular all nucleotides 5' to position 26 derive from the DQB1*0302 allele, those comprised between position 63 and 114 unequivocally belong to the DQB1*0402 allele, while the sequence 3' to position 164 is again that of the DQB1*0302 allele. Due to the sharing of the sequences between the donor and the recipient alleles from position 27 to 62 and from 115 to 163 the cross-over points cannot be unequivocally identified. Considering the sequence pattern of DQB1*0305, to achive a better discrimination of this allele, we suggest the use of the following additional SSO probe to the traditional panel used for the typing of DQB1 locus : DQ-3.5 (5'-GG-ACC-GAG-CGC-GTG-CGG-GGT-3', codons 20-26; washing temperature 72 °C).

Sardinians are an isolated ethnic group believed to be one of the oldest and genetically most deviant population in Europe [12]. The DQB1*0305 allele was found in 10 out of 1000 samples (gene frequency of 0.005) suggesting that it is not an uncommon variant in this population. It is interesting to note that the gene frequency of DQB1*0305 was obtained starting from dried blood spots commonly used for neonatal screening programs and stored for 1-2 years. This approach may offer a simple method to establish the frequency and the intra-regional distribution, from a large number of samples, not only of HLA genes but also of other genetic markers in epidemiological studies

The recognition of the DQB1*0305 alleles has not only implications in evolutionary studies but also for forensic studies, as well as for bone marrow transplantation (BMT) considering also that BMT from unrelated donors is becoming a possible therapeutic option

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MOLECULAR ANALYSIS OF A NOVEL HLA-DQB1 ALLELE AND ITS GENE FREQUENCY AMONG SARDINIANS

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The genetic mechanism of MHC polymorphism generation is a controversial issue and it has been supposed to vary from locus to locus [1,2]. Recombination mechanisms (gene duplication, gene conversion, inter-allelic/inter-locus sequence exchange) and point mutations are thought to have played different roles during the evolution of the various HLA class II loci [3-5]. In this respect, recombination events promoted by conserved χ-like putative recombination signals, found in HLA-DRB1 and -DPB1 genes between the β-sheet and α-helix domains, are believed to have significantly contributed to polymorphism generation at these loci [5,6]. By contrast, the fact that χ-like sequences have not been observed at the DQB1 locus has been put forward to explain why recombination-like events are less frequently seen at this locus where point mutations are considered to have played the major role in polymorphism generation [1]. We indicate that a χ-like sequence is also present within the DQB1 locus (5'-GCTGGGG-3', nucleotide position 156-162) and that this motif may have contributed, through intralocus gene conversion, to the generation of the novel DQB1*0305 allele.

DNA of 1000 randomly selected newborns of Sardinian descent, referred to our centre for Guthrie screening programs, was extracted by the chelex method starting from dried blood spots [7]. Amplification of the polymorphic second exon of DQB1 gene and dot blot analysis of amplified DNA with sequence specific oligonucleotide probes (SSO) was carried out using the procedures and oligonucleotide probes already described by others [8-10].The samples having a novel hybridization pattern were sequenced using the asymmetric PCR method [11] First, DNA was amplified with DQB130 (5'-AGG-GAT-CCC-CGC-AGA-GGA-TTT-CGT-GTA-CC-3') and DQB131 (5'-TCC-TGC-AGG-GCG-ACG-ACG-CTC-ACC-TCC-CC-3') ampli-primers that specifically amplify DQB1 second exon avoiding the

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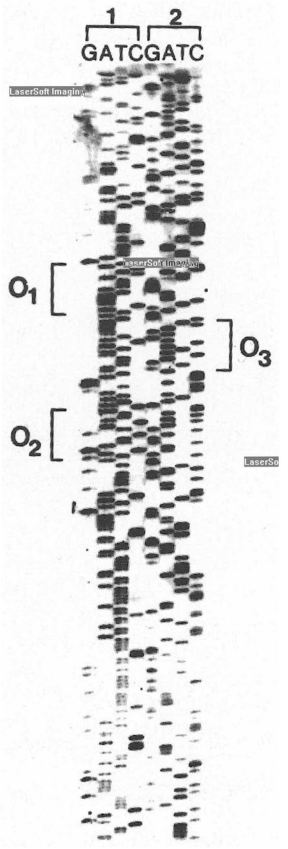

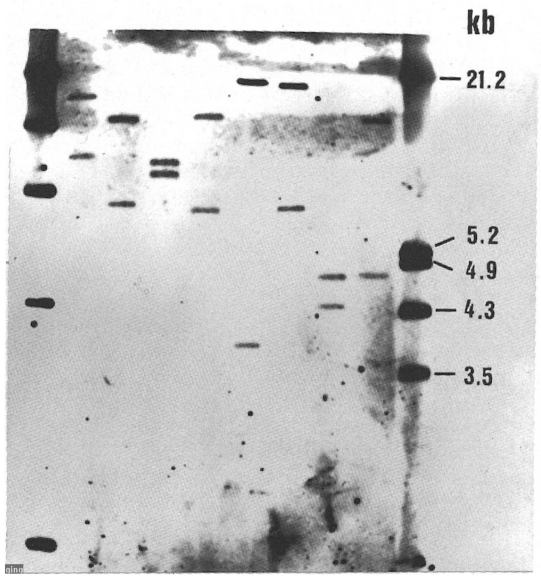

Fig.2: DNA profile of Alu I digested DNA, revealed by Oligo 2 DIG.

Fig.1: Nonradioactive sequence ladder of a recombinant (ATTTT)5 positive clone. (1) sequencing with M13 DIG forward primer, (2) sequencing with M13 DIG reverse primer

Literature:

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Developing a single locus probe, individual (ATTTT)5 bearing DNA loci from the 3 to 4 kb region of such an ALU I digested human DNA fingerprint were isolated. These 3 or 4 kb DNA fragments were cloned in vector pGEM-3Zf(+), which was digested with Sma I. Six (ATTTT)5 positive clones were randomly selected and showed a polymorphic pattern with ALU I digested DNA of unrelated persons.

To identify the sequence of the loci nonradioactive sequencing and directblotting electrophoresis were performed. PCR cycle sequencing was used to produce DNA single strands.A digoxigenated M13 primer was used and given to the DNA of the recombinant vectors. This circular vector was subjected to a normal PCR with therefore dd nucleotides in 4 caps respectively. After 25 PCR cycles the sequencing reaction and DNA single strands were produced at the same time.
PCR cycle sequencing presents many advantages to normal PCR:
- direct sequencing of template DNA is possible without additional effort to make single stranded DNA.
- 25 PCR cycles result in a huge quantity of single stranded DNA, this takes effect on the signal strength of the banding pattern in nonradioactive detection. So it is possible to read 500 bp in most cases.
- cycle sequencing is a very good method to sequence small quantities of ds DNA. Considerably less DNA template is required than in normal didesoxy sequencing.

Direct blotting electrophoresis was used to seperate the sequence reaction. This method requires a special sequencing device (Pohl et al. 1992): During the electrophoresis a DNA binding membrane passes by under the edge of the gel plates. So the DNA fragments leave the gel and automatically bind on the nylon membrane. This method doesn't need the efforts in blotting, is very quick and minimizes the loss of DNA in transfer, because the DNA is carried over directly from the gel to the membrane. With PCR cycle sequencing , direct blotting electrophoresis and nonradioactive detection of the sequence patterns, the results can be obtained within one day.

All of our six recombinant, (ATTTT)5 positive clones were partially sequenced (FIG.: 1) and we found that they contain the same DNA fragment. No repetitive sequence is seen in this part of the DNA fragment, however in this sequence A and T nucleotides appear very often (76,4%). Perhaps we will be able to identify repetitive minisatellites in further sequencing. Comparison of Genebank and EMBL sequences showed that we dealt with unpublished sequences. To verify if the sequence is the flanking region of a new detected minisatellite an oligonucleotide, homologous to parts of the sequenced DNA was sythesized (5'CATTACATGCTTAGCATGA3'). These oligonucleotide (Oligo 2) was reprime labeled with Digoxigenin and then hybridized to Alu I digested DNA. The DNA fragments, isolated and cloned in vector pGEM-3Zf(+) were detected. Every person shows individual specific banding pattern (Fig.: 2). So we discovered a hypervariable DNA locus and found out that the oligonucleotide can be used as a single locus probe for forensic investigations. Now we are sequencing the whole cloned DNA fragment. Future investigations will show the allelic frequency of this new detected minisatellite in different population samples.

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ISOLATION OF A (ATTTT)N POSITIVE LOCUS BY CLONING, PCR CYCLE SEQUENCING AND NONRADIOACTIVE DIRECT BLOTTING ELECTROPHORESIS

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Introduction:

Hypervariable human DNA markers have a profound impact on medicine. Most DNA typing systems use tandem repetitive minisatellites or VNTR loci, which can show extreme levels of allelic variability in repeat copy number and therefore DNA fragment length. To create a single locus probe, we were working with the minisatellite family (ATTTT)n. This is the only repetitive sequence family with a pentanucleotide sequence unit, described in the human genome (Vogel et al. 1990). We want to demonstrate the isolation of a (ATTTT)n positive locus by cloning, PCR cycle sequencing and nonradioactive Direct Blotting Electrophoresis. The isolated locus is hypervariable and can be used for forensic investigations.

Material and methods:

Oligonucleotide synthesis: Synthetic oligonucleotides were synthesized according to the phosphoramidite method, using a Gene Assembler (Pharmacia). The oligonucleotides were deprotected and desalted by NAP 10 columns (Pharmacia).
Isolation and digestion of DNA: Human genomic DNA was isolated according to Miller et al. (1988). The DNA was digested with ALU I (Boehringer).
Electrophoresis: Electrophoresis was performed in 0,9% agarose gels (20x20 cm), 30h, 40 V.
Hybridization: 3'OH digoxigenated oligonucleotides (ATTTT)5 and Oligo 2 were used as hybridization probes (5xSSPE, 5xDenhardts solution, 0,1% SDS, 10 g/ml denatured E. coli DNA).
Detection: Nonradioactive immunological detection was performed according to the Lumigen manual (Boehringer).
Cloning: Alu I digested 3-4 kb DNA fragments were isolated from Agarosegel (Quiaex / Quiagen) and cloned in Sma I digested pGEM-3Zf(+) vector (Promega). Recombinant clones were randomly selected and screened with (ATTTT)5. DNA from (ATTTT)5 positive clones were isolated with QIA prep-spin Plasmid Kit (Quiagen).
Sequence analysis: PCR cycle sequencing was performed with the Dig Taq DNA Sequencing Kit (Boehringer). To separate the sequence reaction we used the direct blotter from MWG.

Results and Discussion:

Synthetic oligonucleotides, which contain very much A and T such as TT(ATTT)5 and (ATTTT)5 were synthesized. Hybridization of (ATTTT)5 on DNA, digested by Alu I, results in a multilocus fingerprint pattern. This oligonucleotide generates individual specific DNA banding pattern in humans.

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which was also present in COL2A1 8 of the father.
Both alterations of the basic repeat were unlikely to be observed by chance in any other repeat of the three individuals. It was therefore deduced that COL2A1 14 was a mutant allele deriving from the paternal line of inheritance, by unequal crossing over.

Several questions remain to be answered, concerning:
1) the position at which recombination took place;
2) the possible role of the sequence alterations in triggering crossing over;
3) the true frequency of mutational events at COL2A1 locus;
4) the precautions to adopt in using this locus for paternity investigations.

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EVIDENCE FOR A MUTATION AT 3'HVR COL2A1 LOCUS - SEGREGATION ANALYSIS AND SEQUENCE DATA

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Type II collagen is the major structural protein of cartilage and its encoding locus (COL2A1, 12q1.23-3.21) is the candidate gene for the heritable chondrodysplasia. Single base mutations and partial gene deletions in this sequence are possibly responsible for the clinical syndrome.
Downstream of this exone, a hypervariable region exists composed of tandemly arranged, imperfect repeats, each spanning 31-34 base pairs (1). This marker is highly polymorphic (2) and is used for forensic applications.
We report a mutational event at this interesting locus, met during a paternity analysis.

CASE OUTLINE

A family trio showed a parenthood exclusion at this locus. A higher molecular weight allele (COL2A1 14), present in the child, was absent in both parents, who shared in turn the only segregating child's allele. Analysed for a variety of conventional systems (ABO, Rh haplotype, MNSs, Gc, PI, Hp) Single Locus Probes (SLPs; D2S44, Ms43a, Ms1, Ms31 and g3), Amplified Fragment Length Polymorphisms (AmpFLPs; APOB, COL2A1, D1S80) and Short Tandem Repeats (STRs; HUMTH01 and MIT-MH26), both parents proved to be perfectly compatible. A high probability of paternity was in fact deduced (99.9999).
This strongly supported the hypothesis that a mutation could have occurred at either the maternal or paternal gamete. Therefore, a closer analysis of COL2A1 fragments of this family was carried out.

BAND CLONING AND SEQUENCE ANALYSIS

Amplified products of the trio were run on agarose, then each individual band was excised, electroeluted and separately reamplified. Reamplified alleles were passed through a Centricon 100 filter (Amicon), cloned in Bluescript vector and sequenced by deoxy chain termination (Sequenase, USB).
Sequence analysis supplied the following indications:
1) COL2A1 12 of the mother was identical to the child's allele;
2) COL2A1 14 of the child showed an unusual base transition (C - T) 467; the same mutation was present in COL2A1 12 of the father.
3) COL2A1 14 of the child exhibited a base insertion (A244),

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Only 6 alleles have been subjected to sequence analysis. The results show that one class of genotypic variant was the presence of the sequence GATTA in the middle of the GATA repeats. Other changes detected represented mostly single base sequence changes. Comparison of two alleles containing the same number of GATA repeats show differences at various places in the sequence. For example:

(GATA)21-1: CAGGTCTCCGA.....GATACATAGATA.....TATAAACT
(GATA)21-2: CAGGTCT- CGA.....GATAGATAGATA.....TATACACT

(GATA)10-1: CAGGTCTTGA..... GATAGATAGATA.....TACCAACT
(GATA)10-2: CAGGTCCTGA..... GATAGATAGATA.....TACAAACT

The results obtained for both loci indicate significant variation at the DNA sequence level for alleles containing equal number of GATA repeats. This allows for the identification of new alleles within the same size class. The effect of this increase in the number of alleles on the frequency calculations will require analysis of a large number of alleles, however, it will result in an increase of the power of identification of these loci.

It is possible that the frequency of mutations may depend on the sequence of STR. However, there has not been enough of this type of loci sequenced to date to draw conclusions.

A study similar to the one described in this paper was reported by Adams et al. (1993) using an Alu-associated STR locus. The results of that study also shows large number of sequence variants among alleles with the potential of an increase in the power of identification of the locus.

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Results and Discussion.

The general strategy for sequencing individual STR alleles involved a primary amplification of genomic DNA, isolation of individual alleles from the gel after electrophoresis, followed by reamplification of each allele with a set of biotinylated primers. Single stranded DNA was isolated and sequenced.

The general property of these loci were:

Locus 1. Heterozygosity: 85%. STR: (GATA)n.

Number of alleles by size (by electrophoresis): 8

Frequency of allele number 1 = 4.2% Size range of alleles: 134 to 162 bp.
2 = 10.4%
3 = 10.4%
4 = 27.0%
5 = 18.8%
6 = 12.5%
7 = 8.3%
8 = 8.3%

Comparison of the sequence of several alleles within the same size class detected the existence of several sequence variants. For example, each of four alleles containing 13 GATA repeats showed sequence variations in the region contiguous to the repeat either due to sequence changes or insertion/deletions of a few bases.

$(GATA)_{13}$GAACGAACAC.....
$(GATA)_{13}$GATGAACAC.....
$(GATA)_{13}$GAACAC.....

Sequence analysis among alleles from the most common classes (i.e. 4, 5, 6) revealed 2 or 3 sequence variants in each group.

Locus 2. Heterozygosity: 85%. STR: (GATA)n.

Number of alleles by size (by electrophoresis): 10

Frequency of allele number 1 = 2.5% Size range of alleles: 220 to 300 bp.
2 = 7.5 %
3 = 5.0%
4 = 2.5%
5 = 17.5%
6 = 30.0%
7 = 15.0%
8 = 12.5%
9 = 5.0%
10 = 2.5%

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GENOMIC SEQUENCING OF ALLELES FROM STR LOCI.

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Introduction.

The traditional procedures for the analysis of VNTR, AMP-FLP and STR loci, for DNA typing rely on the determination of the relative DNA fragment size for the definition of alleles. This type of allele definition provides large amount of information for VNTR loci containing substantial number of alleles. For loci composed of a few small alleles (i.e., a few hundred bases long), that are most suited for analysis by DNA amplification, the power of identity of a locus could be greatly expanded if additional source of variation were found at the level of their primary sequence. This would allow for further sub-classification of alleles for this type of loci. To this objective, we began studies on two tetranucleotide STR loci by sequencing their individual alleles.

Materials and Methods.

Genomic DNA from unrelated North American Caucasoid individuals was isolated from blood using proteinase K/SDS lysis and phenol extraction. Amplification was performed in a 100 μl reaction containing approximately 10 ng of DNA as template plus 200 μM dNTPs, 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$), 2.5 units of Taq polymerase (Perkin Elmer, Norwalk CT.) and 1 μM of primers. Cycling parameters were: 95 °C for 1', 50 °C for 1', and 72 °C for 1' for 30 cycles, followed by a 10 ' final extension at 72 °C. The amplified products were size fractionated in 3% high sieving agarose gel (, FMC Bioproducts) with 1 X Tris-Borate-EDTA buffer at 150 Volts for 5 hours.

The two STR selected for this study contain tetranucleotide repeats of the sequence GATA. The alleles detected contain from 8 to 16 repeats. Heterozygosity, determined from the frequency of different allele sizes was about 85 %.

After electrophoresis, individual alleles stained with ethidium bromide were excised from the gel, melted, diluted with H_2O and 10% used as template for a second amplification reaction. This second amplification was performed in two reactions using 5'biotinylated primers from opposite ends of the allele. The conditions used were the same used previously. The biotinylated amplification product was immobilized onto Streptavidin coated magnetic beads (Dynal, Inc., Glen Cove, NY) and the non-biotinylated strand eluted off with NaOH. The single stranded DNA was sequenced, in an ABI Model 373A automated sequencer, using fluorescent dideoxy terminators (Taq Dye-Deoxy Terminator Cycle Sequencing Kit; ABI, Foster City, CA) according to manufacturers' recommendations.

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daughter and mother was questioned. The AmpFLP loci D1S80 (29), HUMFABP (30,31), HUMARA (30,31), and HUMACTBP2 (SE33)(32,33) were used to screen for maternity, and the daughter shared an allele with the mother at all loci. RFLP testing was subsequently performed by Art Eisenberg at the University of North Texas Health Science Center in Ft. Worth, Texas. A total of eight probes were used (D2S44, D14S13, D10S28, D4S139, D17S79, D1S7, D5S110, and D17S26). Again, the daughter shared at least one allele at each locus tested. A maternity index (using seven of the eight loci) of 197,695 was calculated and a 99.999% probability of maternity was determined. Therefore, the relationship of the daughter was no longer an issue. We have not been able to obtain the paternal reference in this case to determine whether this is an example of paternal inheritance. Assuming maternal inheritance, this would be the first example of two polymorphisms between generations found in humans. Nevertheless, given the two additional examples described above, the observed mutation rate of mtDNA may be higher than expected.

Table 3: Polymorphisms Observed for Three Case References

Reference	MtDNA Sequence Polymorphisms[a]
Mother and Son	16221T, 207A[b], 263G, 309.1C, 315.1C
Daughter	16093C[b], 16221T, 263G, 309.1C, 315.1C

[a]The numbering system represents the position where a polymorphisms has occurred and the nucleotide at that position. Numbers followed by a ".1" indicate an insertion.
[b]The positions where the two sequences differ.

In order to establish a mutation rate for the control region of mtDNA, a preliminary study was performed in our laboratory. Seven CEPH (Center for the Study of Human Polymorphisms, #13291-13294, #1331-1333) families were analyzed. On average, sequence information was generated for six children and two parents. The seven families exhibited a total of 58 polymorphisms at 28 different sites, and an average of 9 polymorphisms between each family. In a total of 46 mother-child comparisons no polymorphisms were observed. In addition, there were no examples of paternal inheritance. Additional studies are underway in our laboratory in collaboration with the Dr. Kevin Sullivan at the Home Office in England and Mark Wilson at the FBI Academy in Quantico, Virginia, to further investigate the rate of single-generation and multiple-generation mtDNA mutations.

In conclusion, comparative mtDNA sequence analysis is a powerful method of human remains identification. With the development of more rapid forms of automated sequence analysis, the rate of generating sequence information could be greater than 3000 nucleotide every hour in the near future (34). This would potentially provide an opportunity to sequence both nuclear and mtDNA on a routine basis in forensic laboratories.

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immediate relatives are not available, an identification can still be made using mtDNA sequencing.

When performing casework, sequence information is confirmed by sequence analysis of both DNA strands, or by multiple sequencing of the same strand. After generating confirmed sequence for both the evidentiary material and maternal references, the sequences are aligned and compared. Polymorphisms are determined with respect to the Anderson sequence (18). Transitions from one purine nucleotide to another purine nucleotide or a pyrimidine nucleotide to another pyrimidine nucleotide (i.e. adenine to guanine or cytosine to thymidine, respectively) are common polymorphisms. Insertions and deletions are also commonly observed. Transversions from a purine nucleotide to a pyrimidine nucleotide, or visa versa, are uncommon.

Once a mtDNA sequence match has been made, determination of the significance is necessary. In order to apply a statistical value to the match, databases have been generated by a number of laboratories (24,25). Our laboratory has a core database of 100 unrelated United States Caucasians. Adding staff, research, and casework, the total database is greater than 140. Within the core 100 Caucasians, we have found 92 unique sequence types, two individuals with one type, two other individuals with a second type, three individuals with a third type, and one individual with the Anderson sequence (18). Polymorphisms were detected at a total of 112 sites within the control region, and 52 of these were unique (only seen once). These data suggest that a significant number of polymorphisms have yet to be revealed. In total, 656 polymorphisms were detected in the 609 base pairs analyzed. Therefore, the average nucleotide diversity for each individual in the database is 1.07%. Overall, the polymorphic character of the control region appears to be excellent.

Combining our database with databases from other laboratories, there are more than 1000 individuals representing a mixture of Caucasians, African Americans, and Afro-Carribians. Most of the databases, however, have not been published. Because the databases have not been analyzed as a whole, there is no accepted statistical model available to calculate the significance of a mtDNA sequence match. Through personal communications, however, a new sequence can be compared to all databases. The frequency of a new sequence may then be reported as one over the size of the combined database. Although this does not take into account confidence limits or the relatively small size of the combined database, a match involving a mtDNA sequence that is unique to the database would strongly support an identification.

The mutation rate of the mtDNA control region is thought to be one polymorphism every 200-400 generations (26). This range was calculated by extrapolating back from sequence information of ancient populations to sequence information from modern populations. It is possible, however, that this approximation is too large. Unpublished data and results from our laboratory suggest that the mutation rate may be higher (25,27). A polymorphism has been observed between individuals separated by 18 generations (25). In addition, a polymorphism giving a heteroplasmic sequence profile was observed in the analysis of the Russian Tsar's remains (12,27). In our laboratory we have observed an example of two polymorphisms in the same individual which may have occurred in a single generation (28).

While completing a case involving skeletal remains, sequence was obtained for three family references (mother, brother, and sister of the deceased). The mtDNA sequences of the mother and brother (son) were identical. However, there were two polymorphisms between the mother and sister (daughter)(Table 3). The sequence analysis was repeated from the original blood sources with the same results. Consequently, the relationship between the

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Prior to loading, the reactions were heated at 90°C for 2 minutes, placed on ice for 5 minutes, and then loaded onto an AB 373A DNA Gene Sequencer. Gel electrophoresis was performed according to the AB 373A Protocol. A summary of the conditions is as follows: a) A pre-run electrophoresis step in 1X TBE (89 mM Tris-HCl, pH 8.3, 89 mM Boric Acid, and 2 mM Na_2EDTA) was executed for approximately 15 minutes; b) The samples were loaded, even numbers first. Electrophoresis was performed for approximately 10 minutes; c) The remaining samples were loaded; d) The electrophoresis was performed for 9-14 hours depending upon the amount of sequence desired (400-800 base pairs, respectively). The run parameters were at a constant power of 30 watts, variable readings of 18-21 milliamps and 980-1600 volts, and a temperature of 40°C. The data was collected and analyzed using AB software. Sequence comparisons were made using AB SeqEd (sequence editing) software.

Table 2: DNA sequencing primers for mitochondrial DNA sequence analysis of highly degraded DNA.

Primer Set[a]	Forward Sequencing Primer	Reverse Sequencing Primer	Sequence Obtained (Approximate)
1	F15971 5'-TTA ACT CCA CCA TTA GCA CC-3'	R16255 5'-CTT TGG AGT TGC AGT TGA TG-3'	16024-16225 HV1[b]
2	F16144 5'-TGA CCA CCT GTA GTA CAT AA-3'	R16414 5'-CAC GGA GGA TGG TGG TCA AG-3'	16180-16365 HV1[b]
3	F29 5'-CTC ACG GGA GCT CTC CAT GC-3'	R270 TGG AAA GTG GCT GTG CAG AC-3'	73-230 HV2[b]
4	F155 5'-TAT TTA TCG CAC CTA CGT TC-3'	R381[c] 5'-GCT GGT GTT AGG GTT CTT TG-3'	200-340 HV2[b]

[a]Primer sequences are based on the numbering system of Anderson et al. (18).
[b]HV1=Hypervariable Region 1, HV2=Hypervariable Region 2.
[c]When sequencing PCR product generated from good quality DNA extracts, the reverse primer R484 may be used in both the amplification and sequencing reactions (5'-TGA GAT TAG TAG TAT GGG AG-3').

SUMMARY

Primers reported in the literature (21,22), as well as novel primers (11), were used to amplify the two hypervariable regions (HV1 and HV2), and to generate sequence information from the products (Tables 1 and 2). The sequence is then compared to maternal reference sources to assist in the identification of human remains. Identifications have been made on a broad range of specimens; from aged skeletal fragments of the Vietnam War era to fresh tissue recovered from recent aircraft accidents (10,11). The high copy number of mtDNA allows for the analysis of only a few picograms of DNA. In addition, maternal inheritance allows for the identification of remains when immediate relatives are unavailable for comparison purposes. Distant maternal relatives can provide reference material for direct comparisons. Therefore, when the quality of the evidentiary DNA is not an issue, but

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seconds, and 72°C X 30 seconds for 38 cycles; and c) 4°C soak. For Primer Sets 2, 3, and 4 - a) 94°C X 30 seconds; b) 94°C X 30 seconds, 56°C X 30 seconds, and 72°C X 30 seconds for 38 cycles; and c) 4°C soak. For good quality templates, the amplification parameters are a) 94°C X 30 seconds; b) 94°C X 20 seconds, 56°C X 20 seconds, and 72°C X 20 seconds for 30 cycles; c) 4°C soak. The PCR product was evaluated on a 1-2% agarose gel. If PCR inhibition occurred and BSA or elevated AmpliTaq® could not overcome the inhibition, no further amplification was attempted and DNA was re-extracted from the specimen source.

Table 1: PCR primers for the amplification of mitochondrial DNA from highly degraded DNA extracts.

Primer Set[a]	Forward Primer Light Strand	Reverse Primer Heavy Strand	Region Amplified
1	5'-TTA ACT CCA CCA TTA GCA CC-3'	5'-TGG CTT TGG AGT TGC AGT TG-3'	15971-16258 HV1[b]
2	5'-TAC TTG ACC ACC TGT AGT AC-3'	5'-CAC GGA GGA TGG TGG TCA AG-3'	16140-16414 HV1[b]
3	5'-CAC CCT ATT AAC CAC TCA CG-3'	5'-TGT GTG GAA AGT GGC TGT GC-3'	15-274 HV2[b]
4	5'-CTC ATC CTA TTA TTT ATC GC-3'	5'-CTG GTT AGG CTG GTG TTA GG-3'	145-389[c] HV2[b]

[a]Primer sequences are based on the numbering system of Anderson et al. (18).
[b]HV1 = Hypervariable Region 1, HV2 = Hypervariable Region 2.
[c]When amplifying good quality DNA extracts, the reverse primer R484 may be used (5'-TGA GAT TAG TAG TAT GGG AG-3').

Automated DNA Sequencing

Purification of the PCR product in a Centricon 100 (Amicon Corporation) spin-dialysis column is necessary before sequence analysis to remove unincorporated PCR primers. Following purification, each sequencing reaction contained 3.2-10 pmol sequencing primer, approximately 1 μg of DNA template, 9.5 μl of Applied Biosystems DyeDeoxy™ Terminator Cycle Sequencing reaction mix (or Prism™ Ready Reaction mix), and QS to 20 μl with sterile deionized water. The sequencing primers for forward and reverse sequencing of mitochondrial DNA are located in Table 2.

The thermal cycling conditions were as follows; a) Place tubes in a Perkin Elmer 9600 Thermal Cycler preheated to 96°C; b) 94°C X 30 seconds; c) 96°C for 15 seconds, 50°C for 5 seconds, 60°C for 2 minutes for 25 cycles; d) 4°C soak (modified with respect to the AB protocol).

The sequencing reaction products were purified using Quick Spin™ Columns from Boehringer Mannheim according to the Applied Biosystems protocol. A volume of 4 μl of loading buffer was added to each purified sample (4 μl of a mixture of 1 μl of 50 mM Na_2EDTA and 5 μl of deionized formamide), the samples were then mixed, allowed to re-suspend for approximately 10 minutes, and loaded within two hours. Samples can be stored for one month at -20°C before adding loading buffer.

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extraction, amplification and sequence analysis can be found below in the Materials and Methods section.

MATERIALS AND METHODS

Extraction of DNA From Tissue and Whole Blood

DNA was extracted from tissue and whole blood using the Chelex 100 method (23). Approximately one cubic millimeter of fresh tissue or 3 μl of whole blood were analyzed. The initial wash step was omitted when extracting DNA from tissue.

Extraction of DNA From Skeletal Remains

DNA extraction was performed using a modification of the method described by Holland et al. (10). In brief, approximately 2 grams of compact bone was extensively sanded and cleaned, crushed into small pieces, and the small pieces ground into a powder using either a Micro-Mill grinder (Fisher Scientific, Pittsburgh, PA), or a Mixer-Mill 8000 mixer (Spex Industries Inc., Edison, NJ). The powder was suspended in extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2% sodium dodecyl sulfate, and 10 mM Na_2EDTA) in the presence of 0.67 mg/ml Proteinase K and incubated at 56°C for 12-18 hours. An additional 0.67 mg/ml Proteinase K was added and the incubation continued for 3 hours. Insoluble material was pelleted by centrifugation and discarded. The supernatant was purified in three steps: 1) organic extraction of protein using chloroform/phenol/isoamyl alcohol, 2) two n-butanol washes (to remove excess phenol and to reduce the extract volume), and 3) two successive washes of the DNA extract with sterile TE (buffer; 10 mM Tris-HCl, pH 7.6, 1 mM Na_2EDTA) in an Amicon Centricon 100 micro-dialysis column, followed by concentration of the sample to a final working volume of 200-500 μl.

Amplification of Mitochondrial DNA

We have found that for highly degraded DNA extracts, amplification of four overlapping regions of the control region, each approximately 250 bp in length, is necessary in order to obtain substantial sequence information (10,11). For convenience, the two hypervariable regions are amplified in their entirety for good quality DNA extracts.

The PCR master mix recipe for a 50 μl reaction is (all concentrations are final based on a 50 μl reaction) 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.4 μM forward amplification primer, 0.4 μM reverse amplification primer, 2.5 units of AmpliTaq® DNA polymerase (Perkin Elmer), QS to 40 μl with sterile deionized water. The PCR Master Mix can be supplemented with 8 μg of DNA grade bovine serum albumin or elevated AmpliTaq® (up to 12.5 units total AmpliTaq®) if inhibition of the amplification occurs. When amplifying good quality DNA, the PCR can be reduced to 25 μl and the master mix can be reduced to 15 μl. The PCR primer sets for mtDNA are listed in Table 1.

The amplification of good quality DNA was performed using the forward primers of Primer Sets 1 and 3, and the reverse primers from Primer Sets 2 and 4. For enhanced success in the DNA sequence analysis of PCR product of good quality DNA, primer R484 (5'-TGA GAT TAG TAG TAT GGG AG-3') can be substituted for the reverse primer of Primer Set 4. Add 10 μl of DNA template (approximately 1-5 ng of total genomic DNA, QS the volume to 10 μl with sterile deionized water) to each tube. Add 40 μl of PCR Master Mix to each tube (15 μl for good quality extracts) and place the tubes in a Perkin Elmer 9600 Thermal Cycler preheated to 96°C. The PCR was performed according to the following parameters; For Primer Set 1 - a) 94°C X 30 seconds; b) 94°C X 30 seconds, 62°C X 30

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Identification of Human Remains Using Mitochondrial DNA Sequencing: Potential Mother-Child Mutational Events

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INTRODUCTION

Determining the primary sequence of DNA is the ultimate form of DNA testing for discriminating between individuals. Various DNA sequencing methodologies are currently available, although the dideoxynucleotide "terminator" chemistry of Sanger is the most widely used in laboratories today (1,2). The development of fluorescence and infrared-based detection methods, coupled with automated DNA sequencing instruments, has greatly improved throughput and efficiency (3-7). Therefore, sequencing technology is readily available to most laboratories, and automation has made the process more cost effective.

At the present time, length variations (RFLP and AmpFLP loci) and sequence site-specific variations (HLA DQαand Polymarker from Roche Molecular Systems) are more than adequate to reliably distinguish between unrelated individuals. Nevertheless, the question often arises in the courtroom whether two alleles with the same length, or the same site-specific sequence, actually have the same primary sequence? There is always a chance that matching alleles will differ at the sequence level. Regardless, an example of such a case where many alleles match at different loci, yet the matching alleles have different sequences, would be an extremely rare event. The more loci tested which result in a match, the more rare the event would become. Therefore, although sequence analysis is available for forensic identification, it is not required to discriminate between individuals. Sequence analysis would be most useful in situations where currently available methods fail. One such area which is receiving attention, and has been generally accepted as a method of human remains identification, is sequence analysis of the mitochondrial DNA (mtDNA) genome (8-12).

Three characteristics of mtDNA are important for human remains identification. First, mtDNA is present in high copy number in each human cell (13). On average, each cell contains hundreds of copies of mtDNA for every copy of nuclear DNA. Second, contrary to classical inheritance patterns, mtDNA is maternally inherited (14). To date, no examples of paternal inheritance of mtDNA in humans has been observed. Finally, mtDNA is polymorphic (15-17). Within the 1200 base pair control region there are two hypervariable segments designated HV1 and HV2. Using the numbering system determined by Anderson (18), most of the polymorphic information is between positions 16024 and 16365 of HV1 and between positions 73 and 340 of HV2. Although sequence analysis of HV1 and HV2 is preferred, techniques for rapid comparison of mtDNA sequence types are being developed for screening prior to sequencing (19,20). Until these are further refined, sequence analysis will be performed without prior screening tests. This manuscript summaries the process of identifying human remains using mtDNA sequence analysis, and describes some of the results from recent research efforts completed in our laboratory. In addition, an example of two polymorphisms between a mother and child will be discussed. The methods of DNA

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8. DNA sequencing

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We established a simple and useful method for removal of water-soluble melanins contaminating DNA preparations from hairs. The method was based on the adsorption of melanins to Bio-Gel. When a Bio-Gel P-60 minicolumn was equilibrated with 10 mM sodium acetate buffer, pH 4.2, water-soluble melanins were completely adsorpted to it whereas DNAs passed through, although the melanins showed incomplete adsorption to the gel when it was equilibrated with TE (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). The filtrates of DNA preparetions gave always excellent response in amplifying mt333DNA by PCR procedure.

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Table 2. PCR-amplification of mt333DNA from hairs stained with an oxidation-type hair-staining agent (Bigen-5Ge). Samples of hair shafts 5 cm long were sampled within a region 6 cm from the hair root

original color	staining time (h)						
	0	0.5	1	2	3	4	5
black	+	−	−	−	−	−	−
white	+	+	+	+	+	+	+

+ : amplified, − : not amplified

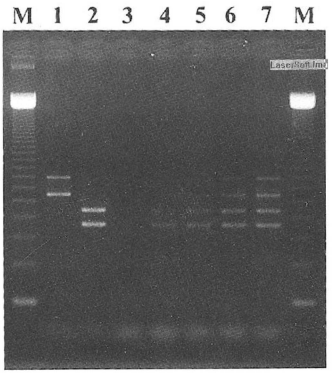

Fig. 2. A possible misdiagnosis of PCR-amplified VNTR (MCT118 locus) fragments by contamination of melanins. Each lane is as follows: (M) 123-bp ladder marker, (1, 2) two heterozygotes, (3～7) mixture of the two which was contaminated by melanin at concentration of 1.6 μg/ml(3), 1.2 μg/ml(4), 0.8 μg/ml(5), 0.4 μg/ml(6) and 0 μg/ml(7), respectively.

of DNA polymerases and DNases, based on the following results: 1) Water-soluble preparations made from chemically synthesized melanin (Sigma products), as well as natural black eumelanins, inhibited the PCR amplification of mt333DNA at concentrations of more than 2 μg/ml. 2) Quantitative measurement of *Taq* and *E. coli* DNA polymerase-catalyzed DNA synthesis in terms of the amount of [α-^{32}P]dCMP incorporated into activated calf thymus DNA showed that both of the water-soluble melanins had the same inhibition activity as represented by the sigmoidal curve derived from a quadratic equation of melanin concentration (Fig. 1). This observation suggested that DNA polymerases combined with two molecules of melanin to form an inactivated complex. 3) Water-soluble melanins gave no effect on neither the thermostability of *Taq* DNA polymerase at 94 ℃, nor the step of primer-annealing to templates. 4) Restriction digestion of λ DNA was inhibited by adding the melanins at concentrations of more than 1 μg/ml in the case of Hind Ⅲ and of more than 4 μg/ml in the case of Hae Ⅲ (the enzymes were at 11 units in 100 μl).

Water-soluble melanins exhibited a stepwise inhibition of DNA amplification of polymorphic DNA with VNTR. In the case of MCT118 (D1S58) locus, amplification degree of individual alleles depended on the numbers of VNTR; i.e. amplification of DNA fragments with a larger VNTR was likely to be more inhibited in comparison with those with a smaller one in the same PCR condition (Fig. 2). Hence, it should be possible that the heterozygous types are misdiagnosed as homozygous ones in DNA typing of criminal cases in the presence of PCR inhibitors.

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Table 1. PCR-amplification of mt333DNA from natural hair shafts at 5-cm intervals without hair root, from five women

Case No.	Color	region (cm) 1～6	6～11	11～16	16～21	21～26	26～31	31～36	36～41	41～46	46～51
1	black	+	+	+	−	−	−	−	−	−	
2	black	+	+	+	+	−	−	−	−	−	
3	black	+	+	−	−	−	−	−	−	−	
4	black	+	+	+	+	−	−	−	−	−	
5	black	+	+	−	−	−	−	−	−	−	
6	black	+	+	−	−	−	−	−	−	−	
7	black	+	+	+	−	−	−	−	−	−	
8	black	+	−	−	−	−	−	−	−	−	−
9	black	+	+	−	−	−	−	−	−		
10	black	+	+	−	−	−	−	−	−	−	
11	black	+	+	+	−	−	−				
12	black	+	+	+	+	−	−				
13	white	+	+	+	+	+	+				
14	white	+	+	+	+	+	−				
15	white	+	+	+	+	+	+				
16	white	+	+	+	+	+	+				

+ : amplified, − : not amplified

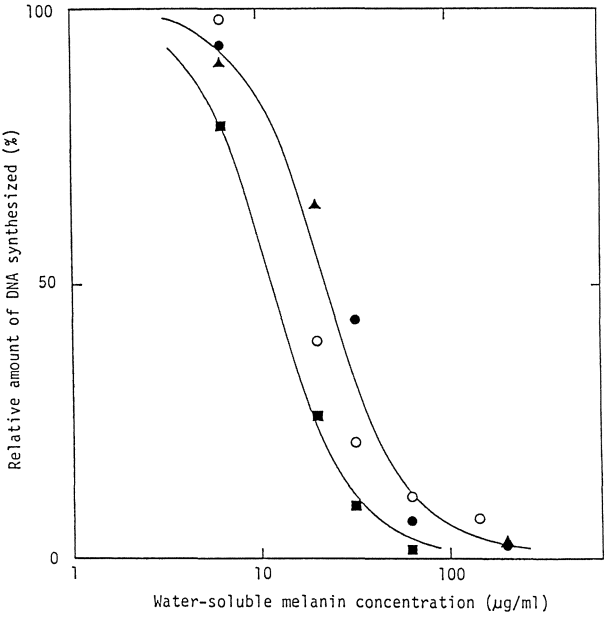

Fig. 1. Inhibition effects of water-soluble melanins (chemically synthesized and natural) on DNA synthesis by *Taq* DNA polymerase. The amount of DNA synthesized was monitored in terms of [α-^{32}P] dCMP incorporated into activated calf thymus DNA. The 100% values correspond to the amount of DNA synthesized in the absence of melanins under each reaction condition. The solid symbols represent the synthesized melanin and the clear symbols natural eumelanin. The circles, triangles and squares represent data obtained at 72℃, 60℃ and 37℃, respectively.

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PCR inhibitor: water-soluble melanin, which inhibits DNA polymerases and DNases

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With the development of methods in current molecular biology, individual identification and paternity testing have been carried out by typing DNA polymorphisms. Analytical methods are divided into two groups. One group includes Southern blot hybridization which detects RFLPs, VNTR polymorphisms and DNA-fingerprint. The other group includes PCR amplification of a polymorphic DNA region, followed by typing the amplified DNA by probe hybridization, RFLP analysis, VNTR detection or sequence determination. In cases when the materials contain only highly degraded DNAs and/or a trace amount of DNA, DNA typing with PCR amplification have provided good resolution. As indicated by various investigations, regions effectively analyzed are HLA-class Ⅱ genes, several short VNTR regions and noncoding D-loop region of mitochondrial DNA (mtDNA). Among them, the mtDNA D-loop region is the most sensitive, because the region can be amplified very efficiently because of numerous copies of mtDNA more than several hundreds per cell.

A PCR system was used to amplify the noncoding 333-bp region of mtDNA (mt333DNA) from single human hairs, and the following results were obtained: 1) Using natural black hairs in the length over 30 cm, mt333 DNA was always amplified from a 5-cm length of hair shaft sampled within a region 11 cm from the hair root, but it was not always amplified from a 5-cm region adjacent to this 11-cm region, and was not amplified in almost all cases when a 5-cm length of hair shaft was sampled from a region more than 16 cm distant from the hair root (Table 1). DNA preparations not responding to PCR were colored dark brown. 2) Using natural white hairs, mt333DNA was amplified from almost all specimens even up to a length of 31 cm (Table 1). 3) When natural black hairs were stained with an oxidation-type hair-staining agent (Bigen-5Ge), mt333DNA could not be amplified always even from the hair root portion, whereas the same treatment of white hairs gave no effect on the amplification (Table 2). In the cases showing no response on PCR, DNA preparations were also colored dark brown. 4) These dark brown DNA preparations inhibited completely the amplification of mt333DNA even after addition of purified DNAs. These results suggest that the dark brown substance in the DNA preparations inhibits the PCR. We therefore investigated the mechanism responsible for the development of this inhibitor. It was found that hydrogen peroxide (a component of hair-staining agent) produced water-soluble melanins from insoluble melanins. Consequently, it is expected that the water-soluble melanins, which occurs spontaneously and gradually in black hairs, may be provoked by oxidation with air.

We confirmed that water-soluble melanins act as an inhibitor

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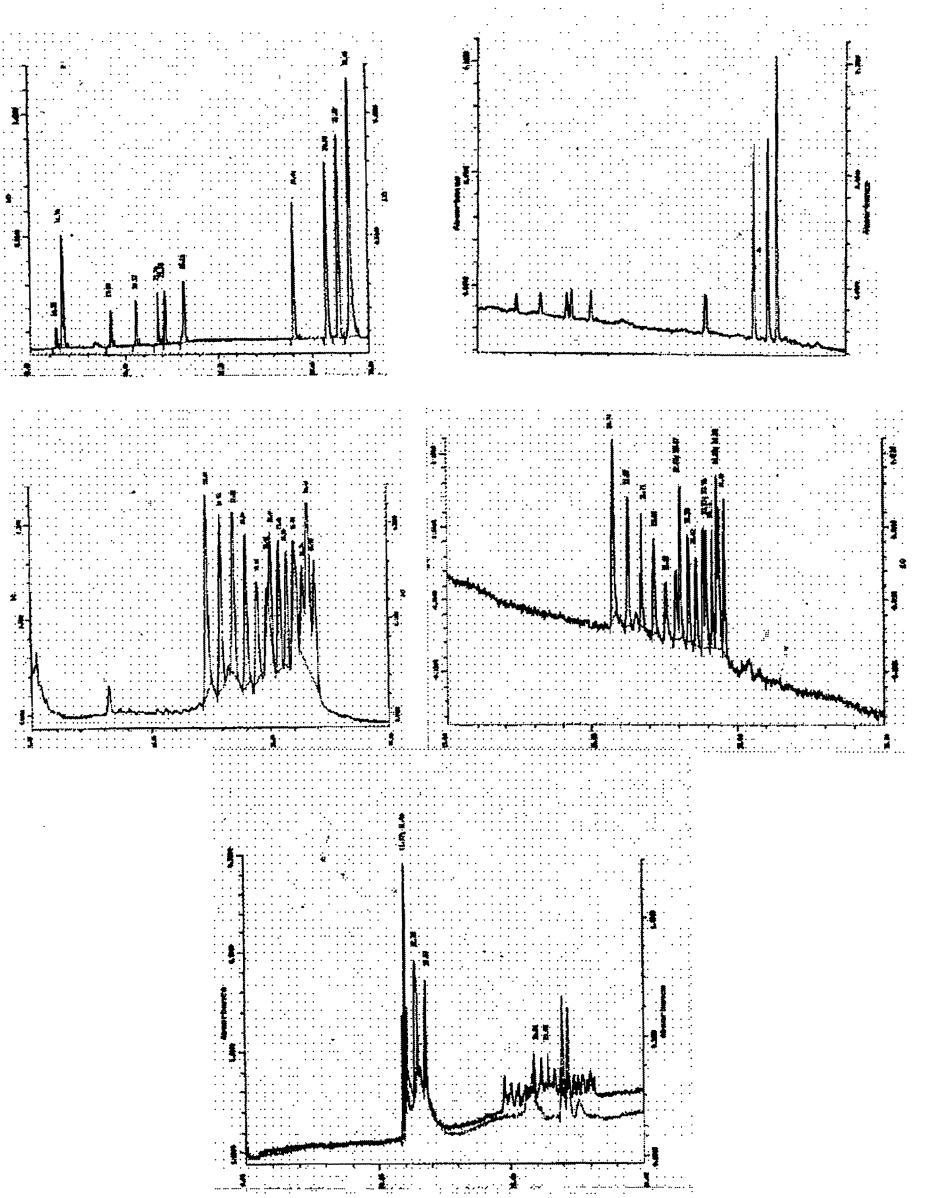

(1) PhiX/Hae III Marker , 5 µl injected at a concetration of 100 µg/ml , UV detection
(2) PhiX/Hae III Marker , 5 µl injected at a concetration of 100 ng/ml , Laser detection
(3) D1S80 Ladder, 5 µl injected , Laser detection
(4) D1S80 Ladder, 5µl injected , after a dluition of 1 to 10 , laser detection
(5) 29/32 D1S80 genotype (dotted) . 5 µl injected after a diluition of 1 to 10, superimposed to the allelic ladder.Laser detection

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Results and Discussion

It is apparent the sensitivity of the method which allows to detect bands of 10 ng/ml of DNA and the reproducibility of the analysis which permits to run not only mix between two different samples in order to show the identity of two alleles, but to run together sample and ladder eliminating the possibility of misinterpretation linked to the shift in migration in the sample.
Moreover, a standard amplification sample can be utilized to repeat the analysis at least 20 or 30 times so to give a direct comparison with other samples everytime.
Limitations are to date, the time consuming analysis for single sample (approximately 30 minutes) and the need to optimize the reproducibility of results during the use of the same capillary.
Better results as specificity and sensitivity can be obtained utilizing fluorescent labelled primers or labelled probes, even if in this case new migration problems will be introduced by the single strand-oligonucleotides hybrid.

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THE USE OF CAPILLARY ELECTROPHORESIS IN FORENSIC DNA ANALYSIS

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INTRODUCTION

Hypervariabilty in sequences of genomic DNA is to date the most reliable way of personal identification from biological samples. Polymerase chain reaction (PCR) solved many of the problems related to the specimen's quantity and degradation, but the accurate evalutation of the dimension of the amplified bands is still a problem. Horizontal polyacrilamide gels give good results with relatively few samples , but this step of identification can be improved .
We tested a system of capillary electrophoresis in order to gain in accuracy of separation and to reduce the quantity of samples required significantly. Electrophoresis was performed utilizing both U.V.and Laser detector with different fluorescent dyes, buffers and viscous media in order to optimize both resolution and sensitivity.

MATERIALS AND METHODS

DNA Extraction and Amplification

DNA was obtained by phenol-chloroform extraction from bloodstains related to criminal cases. The amplification of the D1S80 locus was carried out according to Budowle et al... To better confirm the results an electrophoretical separation of the PCR products was performed using a high resolution horizontal rehydratable polyacrylamide gel and silver staining as described by Budowle et al..

Capillary Electrophoresis

Electrophoresis were performed on Beckman PACE 2100, either equipped with UV or Laser detector . Capillary was Alltech DB17 40 cm x 100 µ and buffer contained 100 mM Tris borate brought to pH 8,7 with CsOH 1% Hydroxyethyl cellulose. When analyzed in UV ethidium bromide 15 µg/ml was added while in Laser ethidium bromide 500ng/ml plus YOPRO 1 (491/509) 13µg/ml were added.
Samples were run at 9 KV 20°C. All amplified samples were purified in microcon filters (30.000 Daltons exclusion) and loaded diluted 1 to 10.

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Table 4. Heterozygosity rate and single and combined mean exclusion chance and DI for 7 PCR systems

	HETEROZY.	MEAN EXCL. CH.	DI
DQα	0.84	0.57	0.09
ApoB	0.80	0.56	0.09
MCT118	0.80	0.69	0.08
YNZ22	0.79	0.69	0.04
COL2A1	0.81	0.58	0.09
HUMTH01	0.76	0.57	0.08
HUMvWA31	0.82	0.61	0.07
COMBINED		0.99	$1.3 \cdot 10^{-8}$

All systems showed a high number of alleles, ranging from 6 for DQα and HUMTH01 to 19 for MCT118. Correspondingly, a high degree of mean exclusion chance and DI was found for all systems, particularly for YNZ22 and HUMvWA31, in which the phenotypes were fairly evenly distributed. The very high combined statistical efficiency for these seven PCR systems makes them a powerful tool in forensics.

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Table 1. DQα allele frequencies

DQα	Allele					
	DQA1*0101	DQA1*0102	DQA1*0103	DQA1*0201	DQA1*0301	DQA1*0501
Freq.	0.164	0.206	0.070	0.16	0.049	0.35

Table 2. Allele frequencies of 4 Amp-FLP's

3'ApoB		MCT118		YNZ22		COL2A1	
Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.
29	-	16	0.017	1	0.062	1A1	0.018
31	0.091	17	-	2	0.208	1	0.079
33	0.061	18	0.225	3	0.153	2	-
35	0.235	19	0.039	4	0.257	3	-
36	0.043	20	0.022	5	0.052	4	-
37	0.409	21	0.034	6	0.032	5	0.248
39	0.052	22	0.045	7	0.01	6	0.262
41	-	23	0.056	8	0.02	7	0.03
43	0.043	24	0.326	9	0.084	8	0.021
45	-	25	0.067	10	0.077	9	0.018
47	0.056	26	0.011	11	0.014	10	0.278
49	0.078	27	0.011	12	0.02	11	-
51	0.087	28	0.045	13	0.01	12	0.032
		29	0.056			13	-
		30	0.006			14	-
		31	0.045			15	0.0139
		32	0.017				
		33	0.056				
		34	0.056				
		35	-				
		36	-				
		37	0.017				
		38	-				
		39	-				
		40	-				

Table 3. Allele frequencies of 2 STR's

HUMTC11		HUMVWA31	
Allele	Frequency	Allele	Frequency
6	0.239	5	0.002
7	0.158	8	0.087
8	0.155	9	0.097
9	0.190	10	0.187
10	0.250	11	0.296
11	0.007	12	0.223
		13	0.085
		14	0.021

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SUITABILITY AND EFFICIENCY OF PCR SYSTEMS IN FORENSICS

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INTRODUCTION

Since the discovery of a high number of VNTR systems (Nakamura et al. 1987) in the non-coding region of DNA and HLA class II sequence polymorphism (Saiki et al. 1986), in the last few years many laboratories have concentrated on the study of these polymorphic systems by means of the polymerase chain reaction (PCR) technique (Saiki et al. 1985).The aims were first to increase the number of polymorphisms investigated and second to demonstrate their power in forensic identification and paternity testing. To the latter end, collaborative research on PCR systems was performed in the laboratories of Ancona and Parma, studying the genetic frequency distribution of several polymorphisms on a sufficient number of Italian subjects. The systems involved in this study were DQα (Saiki et al. 1986), ApoB (Boerwinkle et al. 1989, Ludwig et al. 1989), MCT118 (Kasai et al. 1990), YNZ22 (Horn et al. 1989), COL2A1 (Wu et al. 1990), HUMTH01 (Edwards et al. 1992) and HUMvWA31 (Kimpton et al. 1992). The present work shows the allele frequency distribution of these PCR systems and their efficiency in forensic casework.

MATERIALS AND METHODS

DQα

Two samples of 103 (Ancona) and 40 (Parma) subjects were examined independently by means of the Amplitype Reagent Kit (Cetus) and Dot Blot Hybridization.The two samples showed good agreement (χ^2=3.1534 P=0.6720±0.0148; G statistic=3.2501 P=0.6640±0.0149) when compared for homogeneity, and then pooled as a single Italian population.

ApoB MCT118 YNZ22

115 samples for ApoB, 89 for MCT118 and 202 for YNZ22 were considered. Amplification was according to Boerwinkle et al. (1989) for ApoB, Kasai et al. (1990) for MCT118 and Rand et al. (1992) for YNZ22, with minor modifications. Electrophoretical separation was by means of agarose gel and ethidium bromide staining, allele identification with the local reciprocal method of Elder and Southern (1983) for ApoB and MCT118, and by comparison with a 123 bp BRL ladder for YNZ22.

COL2A1

216 samples were investigated. Amplification was according to Rand et al. (1992) with minor modifications. Electrophoretical separation was by high-resolution PAGE (Budowle et al. 1991) using a discontinuous buffer (Allen et al. 1989), band visualization by silver staining (Budowle et al. 1991), and allele identification by side-to-side comparison with known alleles.

HUMTH01 HUMvWA31

217 samples for HUMTH01 and 211 for HUMvWA31 were considered. Amplification was according to Wiegand et al. (1993a) for HUMTH01 and Wiegand et al. (1993b) for HUMvWA31. Electrophoretical separation was by high-resolution PAGE (Budowle et al. 1991) for HUMTH01 and denaturing PAGE for HUMvWA31 using a discontinuous buffer (Allen et al. 1989), band visualization by silver staining (Budowle et al. 1991), and allele identification by side-to-side comparison with a ladder of known alleles.

The results were analysed to determine the heterozygosity rate. The chance of exclusion was calculated using the equation of Garber and Morris (1983); the discrimination index (DI) according to Sensabaugh (1982).

RESULTS AND DISCUSSION

The allele frequency distributions of DQα, Amp-FLP's and STR's studied in Ancona and Parma are shown in Tables 1-3. Table 4 shows the heterozygosity rate and the single and combined values of mean exclusion chance and discrimination index for all PCR systems investigated.

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ping results from such small amounts of DNA are not necessarily reliable. Dealing with mixed samples in casework, the amplification of different dilutions increases the chances of detection of minor components. Otherwise false results may be caused by excessive DNA (allelic drop out) or too little DNA (unequal distribution of templates). The increased danger of contamination, preferential amplification, allelic drop out and sampling error also concerns other sensitive PCR approaches such as amplifying STR's.

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Results of nested PCR for contaminated stain material

Bloodstains were prepared on one piece of cotton cloth by applying 0,1µl of blood (genotype 39/51) as discrete stains or faint smears and dried. To simulate possible contaminations the cotton cloth was touched (genotype 37/49), coughed on (genotype 31/32) or spitted on (genotype 37/49). For extraction each bloodstain was cut out. The quantitation of the total yields of some extractions gave DNA amounts from 300pg to 20ng/bloodstain. Ten of fourteen bloodstains showed contamination. Sometimes the contamination was only detected by different dilutions of one extraction. It was nescessary to reduce or increase the DNA input to identify the DNA mixtures. After strong contamination with saliva only the contamination could be found and e.g. three different dilutions of one extraction showed three different results: either one or both alleles of the contaminant were detected. Typing the moderately and lightly contaminated material, different genotypes also could be obtained. One extraction showed only the genotype of the contaminant (total DNA amount: 20ng). In four other extractions only the original genotype was found otherwise a combination with one or both alleles of the contaminant was detected.

DISCUSSION

Our results show that, as has been described for other loci (Li et al, 1988), the approach using nested primers for the APO B VNTR increases the sensitivity of the PCR to the single cell level. Working with small forensic samples the higher sensitivity is a big advantage, e.g. we suceeded in typing very degraded DNA from bones that didn't yield signals otherwise. A main problem of nested PCR is the occurence of preferential amplification if overly high total amounts of DNA are used. Probably the second round enhances the amplification of unequal amounts of short and long PCR products that are produced in the first round (Ruano et al, 1991). As a consequence one may get allelic drop out and/or wrong typing results. Exact quantitation of DNA is therefore absolutely necessary to achieve correct typing results.

The correct typing of mixtures is also influenced by preferential amplification, dependent on the respective allele length. In the presence of overly high amounts of DNA even a proportion of 1:1 could not be correctly typed for the longer alleles. The same effect could be demonstrated using 6pg amounts (equivalent to one diploid genome) with the genotype 29/49. Similar results were obtained from the defined numbers of sperm cells.

The high sensitivity causes an increased susceptibility to contamination. Even only superficial contact with bare hands left amplifiable alleles. It was possible to obtain three different results from one piece of evidence: Only the genotype of the blood donor, the genotype of the contaminant, or both genotypes could be detected in separate extractions. The typing of minute quantities of stain material or DNA, as it is possible with nested PCR, is prone to sampling error if working with DNA dilutions from one sample (Navidi et al, 1992). Therefore, DNA-ty-

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Ability to type mixed samples

DNA mixtures in varying degrees of magnitude - see figure 2: DNA of two heterozygous individuals were combined in defined ratios prior to amplification. Genotype 39/51 was always present at higher rate. Genotype 29/49 was added with decreasing amounts down to 6pg.

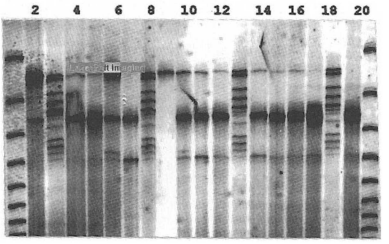

Fig.2 Different mixtures of DNA from two heterozygous individuals
lane 2,4,5:
1ng+1ng;0,1ng;0,01ng
lane 6,7:
0,1ng+0,1ng;0,01ng
lane 9: 6pg;
lane 10-12,14-17,19:
6pg+120;180;240;300;420;600;1200;
1800pg

The DNA typing results for the mixed samples are also influenced by preferential amplification. With a total amount of 2ng of DNA, even with a DNA ratio of 1:1 only the two shorter alleles of each genotype could be detected (lane 2). Using 0,2ng of mixed DNA with a ratio of 1:1, all four alleles were amplified (lane 6). The ability to detect one PCR template with different backgrounds of human DNA also depends on the total amount of DNA in the reaction and on the length of the allele. With a DNA input of 6pg Figure 2 shows how the shorter allele gets fainter with increasing amount of added DNA but still can be detected up to a ratio of 1:200 (lane 9-20). The longer fragment can only be seen up to a ratio of 1:30.

Defined number of sperm cells - see figure 3:

Different numbers of sperm cells (20, 30, 40, 60, 100) from a heterozygous male (Genotype 29/35) were added to single sperm cells that stemmed from a second heterozygous male with a different APO B genotype (37/47). Up to a ratio of 1+40 the allele of the single sperm could be detected. The signal intensity of the PCR products of equal sperm cell numbers showed a high degree of variation, which leads to the conclusion that the true amount of sperm cells in the reaction shows a high fluctuation. Although another cause could be unequal distribution of the two alleles.

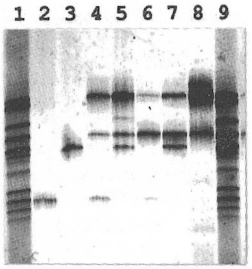

Fig.3 Defined numbers of sperm cells
Mixture of a single sperm and sperms of another genotype
lane 2,3: single sperm
lane 4: 1+20 sperm cells
lane 5: 1+30 sperm cells
lane 6,7: 1+40 sperm cells
lane 8: 1+60 sperm cells
lane 1,9: allelic ladder

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94 °C-1 min., 58 °C-1 min., 72 °C-4 min., 20 cycles
Second round of amplification: (1µl aliquot first round)
as above but 0,5µM each primer (pr.3, pr.4) and 50mM KCl
94 °C-1 min., 58 °C-1 min., 72 °C-4 min., 25 cycles

Primer sequences of the outer set:
Pr. 1: 5′ AACCTCTAGAACACATAGTGTGA 3′
Pr. 2: 5′ CCTCAGGATCAAAGTATGTACAA 3′
Primer sequences of the inner set (Boerwinkle et al, 1989):
Pr. 3: 5′ TGGAAACGGAGAAATTATGGAGG 3′
Pr. 4: 5′ CCTTCTCACTTGGCAAATACAATT 3′

Primer 1 and 2 were located 20bp 5′ to primer 3 and 19bp 3′ to primer 4 respectively and designed based on published sequences (EMBL GenBank, Knott et al, 1986). All amplifications were carried out in a Trio-Thermoblock (Biometra)..

DNA was extracted and quantified following published protocols (Miller et al (1988), Jung et al (1991), Human DNA Quantitation System, Gibco BRL). DNA amplification of DNA from blood and bloodstains was performed as above except the addition of 8µg of BSA to the reaction mix.

RESULTS

Effect of DNA amount on nested PCR - see figure 1 :

DNA of heterozygous individuals was diluted to achieve varied concentrations, which were verified using the dot blot procedure. As shown in figure 1 on the left side, for a DNA sample with 300 bp length difference between the two APO B alleles, a strong tendency towards preferential amplification of the shorter allelic product is noticeable starting at 50pg. For 200pg the longer fragment produces only a faint band in comparison to the shorter allele. Besides, starting from 100pg an increasing amount of DNA causes additional bands, especially outside of the allelic range. Amplifying 6pg of DNA, which corresponds to the size of a diploid genome of a humane cell, at times only one of the two alleles was detected. Because of the random fluctuation of the alleles in DNA dilutions, this was to be expected.

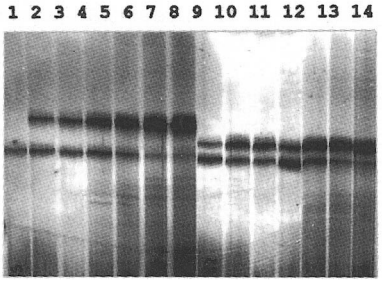

Fig. 1 Effect of DNA amount on nested PCR
lane 1-7, 8 -14:
6, 12.5, 25, 50, 100, 200 and 400pg DNA of different genotype

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ADVANTAGES AND DISADVANTAGES OF THE APPLICATION OF NESTED PCR FOR TYPING OF FORENSIC SAMPLES

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INTRODUCTION

Amplifying the VNTR 3' from APO B using standard PCR procedures with unnested amplification the sensitivity lies between 100-400 pg of genomic DNA. Employing nested PCR after designing a second primer pair that is external to the regular primers but different from the primers described by Vuorio et al (1990) we could increase the sensitivity to single cell level. Using DNA dilutions, DNA mixtures and artificially produced bloodstains we tested the limitations of this method for forensic application. To achieve defined numbers of DNA templates, sperm cells of two heterozygous individuals were purified and combined by micromanipulation.

MATERIALS AND METHODS

Sperm Isolation:
1. Swim-up
-1ml M2-medium or Hepes/Saline to 0,5ml aliquots of semen
-Centrifugation at 600g for 6 min.
-Supernatant discarded, 1ml medium added to the pellet
-Incubation for 60 min. at 37 °C (migration of spermatozoa)
2. Micromanipulation
Isolation of individual sperm or a defined number of sperms from the supernatant of the swim-up under a Leitz microscope (Leitz, Labovert; Germany) equipped with micromanipulators (Leitz, Germany).
3.Microscopic counting and preparation of different ratios of sperm cells
- Counting of the supernatant of the swim-up in a BÜRKER counting chamber.
- Diluting the supernatant to 100 sperms/µl down to 20 sperms/µl
One single sperm was transferred to a PCR tube by micromanipulation. Different dilutions of sperms made by microscopic counting or micromanipulation were added.

Alkaline sperm lysis (Li et al, 1991) and DNA amplification:
-Lysis of the sample (1-2,5µl) with 2,5µl 400mM KOH/ 100mM DTT in 5µl for 10 min. at 65 °C
-Neutralization with 5µl 900mM Tris-HCL pH 8.3/300mM KCL/200mM HCL
First round of amplification:
2U Taq Pol (Promega), 0,5µM each primer (pr.1,pr.2), 150µM dNTPs, 10mM Tris-HCL pH 8,3, 1,5mM MgCl2, 0,1% Triton X-100

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Failure was more frequent with D1S80 than with DQA1. We never obtained positive results with D1S80 if DQA1 was unsuccessful so, routinely DQA1 is always done first.

C. SEMEN

		% SUCCESS
DQA1	SEMEN	67.0
	VAGINAL CELLS	84.6
D1S80	SEMEN	44.4
	VAGINAL CELLS	75.0

COMMENTS: As with blood stains wet conditions were the main cause of failure, especially in blood stained samples.

Variability of stained material was even greater than with blood stains, due to incorrect sampling collection and storage conditions previous to reception in the laboratory.

No significant differences in success rate were found between two different extraction techniques: phenol extraction followed by Centricon-100 ultrafiltration or chelating resin extraction.

CASE REPORTS

CASE 1.- A woman was found dead on a bank. She was completely naked. A preservative found near the corpse and blood from the victim were sent to the laboratory.

DNA typing was successfully carried out in semen and in epithelial cells recovered from the outside of the preservative which coincided with blood typing of the victim.

CASE 2.- A man disappeared and his car was found burnt. Minute blood stains were collected from different objects on the outside and in the surroundings of the car: lateral strip, a plastic bottle and a beer can. Blood from the mother and the brother were also sent for identification purposes.

Analysis: ABO, AcP, Gc, PGM1, DQA1, D1S80, DYZ3.

Results:

	ABO	AcP	Gc	PGM1	DQA1	D1S80	DYZ3
Mother	B	BB	1S1F	1+1+	1.1, 4	T29 T31	
Brother	B	BB	1S1F	1+1+	1.2, 4	T29 T29	
Blood stains	B	B	1S1F	-	2, 4	T29 T29	XY

X/Y= 24685

CASE 3.- A sexual assault was reported.

Samples: Blood from the victim, vaginal swabs, pubic hairs. No suspect.

Spermatozoa were found in the the vaginal swab and DNA typed.

Two pubic hairs were identified different from the victim. DNA typing of one of them coincided with semen, the other one was different. It could be concluded that two different individuals were implicated in the sexual assault.

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B.- CRIMINAL ASSAULTS

	1st year	2nd year
N° Cases	52.10%	52.57%
N° Cases with PCR	21.92%	62.50%

PATERNITY CASES

PCR has been applied routinely since September 1991

25.45% were exclusions

N° PAT. EXCLUSION	CONVENTIONAL	DQA1	D1S80
6*	X		
2	X	X	X
3	X	X	
3		X	X

In two cases there was consanguinity between parents

PCR always confirmed inclusions

SL was only irreplaceable in very conflictive cases

PCR TYPING SUCCESS RATES

(per cent of successfully typed samples in cases where PCR technique was applied)

A.- BLOOD SAMPLES

	PATERNITY CASES	FORENSIC CASES
	% SUCCESS	% SUCCESS
AcP	100.00	100.00
Gc	100.00	94.20
PGM	100.00	97.10
DQA1	100.00	92.95
D1S80	100.00	85.92

COMMENTS: Typing failures in forensic blood samples were frequently due to poor conditions of blood taken from corpses. Recently, whenever possible, PCR amplification is achieved in fixed specimens sent for histopathological study. (DNA extraction is carried out according to Gill et al., 1992). Good results were obtained with samples from exhumations made after several weeks; muscle, skin and hair yielded the best results.

B.- BLOOD STAINS

		% FAILURE CAUSE VERSUS TOTAL FAILURE	
	% SUCCESS	INSUFFICIENT AMOUNT OF SAMPLE	UNDETECTED
AcP	42.80	68.30	31.70
Gc	50.00	52.00	48.00
PGM	29.70	58.70	41.20
DQA1	83.83		
D1S80	58.10		

COMMENTS: The success of PCR was mainly related to the correct collection and preservation of samples. Wet conditions were the first cause of failure.

Difficulties were also found with different kinds of supporting materials such as soils and stones, probably due to adsorption mechanisms. Because of the adherence properties of plastic materials, good results were only obtained when blood crust was discarded and the plastic support was used for the analysis.

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USE OF PCR IN FORENSIC CASEWORK IN SOUTHERN SPAIN.

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INTRODUCCION

For the last two years our laboratory has included PCR amplification techniques in forensic casework as a supplement to traditional diagnostic procedures. Up to this moment the following genetic markers have been used: DQA1, D1S80 and when required, DYZ3 for sex determination. In this paper we present some statistical data from our casework which illustrates the development in PCR implementation and strategy in our Institute. A selection of the most representative cases is also reported.

MATERIAL AND METHODS

Our Institute is located in Southern Spain and covers about one third of the Country (128,859 square Km) and the Canary Islands. (Fig 1). Forensic samples are sent to the laboratory from any town or village in the area by different transport means. Facilities of some local Police or Forensic Services are often precarious. Paternity samples: blood samples are usually taken by venipuncture at our laboratory. Occasionally (i.e. from Canary Islands) they are correctly sent according to a preestablished protocol.

DNA extraction: In general extraction protocols have been taken from AmpliType user guide, Version 2 (Cetus Corporation)
Amplification and typing: Perkin Elmer Cetus DNA Thermal Cycler 480
DQA1: Amplitype HLA DQA1 Forensic DNA Amplification and Typing kit (Perkin Elmer). PCR product was detected by hybridisation with allele specific oligonucleotide probes
D1S80: The D1S80 DNA Amplification Reagent Set (Perkin Elmer) was used, PCR product was detected by flat bed SDS-PAGE followed by silver staining (gel: 8-18% SDS PAA; gel buffer: 0.12 M Tris-Acetate pH 6.4; electrode buffers: anode: 0.3 M Tris-Acetate,4 g/L SDS, pH 6.4, cathode: 0.08 M Tris, 0.8 M Tricine, 4 g/L SDS, pH 7.1; Electrophoretic conditions: 400V, 100 mA, 30 W, 50-55 min).
DYZ3: According to Witt and Erickson (1989). PCR product was detected by agarose gel (3% Nusieve GTG + 1% low EEO), stained with Ethidium bromide, using as size marker Hinf I digested φ x 174 (Promega)

STATISTICS IMPLEMENTATION RATE

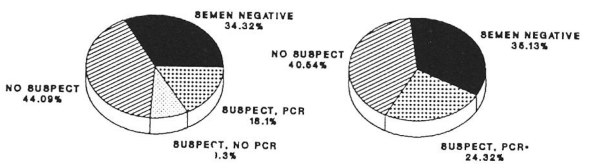
FORENSIC CASES

NUMBER OF CASES WHERE PCR WAS INCLUDED

Global from May 1991 to May 1993	24.4%
1st year	22.54%
2nd year	32.69%

A.- SEXUAL ASSAULTS

N° CASES	47.90%	47.43%



1ST YEAR 2ND YEAR

* in many cases there was no suspect, but some evidence was left (cigarette ends, hairs...)

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As expected the comparison of the amplification results on degraded DNA for Apo B and VWA, showed the lower susceptibility of VWA to degradation. All three individuals were heterozygote for both loci. Their genotypes were VWA (14/16), (14/18), (17/19)
Apo B (31/33), (37/39), (39/51).
There was no influence of the genotype on the VWA typing results. The highest degree of degradation that has been tested here (after 150 minutes of exposure to ultrasonic waves) still contained DNA fragments larger than the longest VWA allele of 167bp. By employing 1ng and 500pg of DNA, reliable results could be obtained. Only with smaller amounts of DNA (for instance 50pg), artefacts as allelic drop out (of the longer and the shorter allele) and additional bands appeared with increasing degradation.

For Apo B, preferential amplification and therefore the genotype of the DNA had a great influence on the PCR results. Both alleles of genotype (31/33) could be detected in DNA fragmented to less than 500bp (60 min degradation time), while the DNA of genotype (39/51) (allele 51 = approx. 900bp) showed strong preferential amplification of the shorter allele (see fig. 4), and, depending on the DNA amount, a single band pattern after 30 seconds to 60 minutes.

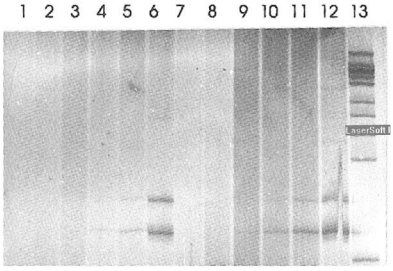

Fig. 4
Amplification test gel for Apo B genotype (39/51)
lane 1-6: 10 ng of DNA
lane 7-12: 100 ng of DNA
150',120',60',4',1',30'' and highmolecular control each
lane 13: 123 bp ladder

Caused by the higher sensitivity, it was possible to somewhat improve the Apo B typing results by employing nested PCR. Probably because the second round of amplification enhances unequal amounts of short and long allele products from the first round (Schmitt et al. subm.), nested PCR is even more susceptible to preferential amplification, so that there was a stronger influence of the allele length. Even closely spaced alleles as (37/39) showed an allele 37 single band pattern for some degradation steps. Increasing the amount of DNA also resulted in an improved allele detection but this was connected to a considerable background smear. Akane et al. (1993) report the separation of high molecular weight DNA from low molecular fragments, which is advisable to solve the background problem.

Our results show that the ability to type degraded DNA is a function of the degree of degradation and the DNA concentration. Since, as we have shown here, the DNA content of a degraded sample cannot reliably be determined, it is therefore, even for a specific polymorphism, not possible to relate a degradation effect, like faint bands or preferential amplification, to the degree of degradation. A more promising approach that has been suggested by Reynolds et al. (1993), is a test amplification of two DNA sequences of different size, which would simultaneously determine the presence of DNA sequences of a specific size range and the amplifiability of the DNA sample.

Because of their short allele lengths the STR polymorphisms will become important tools for analysing degraded DNA. For minute amounts of stain material the requirement of species determination will have to be reconsidered. Even samples that, according to the dot blot results, don't contain any human DNA should be employed for gene amplification, since this result could be caused by degradation.

Acknowledgement: thanks to Carmen Loch for her help with the gels.

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with 400µl of 5% Chelex (Biorad) in aqua bidest. and 5µl (20mg/ml) proteinase K, incubation for 2 h at 56°C, 30 sec vortex, 8 min boiling water bath, 30 sec vortex, and centrifugation at 14.000 xg for 5 min. Parallel samples were extracted using DNA lysis buffer (Gill et al. 1985), proteinase K incubation, phenol/chloroform extraction, and Centricon 100 (Amicon) concentration.

RESULTS AND DISCUSSION

The exposure to ultrasonic waves resulted in severe degradation with no high molecular DNA left after only 30 seconds (fig. 1). As is shown in fig. 2 using the dot blot quantification, the degraded DNA produced only faint signals which completely disappeared for increasing degrees of degradation. The DNA probe contained in the kit (D17Z1) is based on probe p17H8, that has been developed by Waye and Willard (1986) and hybridizes with an α-satellite repeat on chromosome 17 consisting of 500-1000 copies of a 2,7kb EcoRI fragment, that is composed of 171bp repeat units. Considering the short length of the repeat unit and the fact that D17Z1 is an oligonucleotide probe, the system shouldn't be as sensitive to DNA degradation as, according to our results, it is. The effect of the reduced signal intensity is an underestimation of the true DNA concentration. For the PCR this could cause background smear or inhibition if overly high amounts of DNA are used. Definitely, depending on the PCR system, samples that don't show hybridization signals can still be amplified. For all degradation steps, aliquots containing 500pg or 1ng of DNA could not be detected on the dot blot, but showed correct VWA genotypes.

This phenomenon and preferential amplification have also been noted for samples that had been extracted using the Chelex method. Since Chelex extraction yields single stranded DNA, it is not possible to test the molecular weight by yield gel electrophoresis and ethidium-bromid staining. Therefore a yield gel has been blotted and hybridized with probe D17Z1 (see fig. 3). The DNA extracted with phenol/chloroform was of high molecular weight and showed only slight degradation for the 10 week old stains. For both storage times the DNA extracted with Chelex was severely degraded to a fragment size of smaller than 5kb. Singer-Sam et al. (1989) postulated that Chelex as a chelating agent would prevent DNA degradation that could be catalyzed by metal ions during boiling the sample in a low ionic strength solution. Maybe due to the presence of, e.g., high amounts of Fe^{++} ions stemming from the heme components, this could be especially important for the extraction of bloodstains. Chelex extraction of saliva stains yielded high molecular weight DNA (data not shown). Therefore the degradation shown here for 20µl bloodstains may be less for other materials and smaller bloodstains. This will be further investigated.

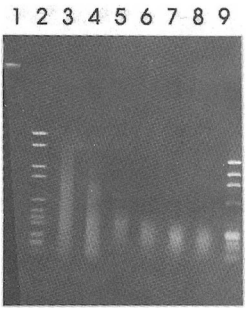

Fig. 1 Degradation test gel
lane 1: high molecular weight control
3 - 9: 30'',1',4',60', 120',150' exposure to ultrasonic waves
lane 2: Boehringer VI
lane 9: PhiX174/HaeIII

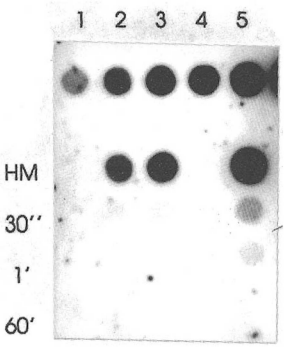

Fig. 2 Dot blot results
lane 1-5: 0.2,0.4,1,2,4ng control, 4,1 and 0.4ng of degraded DNA were spotted

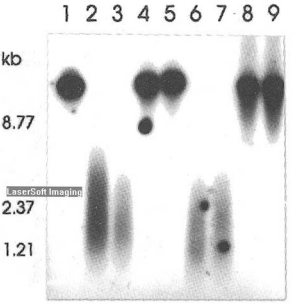

Fig. 3 Yield gel hybridization
lane 1 high molecular reference
lane 2,3,6,7 Chelex extraction
lane 4,5,8,9 phenol/chloroform ex.

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EFFECT OF DEGRADATION ON PCR BASED DNA TYPING

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INTRODUCTION

In almost 50% of all cases, DNA extracted from forensic evidence turns out to be either not enough to be tested for molecular weight or severely degraded (Perlee and Balasz 1993). Using DNA polymorphisms with short alleles and the PCR approach it is possible to achieve results from minimal amounts of DNA and degraded samples. But, as has been described e.g. by Reynolds et al. (1993) for D1S80, the degradation can lead to dubious or even wrong typing results through causing preferential or sole amplification of the smaller allele.

In cases where the degree of degradation cannot be tested beforehand, degradation artefacts will not always be expected and recognized. In order to provide a basis for accurate interpretation of these artefacts using artificially degraded DNA, we have systematically studied the effects of different degrees of degradation on PCR typing results. To get information for different size allele ranges, the results for one AmpFLP system (Apo B, standard and nested) were compared to results for the STR system VWA.

Furthermore, we investigated the effect of DNA degradation on quantification results achieved by the dot blot approach and chemiluminescent probe D17Z1, and tested the molecular weight of DNA from bloodstains after Chelex extraction.

MATERIAL & METHODS

DNA degradation:
DNA of three individuals was diluted to 40µg/ml. The concentration was reexamined, and if necessary, adjusted using the "Human DNA Quantitation System" (Gibco/BRL) according to the manufacturers instructions. Aliquots of the DNA solutions were subjected to ultrasonic waves (Rinco Ultrasonics 9320 Arbon) for different time spans, which resulted in increasing degrees of degradation as is shown in fig. 1. After degradation the aliquots were diluted further and employed for dot blot quantitation and gene amplification.

Amplification:
The two loci chosen for amplification were:
Apo B - VNTR (Boerwinkle et al. 1989)
allele range: 570-900 bp, length of repeat unit 14-16 bp
VWA - STR (Kimpton et al. 1992)
allele range: 135-167 bp, length of repeat unit 4 bp
Apo B was amplified as described elsewhere (Prinz et al. 1993) using the original primers by Boerwinkle et al. (1989). For nested PCR the DNA was first amplified for 20 cycles using external primers by Schmitt et al. (subm.). A second round was performed with 1µl of the first round, the original primers, and 25 cycles. VWA primers were synthesized according to Kimpton et al. (1992), the PCR conditions were as given in Schmitt et al. (subm). Electrophoresis and silver-staining have been modified after Allen et al. (1989).

Chelex extraction:
Bloodstains on cotton cloth (2 days and 10 weeks old) that were equivalent to 20µl of fresh blood were shredded and subjected to Chelex extraction following Singer-Sam et al. (1989),

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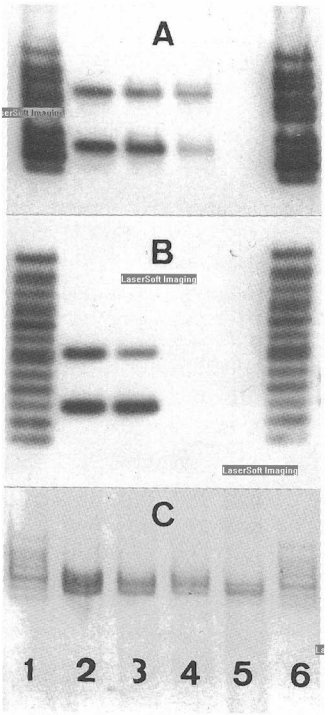

Fig. 2:

Successive amplification of Apo B (A), D1S80 (B) and TC 11 (C) fragments on nylon-fixed DNA: marker consisting of a mixture of different allele fragments (1, 6), control DNA (2), dots with 50 ng (3), 5 ng (4) and 500 pg (5) of the same DNA fixed on a nylon membrane.

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In our experiments Apo B was amplified in a final volume of 50 µl containing 40 pmol of each primer, 200 µM dNTPs, 0,1 % BSA (Pflug et al.), the reaction buffer (10 mM Tris/HCl pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl) and 2 U Taq-Polymerase. 27 cycles with the following steps were carried out: denaturation 90 sec at 94°C, primer annealing 90 sec at 61°C and chain extension 120 sec at 72°C.
Amplification of D1S80 was carried out with the Forensic DNA Amplification Reagent kit from Perkin-Elmer according to the instruction of the manufacturer. TC 11 (HUMTH01) was amplified according to the conditions described for Apo B except the cycling parameters: denaturation 45 sec at 94°C, primer annealing 30 sec at 60°C and chain extension 30 sec at 72°C, 32 cycles.
Electrophoresis of Apo B and D1S80 products was performed on 1,5 % agarose gels with subsequent blotting and hybridization. As probes we used homologous DNA fragments amplified in the presence of Dig-UTP (Boehringer, Mannheim). Detection of the PCR fragments was carried out by chemiluminescence on x-ray films. TC 11 products were separated on polyacrylamide gels and silver stained.

RESULTS AND DISCUSSION

In our tests we started PCR with the AmpFLP system Apo B followed by D1S80 and the STR system TC 11. Changing the succession didn't affect the results significantly. Among the different membranes we tested (Biodyne B, Pall, Nytran 12 and NC, Schleicher u. Schüll) Nytran 12 seems to be superior. If the dots remained in the reaction cap during the whole amplification procedure we normally could detect amplification products only at the highest level of 50 ng of DNA in the case of Apo B and D1S80 and at 50 ng and 5 ng in the case of TC 11. Better results were obtained doing a preamplification of 5 - 11 cycles with 1 : 40 decreased amounts of primers and nucleotides. Then we started a new amplification cycle without the dots but with an aliquot of 5 - 10 µl of the preamplification mixture. Under these conditions we got PCR products even with the 5 ng dots in case of Apo B and even with the 500 pg dots in case of TC 11 as it is shown in Figure 2. The consistently better efficiency of the amplification of the TC 11 system is probably due to its much shorter fragments compared to Apo B and D1S80.
Our results show that successive amplification of three different PCR systems on the same matrix-fixed DNA is possible. This offers the possibility to do several PCR systems even with very small amounts of DNA.

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PCR ON REUSABLE MATRIX-FIXED DNA

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INTRODUCTION

In forensic science case work DNA analysis has become the most powerful method. In the future the use of amplified fragment length polymorphisms like AmpFLPs (Boerwinkle et al., Kasai et al.) and STRs (Edwards et al., Polymeropolous et al.) will be mainly used in forensic stain analysis. But in contrast to the RFLP analysis with its high discriminating power the PCR method needs more than four or five different systems for individualization. In many cases when small stains of blood or other specimen have to be analyzed the amount of isolated DNA is only sufficient for the amplification of one or a few different PCR systems. On the other hand coamplification of several PCR systems (multiplex PCR) often does not lead to satisfactory results because optimal conditions for each system cannot be realized. Therefore we tried a different approach by using matrix-fixed DNA for repeated amplification of different PCR systems.

MATERIALS AND METHODS

Aliquots (1 - 2 μl) containing 50 ng, 5 ng and 500 pg of human DNA respectively were fixed on a immobilizing membrane. After cutting out these dots, remaining free charges were blocked with 1 % blocking solution (Boehringer, Mannheim) for about 1 h. The dots were then used for successive amplification of three PCR systems as shown in Fig. 1.

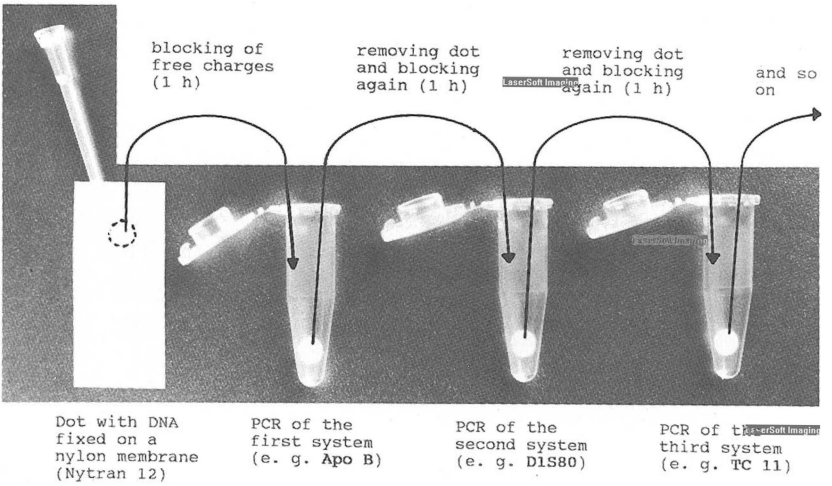

Fig. 1: Principle of repeated PCR with matrix-fixed DNA

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RESULTS AND DISCUSSION

Both the AMGL Y-specific fragment (788 bp) and the AMG X-specific fragment (977 bp) or the DYZ3 Y-specific fragment (170 bp) and the DXZ1 X-specific fragment (130 bp) were detected when male DNA was analyzed, while only the X-specific fragment (AMG or DXZ1) was observed from female samples (See Figure 1).

Both methods were employed in parallel for sex determination in different forensic samples (bloods, bloodstains, single hairs, cigarette butts and paraffin embedded tissues) and were found to be reliables (See table 1 and Fig. 2). However, the DYZ3/DXZ1 PCR-method was found to be a more sensitive method than the AMGL/AMG PCR-method as can be deduced from the rate of success obtained in critical samples (single shed hairs and cigarette butts). On the other hand, the amplification of the DYZ3/DXZ1 region was less susceptible to DNA degradation than the amplification of the amelogenin gen (See Fig. 3).

Table 1. Successful rate in the amplification of AMG/AMGL or DYZ3/DXZ1 from different forensic samples.

	AMGL/AMG			DYZ3/DXZ1		
	N		Successful	N		Successful
SAMPLES	Males	Females	rate (%)	Males	Females	rate (%)
Fresh blood	35	6	100	50	10	100
Bloodstains	29	19	100	25	15	100
Cigarette butts	21	21	47.6	40	28	95.6
Puckled single hairs	16	16	87	16	25	100
Shed single hairs	10	9	0	25	16	73.2
Paraffin embedded tissues	4	3	44.4	5	4	72.7

In conclusion, although both methods are specifics and reliables, it is recommended the use of the DYZ3/DXZ1 method for sex determination in critical and degraded samples.

ACKNOWLEDGEMENTS

The authors are grateful to Angela Zamora for the photographic work.

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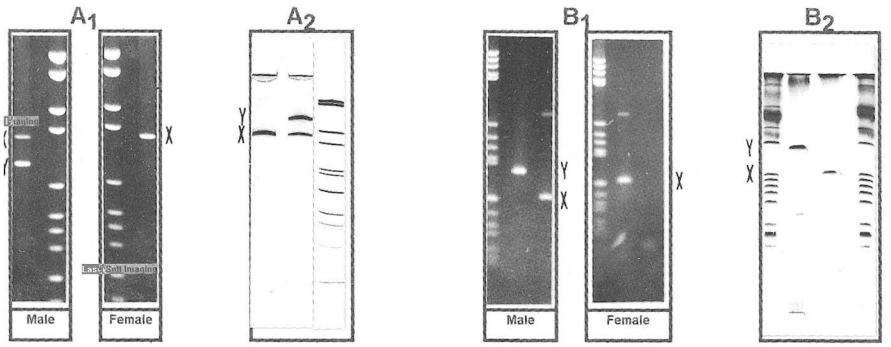

Fig. 1. Specificity of two PCR sex-determination methods. A: Electrophoretic analysis of a 788 bp PCR-amplified fragment (AMG) and a 977 bp PCR-amplified fragment (AMGL). B: Electrophoretic analysis of a 130 bp PCR-amplified fragment (DXZ1) and a 170 bp PCR-amplified fragment (DYZ3). A_1 and B_1 agarose gel electrophoresis and ethidium bromide staining. A_2 and B_2 discontinuous polyacrylamide gel electrophoresis and silver stain.

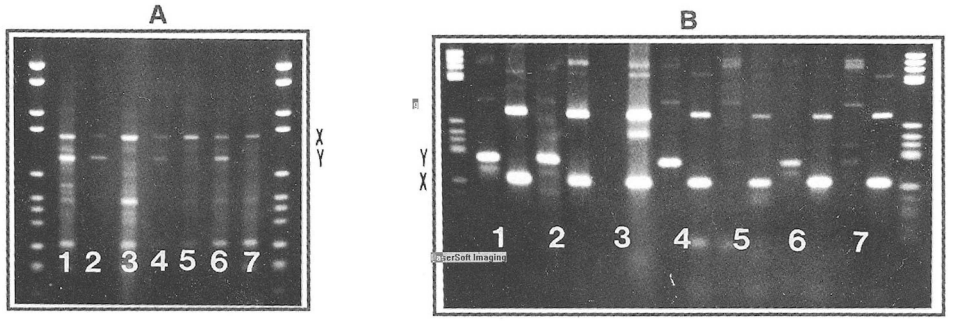

Fig. 2. Analysis of AMG/AMGL (A) and DYZ3/DXZ1 (B) from different forensic samples: 1. Male fresh blood; 2. Male bloodstain; 3. Female bloodstain; 4. Male hair; 5. Female hair; 6. Cigarette butt (smoked by a male); 7. Cigarette butt (smoked by a female).

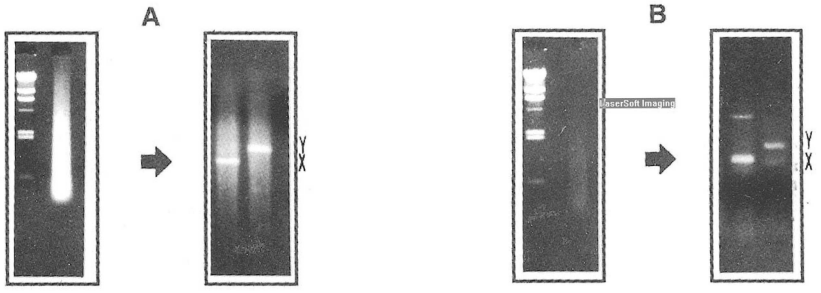

Fig. 3. Analysis of DYZ3/DXZ1 from degraded DNA samples that were unsuccessfull for AMG/AMGL amplification. (A): degraded DNA extracted from a paraffin embedded tissue. (B): degraded DNA from a ten years old bloodstain.

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SEX DETERMINATION IN FORENSIC SAMPLES BY PCR: A COMPARISON OF TWO ALTERNATIVE (AMGL/AMG AND DYZ3/DXZ1) MARKERS.

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INTRODUCTION

Sex determination in forensic samples is of crucial importance in some cases. Recently, two sex-determination methods that exploit the PCR technique has been reported. The first is based on amplification of the repetitive DYZ3/DXZ1 sequences [1] while in the second the simple copy homologous AMGL/AMG genes are amplified [2].
The purpose of this study is to compare the specificity, sensibility and reliability of these two alternative PCR methods for sex determination in forensic samples.

MATERIALS AND METHODS

Samples and DNA extraction

A total of 60 fresh blood samples, 48 bloodstains, 68 cigarette butts, 82 single hairs (41 puckled and 41 shed) and 9 paraffin embedded tissues has been analyzed (Table 1). The methods used for DNA extraction were a follows:

- Standard phenol-chloroform method [3]: bloods and bloodstains.
- Chelex resin extraction approach [4]: bloodstains, single hairs and cigarette butts.
- Phenol-chloroform extraction and ultrafiltration by Centricon-100 concentrators:single hairs, cigarette butts and paraffin embedded tissues.

Amplification and electrophoresis

Amplification of AMGL/AMG [2] and DYZ3/DXZ1 [1] was performed with the followig primers secuences and conditions:

AMGL/AMG	DYZ3/DXZ1
5´-CTG ATG GTT GGC CTC AAG CCT GTG-3' 5´-TAA AGA GAT TCA TTA ACT TGA CTG-3'	X1: 5'-AAT CAT CAA ATG GAG ATT TG-3' X2: 5'-GTT CAG CTC TGT GAG TGA AA-3 Y1: 5'-ATG ATA GAA CGG AAA TAT G-3' Y2: 5'-AGT AGA ATG CAA AGG GCT CC-3'
Preincubation: 94ºC (7 min.)/72ºC (6 min.) Cycles: 30 Temp: 94/55/72ºC Time: 1 2 2 min. Aditional extension: 72ºC (10 min.)	Cycles: 30 Temp: 94/55/72ºC Time: 1 1 2 min.

After amplification the samples were electrophoresed in 2% agarose (NuSieve 3:1) (AMGL/AMG) or 3.5% agarose (NuSieve 3:1) (DYZ3/DXZ1) gel containing ethidium bromide. Alternately amplified samples were separated by semi-dry discontinuous gel electrophoresis (8%T, 3%C for AMGL/AMG and 12%T, 3%C for DYZ3/DXZ1) using the Tris-chloride/Tris-glycine buffer system followed by silver stain [5].

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Amplification.-
- Cycles: Good results are obtained between 30-40 cycles. Below 25, weak or negative results are observed (Fig 2a).
- d-NTPs: Positive results in a range comprised between 10 μM to 200 μM. Higher or lower concentrations lead to negative results (Fig 2b).
- $MgCl_2$: Positive results are observed in a relatively high range (1.5 mM-4 mM). Concentrations of 1 mM or less, gave negative results (Fig 2c).
- Taq DNA Polymerase: Unlike standard higher concentrations, 1 unit of DNA polymerase/50 μl of total amplification mixture is sufficient to achieve reliable reproducible results (Fig 2d). In our experience, many of the commercially available Taq DNA polymerases allow a substantial reduction of the concentration without an appreciable reduction of the yield of amplification.
- Primers: Concentration ranging between 2-0.25 μM displayed efective amplifications. Lower concentrations lead to poor results.

Population and formal genetic studies.- Provisional results in 125 individuals (250 chromosomes) of the Galician population are summarised in Table I. This STR configures thus a value of h=0.655 and PIC=0.605. Genetic studies in 20 family groups show a codominant manner of inheritance for the hTPO alleles without exceptions.

TABLE 1.- Genotype and allele frequencies distribution of the hTPO.

GENOTYPE FREQUENCIES	
GENOTYPE	OBS (EXP)
106bp-114bp	1 (0.54)
114bp	31 (36.45)
114bp-118bp	16 (11.34)
114bp-122bp	9 (10.26)
114bp-126bp	42 (34.56)
114bp-130bp	5 (5.40)
118bp-126bp	5 (5.38)
122bp	1 (0.72)
122bp-126bp	6 (4.86)
122bp-130bp	2 (0.76)
126bp	4 (8.19)
126bp-130bp	3 (2.56)

ALLELE FREQUENCIES
106bp = 0.004
114bp = 0.540
118bp = 0.084
122bp = 0.076
126bp = 0.256
130bp = 0.040

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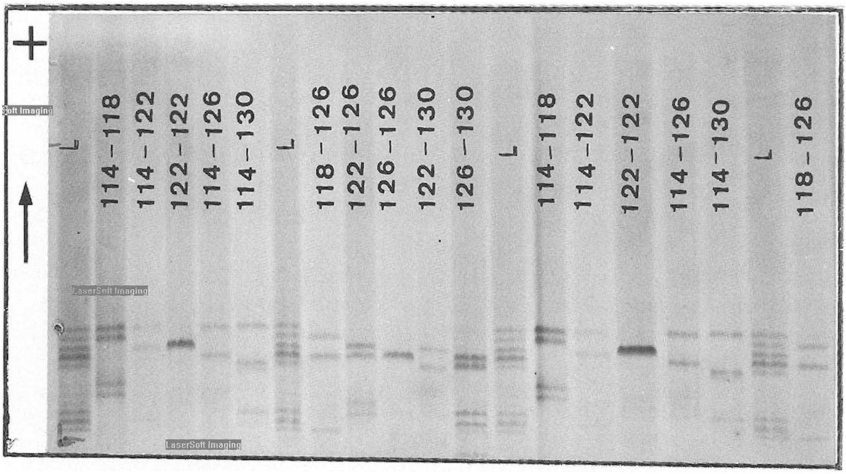

Fig.1.- hTPO phenotypes in semi-dry polyacrylamide gel electrophoresis.

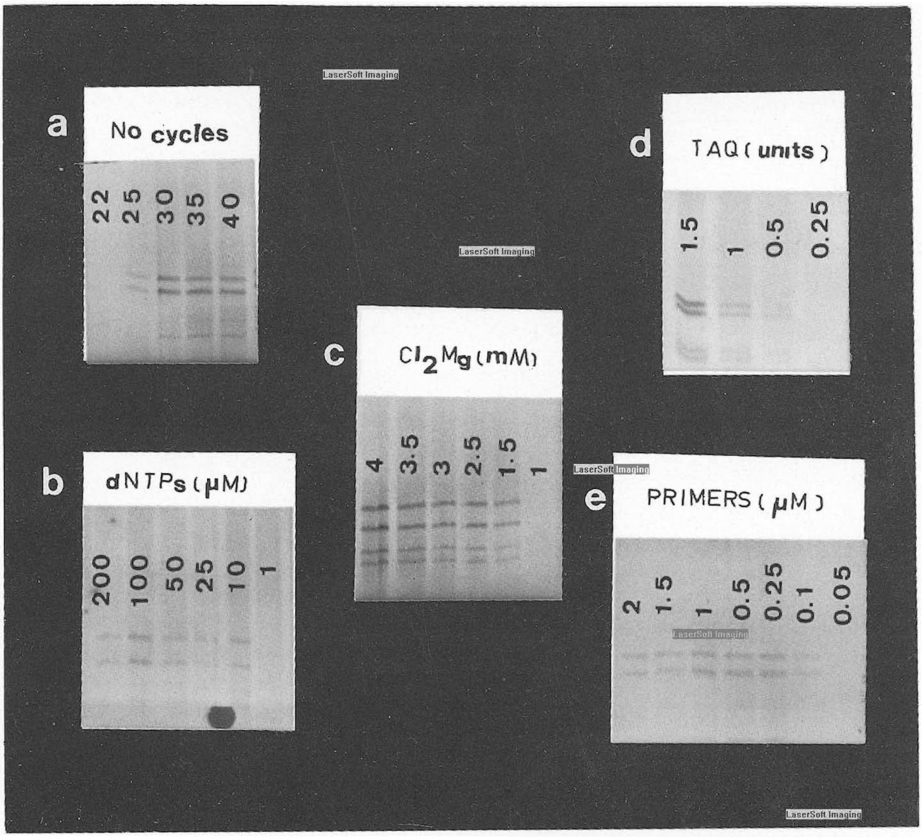

Fig. 2a-2e.- Effectiveness of amplification parameters for hTPO phenotype analysis. Fig.2a: No. cycles. Fig.2b: d-NTPs concentration. Fig.2c: $MgCl_2$ concentration. Fig.2d: Taq Polymerase (Units/25μl). Fig.2e: Primers concentration.

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IMPROVED CONDITIONS FOR GENOTYPE DIAGNOSIS OF A STR OF THE hTPO LOCUS

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INTRODUCTION

The human genome contains numerous multidisperse non-coding regions composed by core units repeated in a variable number of times. The so called Short Tandem Repeat (STRs) correspond to tandem repetitions of only 1-5 nucleotides units [1,2,3]. Given the condition of non-coding DNA, these regions usually exhibit a high degree of variability which confers them an increasing interest in human genetic studies.

This work deals with the analysis of a tetranucleotide repeat (AATG) in the intron 10 of the human thyroid peroxidase gene (hTPO) [4] placed on the chromosome 2p. Thus, in an initial step, a reliable protocol for routine hTPO genotyping is proposed, paying particular attention to PCR-amplification and electrophoretic conditions. Formal and population genetic studies have also be carried out.

MATERIAL AND METHODS

Biological samples were obtained from whole blood from autochthonous individuals from the Galician population (NW Iberian Peninsula). The DNA was extracted by standard methods: (Phenol/chloroform)[5] or chelating resin [6]. The statistical sample consisted of 125 unrelated healthy Galicians, for genotype and gene frequencies analyses, and 20 family groups.

The hTPO amplification took place under the following conditions:

Primer sequences [4]:
AATG strand: 5'CAC TAG CAC CCA GAA CCG TG 3'
TTAC strand: 5'CCT TGT CAG CGT TTA TTT GCC 3'
Temperature profile: 30 cycles
Denaturing: 94°C for 1 min. Annealing: 63°C for 30 s. Extension: 72°C for 90 s.

The amplification mixture consisted of 20-50 ng of DNA, 200 μM dNTPs, 1 μM of both primers, 0.5 units of Taq DNA Polymerase (Promega), $MgCl_2$ 1.5 mM, in 10 mM Tris-HCl, pH 9.0, containing 50 mM KCl and 1% Triton X-100, in a total volumen of 25 μl.

Electrophoretic separation of amplified fragments was made in polyacrylamide gels (T=10, C=5, 0.4 mm thickness and 12 cm long). 0.375 M Tris-HCl pH 8.8 was used as gel buffer and 0.125 M Tris-Glycine pH 8.8 for the bridge. Electrophoresis was carried out at constant 11 V/cm for 4 h, and the detection of the hTPO bands was accomplished by Silver Staining [7].

RESULTS AND DISCUSSION

Electrophoresis.- The electrophoretic patterns of hTPO following the above conditions are displayed in Figure 1. Electrophoresis under non-denaturing and 6% urea denaturing gels lead to an identical interpretation of the hTPO genotypes (data not shown). Bands differing in 4 nucleotides can readily be distinguished. 12 cm long gel plates display sufficient separation between bands. As compared with longer gels (25 cm) a higher separation is obtained but simultaneously lesser definition of the bands is observed . Thus, although running in sequencing gels are not particularly necessary, electrophoretic separations in miniaturized gels are not advisable.

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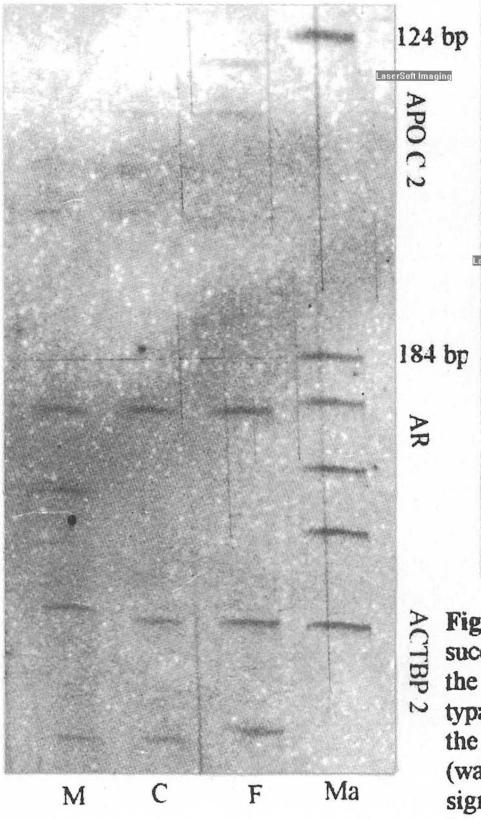

Fig. 1
A mother (M)- child (C)-father (F)-trio
sex of child: male
Ma: marker

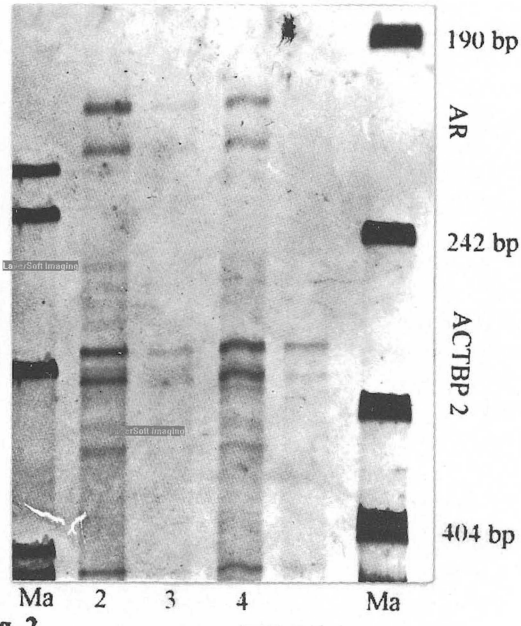

Fig. 2
successful application of random priming: the chelex extract of a cigarette butt (lane 5)was typable after PEP. The extract was prepared from the outer paper of the filter. Two negative controls (water and extract of the filter inlay) gave no signal after treatment with PEP.
lane 2-4: hair, blood and a bone fragment of the victim involved in the same case
Ma: marker
The samples shown here are typed with a duplex PCR (except the PEP product)

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RANDOM PRIMING - PRIMER EXTENSION PREAMPLIFICATION (PEP)

Definition:

PEP is a PCR with an ensemble of 15-mer random oligonucleotides, in which anyone of the four possible bases could be present at each position. The random primers are maximally composed of a mixture of 4^{15} sequences. The probability of amplifying any sequence in the genome to a minimum of 30 copies is not less than 0.78.
After pretreatment with PEP an aliquot of the reaction mixture is used as template in a single locus-specific PCR. A multiplex PCR with the PEP-product is not possible.

Collection of samples, which are typable only after PEP:

- lung tissue, stored one year in non-buffered formalin

- chelex-extract of a cigarette butt (fig. 2)

- diluted DNA-extracts derived from blood and sperm samples (10-20 pg DNA)

- morphologically sorted single sperms

Different extraction-procedures and modified locus-specific PCR without PEP do not yield PCR products.

PCR-based systems tested after preamplification with random primers:
PCR suceeded: HLA-DQ A1, ACTBP 2, APO C II, CTLA 4, HLA CLASS I fragment (620 bp) [5]
PCR not suceeded: D1 S80, Tri-repeat at the androgen receptor gene

PCR protocol and cycling parameters of the PEP-reaction are described elsewhere [4]

RESULTS and DISCUSSION

Our multiplex PCR has two advantages, which will be helpful in routine caseworks:
1. the discrimination power achieved in one working process is very high (probability, calculated with maximal allele frequencies, is lower than 1 in 10.000)
2. the distinct bands are detected by silver staining; fluorescence- or radioactive labelling is not required
An optimization of the PCR-conditions seems to be much more important for multiplex PCR than for a single one.
Until extensive mutation studies are not available, the system should be used preferably for identity testing. We find one mutation at the androgen receptor gene locus in a panel of 20 mother,child,father-trios.
Our experiences with random priming demonstrate the usefulness in case of otherwise unamplifiable template DNA. "PEP-supported" typing of formalin fixed tissue could be applicated for clinical purposes too. Forensic issues concerning mixed samples may be solved by sorting cells morphologically and use them as substrate in a PEP-reaction followed by some locus-specific PCR.
This procedure allows to generate enough template DNA for several PCR-reactions in case of limited DNA-amount (< 1 ng DNA).
An amplification of PCR-products up to a size of 620 bp is possible.
Allelic drop out or mutation caused by the PEP-treatment are not observed.

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RANDOM PRIMING AND MULTIPLEX PCR WITH THREE SHORT TANDEM REPEATS IN FORENSIC CASEWORKS

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INTRODUCTION

In our work we present two new tools for forensic caseworks. Firstly a multiplex PCR with three short tandem repeats (STRs): an easy and rapid method for individual identification with high probability. After optimization of the PCR conditions (especially temperature profile and primer concentrations) slippage bands are avoided and thereby the STRs are adapted to forensic demands. The PCR products of the chosen loci (characteristics see table 1) are simultanously detected on a single PAA-gel.
Secondly we demonstrate the usefulness of preamplification with random primers. The procedure, named Primer Extension Preamplification (PEP), was developed by Zhang et al. [4] for preimplantation purposes. Our studies show that PEP is helpful in case of hampered PCR caused by minute amount of template DNA.To our knowledge this is the first report of forensic application of PEP.

MULTIPLEX AMPLIFICATION OF THE STRs (fig. 1)

Table1

Locus	Chromosomal Location	Number of Alleles	Polymorphic Repeat	Range of Allele size
Human beta-actin related pseudogene H-beta-Ac-psi-2 (ACTBP 2, SE 33)	Chromosome 6	26	$[AAAG]_n$	226-330 bp
Androgen receptor gene [2] (AR)	X-Chromosome	11	$[CAG]_n$	195-225 bp
Apolipoprotein C-II gene (Apo C II) [3]	Chromosome 19	15	$[CA]_n$	127-171 bp

CRITERIA FOR THE SELECTION OF THESE LOCI
- compatibility of the three systems in multiplex PCR
- high polymorphism
- balanced distribution
- non overlapping allele sizes

PCR-protocol:
1-10 ng template DNA, 200 µM each nucleotide, 0.2 µM each primer- APO C II, 0.4 µM each primer-Androgen receptor gene, 1 µM each primer-ACTBP 2, 2.5 µl 10x buffer (Boehringer) diluted to a total volume of 23 µl with distilled water.
5 min Hot Start at 94 °C, 0.8 U Taq polymerase (Boehringer), diluted in 2 µl water, were added after the Hot Start denaturation (at 80 °C).
Cycling parameters:
denaturation 1 min 94 °C, annealing 1.5 min 64 °C, extension 1.5 min 72 °C, 33 cycles final extension 10 min 72 °C

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Alkaline Transfer and hybridization: Alkaline transfers were performed by rocking the gels in 0.4 M NaOH, 0.6 M NaCl for 15 minutes. The gels were then placed in a shallow dish and overlaid with A Nytran® MS membrane wetted in transfer buffer. Two sheets of wet GB002 filter paper were then placed on top of the membrane followed by 5 sheets of dry GB002. The entire sandwich was then covered with plastic wrap. Experiments have shown that transfer of fragments less than 600 bp long is virtually complete in 1 hour. Following transfer the membrane was fixed by UV irradiation and then washed with 5XSSC. Biotin labeled, exonuclease III treated pMCT118 was added to membranes at 125 ng/ml in a hybridization solution consisting of 5XSSC, 50% formamide, 5% casein and 1.0% SDS. Hybridization was allowed to proceed overnight at 42°C. Following stringency washings hybridization was detected by reacting the membrane with 60 ng/ml alkaline phosphatase conjugated streptavidin. The reaction was developed by placing the membrane on a Schleicher & Schuell substrate pad containing Lumi-Phos® 530 and exposing to X-ray film.

RESULTS AND DISCUSSION

The precast horizontal polyacrylamide gel system provides a high resolution, reproducible means of separating DNA fragments from 2000 bp to 50 bp. In the 100-200 bp region we have been able to routinely resolve 2 bp differences. Using 25 cm long gels we have been able to resolve all of the alleles of the D1S80 locus. The higher molecular weight alleles from this locus can be resolved by as much as 1-2 mm.

Using a modification of the Schleicher & Schuell Elu-Quik® system we have been able to rapidly isolate DNA from whole blood samples, which reproducibly serves as template for PCR amplification of the D1S80 locus. DNA from whole blood isolated with this procedure, has a 260/280 absorbance ratio of 1.65-1.80 and is obtained in near theoretical yields. Without further purification or precipitation this DNA will support PCR amplification.

We have also demonstrated a nonisotopically labeled hybridization system to detect amplified product fractionated on these gels. Alkaline transfer from these horizontal gels to Nytran® nylon membranes can be achieved in as little as one hour. In order to detect PCR amplified product, pMCT118 was rendered partially single stranded using *E. coli* exonuclease III and labeled with a psoralen-biotin derivative. Following hybridization the blots are reacted with a streptavidin alkaline phosphatase conjugate and the enzyme reaction detected using Lumi-Phos® 530 as substrate.

In conclusion we have described a sensitive, rapid system for the purification, separation and detection of PCR amplified products starting with whole blood samples. This system has been applied to genetic identification using the D1S80 locus as a model.

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Using a modified rapid alkaline transfer system, transfer of PCR products was complete in less than 1 hr. Transferred products were hybridized to pMCT118 labeled with a psoralen-biotin derivative and detected with alkaline phosphatase conjugated streptavidin employing Lumi-Phos® 530 as substrate.

METHODS

DNA Purification: DNA was purified from fresh whole blood samples utilizing a modification of Schleicher & Schuell's Elu-Quik® system. Blood from finger pricks was collected into an EDTA containing blood collection tube. One hundred microliters of blood was transferred to an Eppendorf tube containing 60 mg of guanidine thiocyanate and followed by the addition of 6 mg of Elu-Quik® glass particles. The tube was mixed and incubated for 5 minutes at room temperature and then spun for 30 seconds in a microfuge at 10,500 RPM. The pellet was washed 2 times with 1 ml of 3.3 M guanidine thiocyanate and 2 times with 1 ml of 0.3 M NaCl, 1 mM EDTA, 50 mM Tris in 50% ethanol. The pellet was then washed with 1 ml of 70% ethanol. Bound DNA was eluted by dispersing the pellet in 100 µl of H_20 at 68° C for 15 minutes. The glass was pelleted and the DNA containing supernatant removed and used directly as a source of template in the PCR reaction.

PCR Amplification: PCR amplification of the D1S80 locus was carried out using a AmpliFLP® D1S80 PCR Amplification Kit from Perkin Elmer. Briefly ten to one hundred nanograms of isolated DNA was brought to 20 µl with water and added to 10 µl of 5 mM $MgC1_2$ followed by the addition of 20 µl of PCR mixture and the amplification reaction carried out according to the manufactures instructions.

Rehydratable Precast Horizontal Polyacrylamide Gels: 7%T2%C containing polyacrylamide gels were cast onto GelBond® PAG Film. Following polymerization the gels were washed to remove polymerization byproducts and buffer components and then dried. Gels of various lengths were cut and rehydrated by submersing them in either 30 mM Tris Formate or 60 mM Tris Formate pH 8.5-9.0 and rocking the gels for 1 hr. Following rehydration 1-3 µl of sample was loaded onto a 4 mm x 4 mm sample application tab placed on the surface of the gel near the cathode. Paper wicks were soaked in 1.5 M Tris Serine pH 8.5-9.0 containing 0.004% bromophenol blue to serve as a tracking dye. The gel was placed in an ICE isoelectric focusing unit (EC Apparatus) and the temperature maintained at 17°C throughout the run. In most cases 300 volts was applied to the gels until the tracking dye stacked and then the voltage was increased to 600 volts and held constant for the remainder of the run. Electrophoresis was stopped when the dye front reached the anodal wick.

Silver Staining: Following electrophoresis the gels were fixed in 200 ml of 2% nitric acid for 10 minutes. The gels were then stained using a modified silver staining procedure.

Labeling of pMCT118: pMCT118 plasmid was isolated from *E. coli* using Schleicher & Schuell's Elu-Quik® plasmid isolation system. Isolated plasmid was digested with Hind III and purified by phenol chloroform extraction. Linear plasmid was digested with *E. coli* exonuclease III for 30 minutes at 37°C. The reaction was stopped by heating at 70°C for 15 minutes. Plasmid treated in this way contained about 25% double strandedness.

Five micrograms of exonuclease III treated pMCT118 was mixed with 1.4 µg of psoralen-biotin and intercalation allowed to proceed for 15 minutes at room temperature in the dark. The reaction mixture was then illuminated for 1 hour at room temperature with a 365 nm source lamp (EA-180, Spectronics, Inc.). Following irradiation unreacted psoralen-biotin was extracted with n-butanol.

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PCR AMPLIFICATION OF THE D1S80 LOCUS: ANALYSIS USING REHYDRATABLE HORIZONTAL POLYACRYLAMIDE GELS.

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ABSTRACT

Much of the human genome is composed of repeated DNA segments that display a high degree of polymorphism. In many cases, this DNA is composed of short repeated sequences referred to as variable number tandem repeats (VNTR). Many of these VNTR loci offer sufficient polymorphism to be suitable for genetic characterization. One such locus, D1S80, has proven useful for parental testing and forensic identification. In order to determine the particular D1S80 alleles present in a given sample, the DNA must be at least partially purified, subjected to PCR amplification and the amplified allele accurately identified by electrophoresis separation and/or hybridization.

We have investigated the isolation of DNA from whole blood samples for PCR amplification of the D1S80 locus. Identification of the amplified allele has been accomplished by employing a high resolution ultrathin rehydratable polyacrylamide gel system (Allen et al, 1989, BioTechniques 7:736-744). These gels utilize a discontinuous buffer system which offers fractionation of DNA fragments from 4kb to 50bp and yields extremely high resolution (2-4bp in the 200bp range). Total run time for these gels is about 1 hour and detection of as little as 300 picograms of DNA can be accomplished using silver staining. Southern blots from these gels can be performed in as little as 1-2 hours, and identification of alleles carried out by probing with nonisotopically labeled pMCT 118.

INTRODUCTION

Analysis of polymorphic loci by amplification using the polymerase chain reaction (PCR) has become an increasingly important method for genetic characterization. In order to reproducibly employ and successfully analyze the products of this technique requires a reliable means of DNA purification, often from difficult samples, a reproducible high resolution electrophoresis system and a sensitive detection method.

We have used a simple purification method for the isolation of DNA from whole blood samples using a modification of our Elu-Quik® procedure. This DNA was then used as template for PCR amplification of the D1S80 locus. Separation of the PCR amplified products has been performed on a precast rehydratable horizontal polyacrylamide gel system (Allen et al, 1989, BioTechniques 7:736-744) employing a discontinuous buffer system. Detection of the amplified products has been accomplished by either silver staining or transferring amplified products to Nytran® membranes followed by hybridization with nonisotopically labeled pMCT118.

DNA isolated from blood in this way proved to be a reproducible source of template for the PCR reaction. Two microliters of PCR reactions was sufficient for analysis using the polyacrylamide gel system. Depending on the length of the gel, analysis could be completed in 1-2 hrs and resolution of all of the D1S80 alleles could be achieved. Silver staining could detect as little as 300 pg of DNA/band.

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distilled water. Ten microliters (5-10 ng) of DNA is added into the PCR reaction.

Currently we utilize one or both of the following multiplex amplification systems on all of our cases. Multiplex 1 (see Figure) is a coamplification of D1S80, SE33 and HUMTHO1. Multiplex 2 (not shown) is a coamplification of D1S80, FGA and HUMP450.

All of the PCR systems have undergone extensive validation that has included: optimization of amplification conditions, preparation of composite amplifiable allelic ladders, construction of population databases (initially n = 400 alleles), mutation studies (to determine a known mutation rate), family studies (to show Mendelian Inheritance) and Southern analysis (to show the PCR product is a faithful representation of the VNTR within the genome. In addition, the DQ alpha, D1S80 and HUMTHO1 loci have been validated for forensic casework according to the TWGDAM guidelines.

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3. Mills et al. (1993). Tetranucleotide repeat polymorphism at the human alpha fibrinogen locus (FGA). Human Mol. Genet. 1:779.

Figure 1.

Multiplex 1, PCR products from parentage trios were electrophoresed on a 8% Tris-sulphate polyacrylamide gel on a vertical format apparatus (BRL SA32). M= mother, C= child, Af= alleged father. Marker= mixed amplified ladder for D1S80 (top system), SE33 (middle system) and HUMTHO1 (bottom system).

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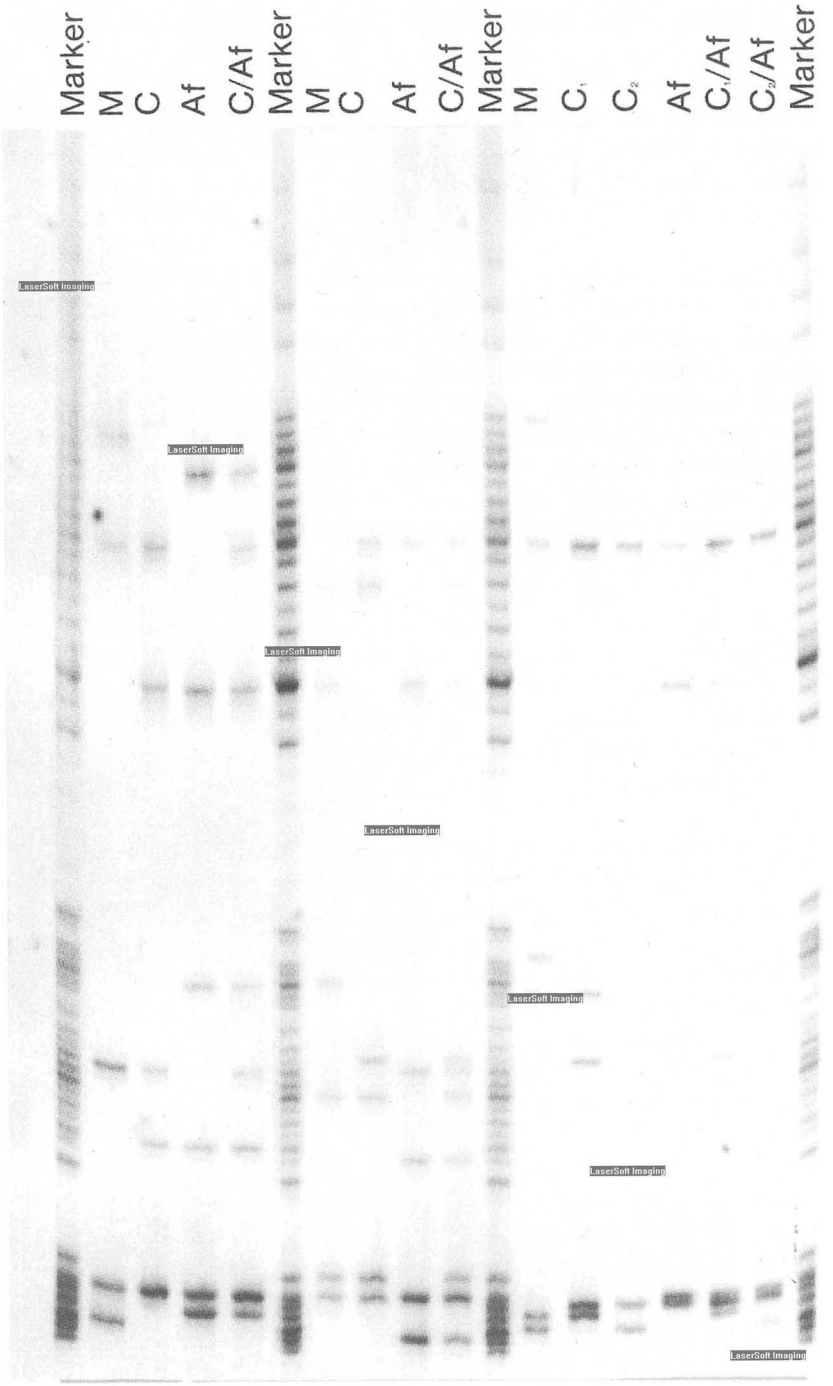
Marker
M
C
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C/Af
Marker
M
C
Af
C/Af
Marker
M
C1
C2
Af
C1/Af
C2/Af
Marker
LaserSoft Imaging

Multiplex 1

PCR-Based Analyses for Identity Testing

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Abstract

Roche Biomedical Laboratories has used PCR based testing for thousands of paternity and identity cases. The loci and amplification conditions for the D1S80, D17S5, APO B, HUMTHO1[1], SE33[2], DQ alpha, HUMFABP[1], FGA[3] and HUMP450[1] have all been validated and optimized. Validation experiments have included identification of alleles, production of allelic ladders, development of databases for the caucasian, black and hispanic races, studies to determine the mutation frequency, family studies to demonstrate Mendelian inheritance and Southern analysis to confirm that the PCR product is a faithful representation of the polymorphism within the genome. Those loci which are used for forensic casework have had additional validation studies performed as described in the TWGDAM guidelines. The loci D1S80/SE33/HUMTHO1 and D1S80/FGA/HUMP450 have been multiplexed and are successfully being coamplified for paternity casework. We have developed conditions for the isolation of DNA from a variety of specimens including bone, hair, paraffin-embedded tissue, fixed and stained tissues on slides and urine. DNA from all of the various sample types has been successfully amplified for the loci listed above.

Roche Biomedical Laboratories has utilized PCR-based analyses in our parentage testing laboratory for two years. It is currently run on every case received for testing. This corresponds to the amplification and analysis of approximately 3000 specimens a week.

All liquid blood samples are spotted onto S&S 903 paper when they enter the laboratory. A standard size hole punch is removed from the stain card and extracted in lysis buffer containing Proteinase K. This is followed by a single extraction with phenol/chloroform and then ethanol precipitation. The DNA is resuspended in 250 ul sterile

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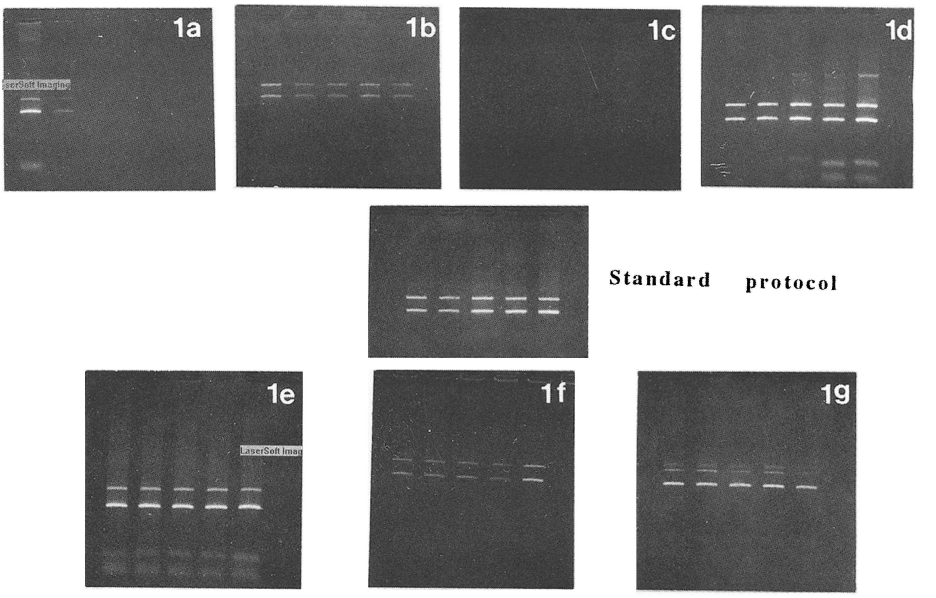


Fig 1. PCR parameters investigated. From left to right : 100, 80, 60, 40 and 20 ng of K562 genomic DNA compared with the standard protocol.

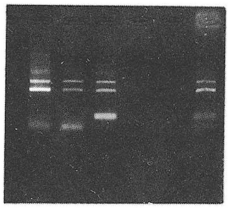
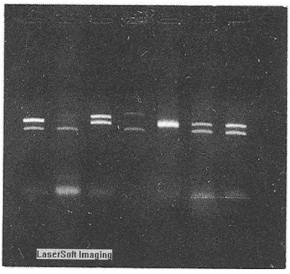


Fig 2. D1S80 amplification with additives. From left to right : 0.2% Triton, 4% DMSO, 4% PEG 8000, 4% Formamide, 0.7% Glycerol, 2% Tween 20.

Fig 3. Air cycler D1S80 products from two sexual assaults and one burglary obtained with the standard protocol.
From left to right : **Rape case** : victim blood, semen stain, suspect blood; **Burglary case** : blood stain; **Rape case** : victim blood, semen stain, suspect blood.



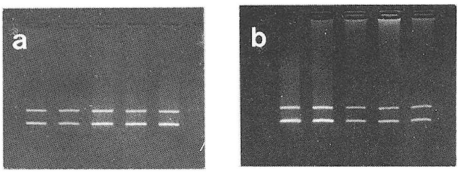


Fig 4. Comparison between the air cycler and the metal block cycler with 100, 80, 60, 40 and 20 ng K562 genomic DNA : a) D1S80 products obtained with the air cycler, b) D1S80 products obtained with the metal block cycler.

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Results and Discussion

The following parameters were investigated with respect to the D1S80 PCR product :

Pre-denaturation
- a brief nucleic acid pre-denaturation lasting 2-5 min at 100 °C was necessary before rapid cycling if a short 10 sec denaturation time at 94 °C was used (Fig 1a).

Number of cycles
- the utilisation of 40 cycles compared to 30 cycles increased the D1S80 product without any problem of non-specificity or background (Fig 1b).

Annealing temperature
- the optimal annealing temperature was found to be about 64-65 °C, while at 60 °C no PCR product was obtained (Fig 1c).

Taq polymerase quantity
- the use of 2U Taq polymerase gave good results while 4U Taq polymerase increased the PCR product but also the non-specificity and the background. This non-specific priming and a primer dimerization especially occurred with 20 to 60 ng K562 template (Fig 1d).

Extension temperature
- the optimal extension temperature was about 77° C while an extension at 70° C resulted in a decreased PCR product of the larger fragment. Furthermore, a slight background and a dimer polymerization were also present (Fig 1e).

Final extension
- the 10 min final extension was necessary to increase the PCR product about twofold (Fig 1f).

Additives
- the use of 1 µL Perfect Match Polymerase Enhancer (BRL) produced a preferential and non-specific amplification with a three bands product (Fig 1g).
- Formamide and Glycerol inhibited the reaction while 0.2% Triton seemed to increase the D1S80 product. Tween 20, DMSO and PEG had little effect on the result (Fig 2).

Air cycler D1S80 amplification of routine cases
Casework examples involving sexual assault and burglary were successfully amplified with the air cycler after Chelex extraction. The denaturation step before the PCR was not necessary since the template was already single strand DNA (Fig 3). The genotypes were easily determined by vertical hydrolink minigel electrophoresis using a glycine/chloride buffer system.

Conclusion

With 40 cycles of 1 min 20 sec using the Idaho air cycler, D1S80 products were increased compared to the products obtained with 30 cycles of 11 min on a metal block cycler. Furthermore, in contrast to the conventional cycler, the air cycler amplification was more efficient with less template : the D1S80 product was higher with 20, 40 and 60 ng compared to 80 and 100 ng K562 DNA (Fig 4).

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RAPID AIR CYCLING OF D1S80 AMPLIFICATION

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Introduction

In order to obtain a suitable PCR product, the DNA amplification method is currently performed over a period of 2 to 6 hours with metal block PCR machines. The length of the procedure is partly due to the time needed to heat or cool the samples.
A new rapid cycling system "Idaho ATC 1605" is now available. It is based on heat transfer by air to samples contained in thin glass capillary tubes. Air is an ideal heat transfer medium and the temperature changes quickly because of its low density. The glass capillary tubes have a better surface-to-volume ratio and the heat conductivity is higher than with polypropylene microfuge tubes. Thus, the total time required for the polymerase chain reaction is significantly decreased (1, 2, 3). Some authors have already used the air cycler to rapidly amplify DNA from E. Coli (4), from Aeromonas Salmonicida (5), the ΔF508 locus in patients with Cystic Fibrosis (6), and mRNA (7).
Results of assays aimed at the optimization of D1S80 amplification using the air cycler are presented here. Different parameters including temperature and time of denaturation, annealing and elongation were tested. Furthermore, the effects of additives such as Perfect Match polymerase enhancer, Triton, DMSO, PEG, Formamide, Glycerol and Tween 20 on the PCR efficiency were also investigated. The application is illustrated through casework examples.

Materials and Methods

PCR reaction parameters

D1S80 amplification of 20, 40, 60, 80 and 100 ng of K562 genomic DNA was performed with the Idaho air cycler. Each 50 µL sample contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatine, 0.4 µM of each primer, 200 µM of each dNTP, 0.016% BSA, and 2U Taq polymerase (Promega). Each sample was placed in 10 cm long, thin-walled capillary tube, the end of which was fused with an oxygen-propane torch.

Time cycling conditions for the metal block cycler (Prem) over 30 cycles were as follows: denaturation 2 min at 94° C, annealing 1 min at 65° C, extension 8 min at 70° C, and final extension 10 min at 70° C.
Time cycling conditions for the air cycler over 40 cycles were as follows : denaturation 10 sec at 94 °C, annealing 10 sec at 65 °C, extension 60 sec at 77 °C, with final extension 10 min at 77 °C.
This standard protocol was used to investigate the effects of certain parameters with respect to the D1S80 PCR product.

PCR parameters investigated

Amplification was performed:
- with and without 2 min template denaturation at 100 °C before PCR
- with 30 or 40 PCR cycles
- with annealing at 60° C or 65 °C
- with 2U or 4 U Taq polymerase (Promega)
- with extension at 72 °C or 77 °C
- with and without final extension for 10 min
- with 1U Perfect Match polymerase enhancer (BRL)
- with 0.2% Triton, 4% DMSO, 4% PEG 8000, 4% Formamide, 8.7 % Glycerol or 2% Tween 20.

Each K562 amplified sample was tested by electrophoresis at 100 V for 45 min on a 2% agarose minigel with ethidium bromide.

Amplification of casework samples

DNA was isolated from blood and semen stains by Chelex extraction after slot-blot quantification : 5-10 ng were used for D1S80 amplification with the air cycler. Five µL of each amplified sample was tested by electrophoresis at 100 V for 45 min on a 2% agarose minigel with ethidium bromide. Genotypes were then determined by vertical hydrolink minigel electrophoresis using a discontinuous glycine/chloride buffer (8).

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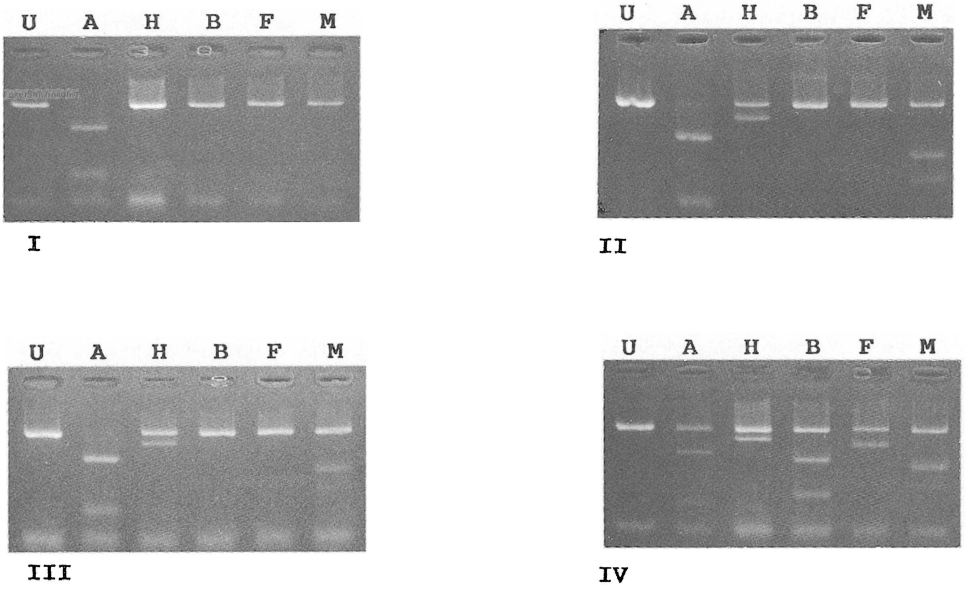

Figure 1. Restriction patterns of the PCR-amplified HLA-DQA1 exon 2 region from persons with the phenotypes HLA-DQA1*0101,0102 (I), HLA-DQA1*0101,0103 (II), HLA-DQA1*0102,0103 (III) and, HLA-DQA1*0103,0401 (IV) when digested with the 5 enzymes ApaLI (A), HphI (H), BsaJI (B), FokI (F), and MboII (M); U denotes undigested DNA. Digestion with these 5 enzymes allowed for the discrimination of all phenotype combinations of the HLA-DQA1 alleles (0101/0102), 0103, 0201, 0301, 0401, 0501, and 0601. Discrimination between the 0101 and 0102 alleles was achieved by the ASA technique.

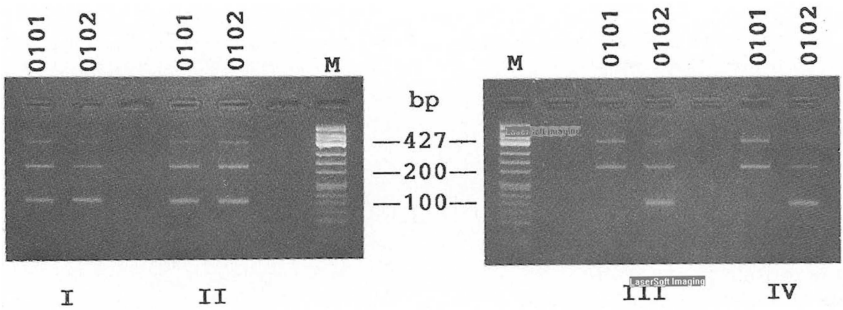

Figure 2. Allele-specific amplification bands from persons with the phenotypes HLA-DQA1*0101,0102 (I), HLA-DQA1*0101,0103 (II), HLA-DQA1*0102,0103 (III), and HLA-DQA1*0103,0401 (IV). The two bands of 441 and 230 bp are internal controls of the reaction which derive from the intron 3 region of the HLA-DQA1 gene and HLA-DQA2 pseudogene, respectively. The 0101/0102 allele sub-type was determined from the appearance of an 101 bp exon 2-specific band in the 0101 and/or 0102 reactions. The 0103 allele was also amplified in the 0102 reaction, but as the existence of this allele is known from the PCR-RFLP data this did not influence the interpretation of the results. The molecular weight marker (M) was ϕX174/HinfI.

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RESULTS AND DISCUSSION

Complete concordance of the typing results was found for all 109 persons tested. Every third person was typed a second time by both methods and again complete concordance was observed, both between the first and second typing by each of the two methods and when comparing the typing results of the two methods to one another. The PCR-RFLP/ASA method was found to be as reliable and easy to handle as the AmpliType kit. As the AmpliType method relies on the principle of hybridisation for the detection of the HLA-DQA1 phenotypes and the PCR-RFLP/ASA method on restriction analysis of the PCR product and selective amplification of a subset of HLA-DQA1 alleles, a higher level of certainty about the typing result is expected if the two methods are used in conjunction compared to using only one of the two typing techniques. The PCR-RFLP/ASA method is well suited for paternity testing but problems may arise if it is used for forensic investigations of mixed samples. In such instances, more than two HLA-DQA1 alleles may be present in the amplification product and the restriction pattern might be too complex for reliable deduction of the HLA-DQA1 types.

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Table 1. PCR primers for allele-specific amplification

Primers	Sequences
DQA1.1-5′	5′-ATGAATTTGATGGAGATGAGG-3′
DQA1.2-5′	5′-ATGAATTTGATGGAGATGAGC-3′
DQA1-3′	5′-CTGCGGGTCAAAACCTCC-3′
DQA5′-int3	5′-AGCCTCTTCTGAAACACTGGG-3′
DQA3′-int3	5′-TCTCTGTGAGCTCTGACATAGG-3′

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Comparison of two methods for HLA-DQA1 typing

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INTRODUCTION

PCR-based typing for the HLA-DQA1 gene is widely used in cases of disputed paternity and for forensic purposes. With the introduction of the Amplitype™ HLA DQα Forensic DNA Amplification and Typing Kit (1,2) this method of HLA-DQA1 typing has been adapted by most forensic laboratories. This analysis relies on the hybridisation of the PCR product to fixed oligonucleotide probes by the reverse dot-blot principle. As is the case for any typing method which relies on hybridisation the outcome of the reverse dot-blot method is sensitive to small changes in the temperatures and salt concentrations of the hybridisation and stringency washing buffers. A second typing method, which relies on an entirely different principle of analysis, may therefore be advantageous and may help to increase the overall level of certainty about the typing result. Here, we present a modification of the PCR-RFLP method published by Ota and coworkers (3) which in combination with allele-specific amplification (ASA) gives even more information about the HLA-DQA1 alleles than the AmpliType kit. The two methods of HLA-DQA1 typing were compared and found equally suited for paternity testing.

MATERIALS AND METHODS

Genomic DNA from 109 healthy Danes was purified as described (4). For the reverse dot-blot method, 50 ng of genomic DNA was amplified and analyzed according to the manufacturers instructions (2). For the PCR-RFLP method, 50 ng of DNA was amplified with the primers GH26 and GH27 (3) in a total volume of 100 µl. The amplification conditions were: 2 min at 94°C, 32 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, and finally 5 min at 72°C. Seven µl of the amplification mixture was digested with each of the enzymes ApaLI, BsaJI, FokI, HphI, and MboII and analyzed on a 4% MetaPhor™ (FMC) agarose gel alongside 7 µl of undigested DNA (fig 1). The HLA-DQA1 phenotypes were assigned as described (3). In order to discern between the HLA-DQA1*0101 and -0102 subtypes, two allele-specific amplification reactions were set up: One which amplify the HLA-DQA1*0101 allele (with the primers DQA1.1-5´ and DQA1-3´), and another which amplify the HLA-DQA1*0102 allele (with the primers DQA1.2-5´ and DQA1-3´). The DNA was amplified in a volume of 100 µl using a hot-start technique. The amplification conditions were as for GH26 and GH27 except for the initial denaturation step being extended from 2 to 4 min. The primers DQA5´-int3 and DQA3´-int3, which amplify intron 3 of the HLA-DQA1 gene, was included as internal controls of the reaction. The amplification product was analyzed on a 4% NuSieve® 3:1 (FMC) gel (fig 2).

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It is notheworthy that the use of the conditions here described are sufficient not only to extract DNA form a single hair shaft (without bulb), but also to permit the extraction process to be repeated from the same sample (Fig.1).

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Fig.1 Amplification of HLA DQα system.
Lane 1: Marker VI Boehringer; lane 2: control DNA; lanes 3, 4 and 5: hairs with bulbs: 2, 3 and 4 hairs respectively; lane 6: hair without bulb; lane 7: repetetive extraction of sample in lane 6.

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HLA-DQα TYPING BY DNA AMPLIFICATION OF SINGLE HUMAN HAIR

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INTRODUCTION

One of the best characterized marker systems in the forensic identification with PCR is the HLA-DQα with the Amplitype™ kit.

The possibility of detecting this system in a single human hair was first described by Higuchi (1). Threafter, a number of Authors have discussed the system's power and methods of extraction (2-6). In fact, the choice of extraction method represents the crucial step in the determination of the efficacy of the amplification, particularly when consisering a single human hair without bulb.

This paper describes the results of a simple and pratical method for the extraction of PCR-amplifiable DNA from human hair for the HLA-DQα gene.

DNA from single human hair, with or without bulb, was extracted by incubation with 0.85% NaCl and Proteinase K and then amplified and typed with the Amplitype™ HLA-DQα kit.

MATERIALS AND METHODS

Sample preperation

The samples were obtained from hairs which had been pulled out directly from the scalp, or had fallen spontaneously. In either case, hairs with and without bulb were present. Samples were kept in nylon bags for up to six months. The amount of sample extracted ranged from a single, 1 cm-long hair to 4 hairs with or without bulb.

Extraction procedure

Each hair sample was first washed in water with soap, rinsed in dH_2O, then cut in small pieces and introduced into an eppendorf tube. Subsequent incubation was with the following buffer:

1 ml 0.85% NaCl +

100 µl buffer K (containing 25 mM $MgCl_2$ 20 mM Tris pH 8.5,0.5% Tween 20)+

10 µl Proteinase K (10mg/ml).

The tubes were then shaken vigorously and allowed to stand in a water bath at 65°C for 3 h.

Following incubation, the supernatant was removed and dispensed into another eppendorf tube, while the pieces of hair were rinsed with 500 µl of 0.85% NaCl before being added to the eppendorf tube containing the supernatant. The solution was centrifuged at 13000 rpm for 15 min., the supernatant discarded, and the eppendorf tube inverted to allow the pellet to dry. 40 µl of dH_2O were subsequently added to the tube.

The contents of the eppendorf tube were then boiled for 10 min. and kept at -20°C until use.

Amplification and typing

30 µl of extracted DNA were amplified by PCR using the Amplitype™ DQα kit (CETUS) according to the recommended protocol. Positive and negative controls were used.

Before typing with reverse dot blot each amplification was checked by employing 10% polyacrylamide gel electrophoresis in 1x TBE buffer.

The amount loaded for each sample was 10 µl of amplified material.

The band of 239/242 amplified fragment was visualized with ethidium bromide.

It was noted that if the band was visible, 35 µl of amplified material, were sufficient to achieve dot blot typing.

RESULTS AND DISCUSSION

DNA was extracted from locks containing up to 4 strands (from 1 to 6 cm), as well as from single shafts with or without roots.

Best results were achieved using by incubating the sample with 0.85% NaCl, buffer K and Proteinase K at 65°C for 3 h.

Each extraction required the final addition of 40 µl of dH_2O. Obviously, under the same conditions more DNA is extracted when the hair root is also avaible, but even a single 1 cm-long hair shaft will provide enough extract for one amplification.

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In the Figures 3a–3d the vWF phenotypes from different biological sources and conditions are presented.

- Bloodstains: vWF genotype was easily identified even from a 1 mm^2 bloodstain kept at room temperature for 5 years. (Fig. 3a). Other stains and enviromental conditions are under study.
- Whole blood: positive identification from 0.01 microliter of whole fresh blood (Fig. 3b).
- Whole saliva: positive results from 1 microliter (Fig. 3c).
- Plucked hairs: positive identification from 1 root, and 3 cm hair shafts (Fig. 3d).

Without doubt the promising possibilities od this DNA marker merit further studies in order to asses its exact usefulness in individual characterization, paternity testing and population profiling.

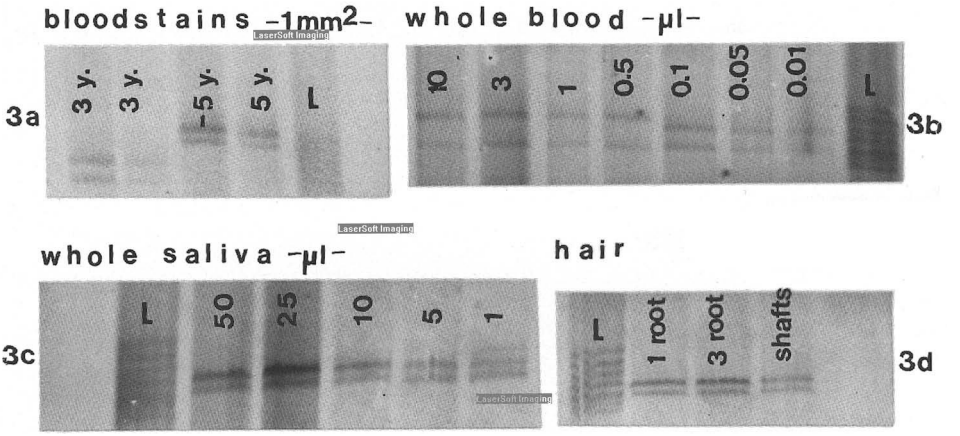

Fig.3a–3d.– vWF phenotypes in minute biological samples.

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ACKNOWLEDGEMENTS

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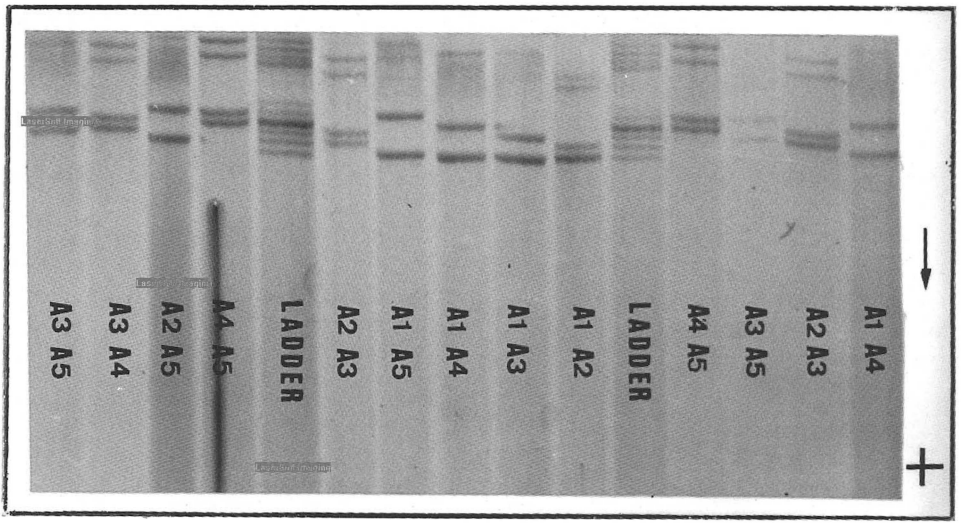

Fig.1.– Electrophoretic patterns of the vWF phenotypes.

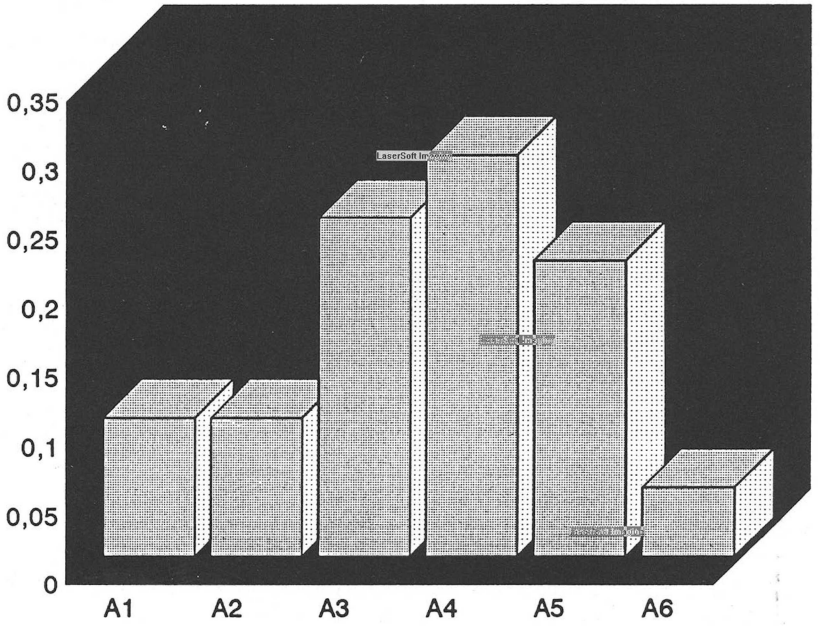

Fig.2.– Allele frequencies distribution at the vWF locus.A1: 0.1, A2: 0.1, A3: 0.245, A4: 0.291, A5: 0.214, A6: 0.05

Family analyses are underway. Preliminary results in 20 groups are in agreement without exception with an autosomal codominant model of inheritance.

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GENETIC STUDIES OF A STR AT THE vWF LOCUS AND ITS APPLICATION TO INDIVIDUALISATION

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INTRODUCTION

The feasibility of DNA analysis from minute and even partially degraded samples, may explain in some extent the increasing interest in PCR technology in the characterization from human biological remains [1,2]. The introduction of the STR ("Short Tandem Repeats") [3,4] is substantially enlarging the battery of possible DNA markers susceptible to application in these types of studies with this finality. This work concerns preliminary formal and population genetic studies on a tetranucleotide repeat in the intron 40 (between nt 1640–1750) in the von Willebrand Factor locus, vWF [5]. Further analyses in human biological remains are carried out with the objective of evaluating the usefulness of this STR in genetic individual profiling.

MATERIALS AND METHODS

The DNA was extracted from 20 family groups, 110 unrelated healthy individuals from Galicia, and in minute biological samples: whole blood, hair (roots and shafts), whole saliva, bloodstains, using standard phenol/chloroform [6] and chelating resins [7] depending on the case.

For vWF PCR–amplification, the primers were synthesised according to [5]:

vWA1: 5´ CCC TAG TGA ATG ATA AGA ATA ATC 3´
vWA2: 5´ GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG 3´

The PCR reaction parameters (total 25 µl) consisted of: 20–50 ng DNA, 200 µM dNTP´s, 0.25 µM each primer, 0.5 units of Taq DNA Polymerase, $MgCl_2$ 1.5 mM, in 10 mM Tris–HCl buffer pH 9, containing Triton X–100 (1%) and KCl 50 mM.

The temperature cycling conditions were as follows: denaturation 45 s, 94°C; annealing 30 s, 50°C; extension 30 s, 72°C; 30 cycles.

Amplified vWF was electrophoresed in horizontal gel electrophoresis in a Tris–HCl/Glycine buffer pH8.8, according to [8] with modifications. Electrophoresis was accomplished in horizontal polyacrylamide plates, at 12.5 V/cm for 3 h, at constant cooling at 6 C, followed by a specific DNA Silver Staining [2].

RESULTS AND DISCUSSION

Polyacrylamide gel electrophoresis, in non–denaturing thin layer gels achieves a reliable separation between the bands of vWF (Fig 1). Unlike other AMPLPs (such as COL2A1) [9], urea/formamide denaturing and non denaturing gels lead to the same genotype diagnosis. The inclusion of composite vWF allelic ladder is of crucial importance for an accurate interpretation. Non–radiactive modalities of detection such as Silver Staining, achieve sufficient sensitivity for a clear visualisation of vWF phenotypes.

An initial population survey in 110 individuals from Galicia gave the allele frequencies displayed in Figure 2. Up to 6 allelic variants have been detected with frequencies ranging between (0.05 and 0.291). The heterozygosity index configures a value of h=0.816 and the PIC was 0.771.

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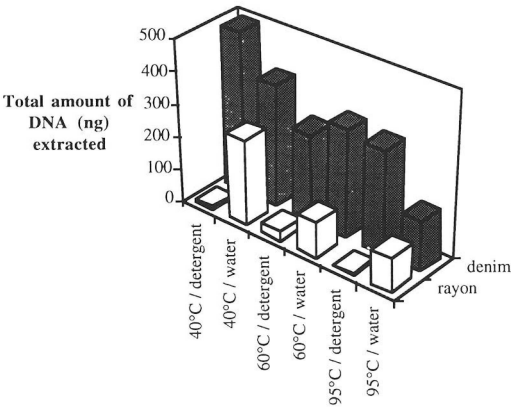

Fig.3 Influence of the use of washing powder on the DNA yield from washed 10µl semen stains on rayon and denim. On denim, the washing with water seems to be curiously more effective than the washing in the presence of detergent.

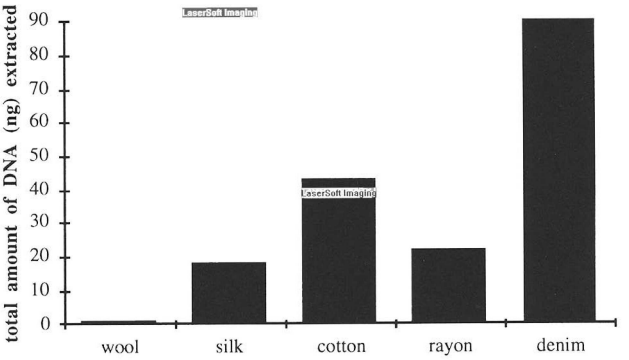

Fig.4 Influence of the support of the stain on the DNA yield from 10µl blood stains washed at 40°C with water.

Washed stains are definitely a useful source of DNA for forensic identification. Even tiny washed stains allow successful PCR DNA typing (data not shown). The main remaining problem consists in the mere detection of the presence of the stains.

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- The yield was inversely proportional to the washing temperature. Several 95°C washed stains did not yield any DNA (Fig.2).
- The use of washing powder substantially reduced the yield, with a few unexplained exceptions (Fig.3).
- The support of the stains is of great importance. The larger yields were obtained with stains on cotton and especially on blue denim (Fig.4).

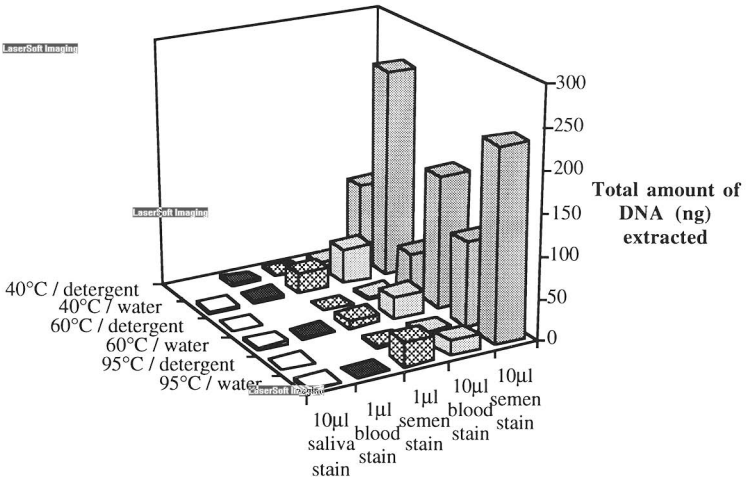

Fig.1 Overview of the DNA yield from washed stains of various types and sizes on cotton

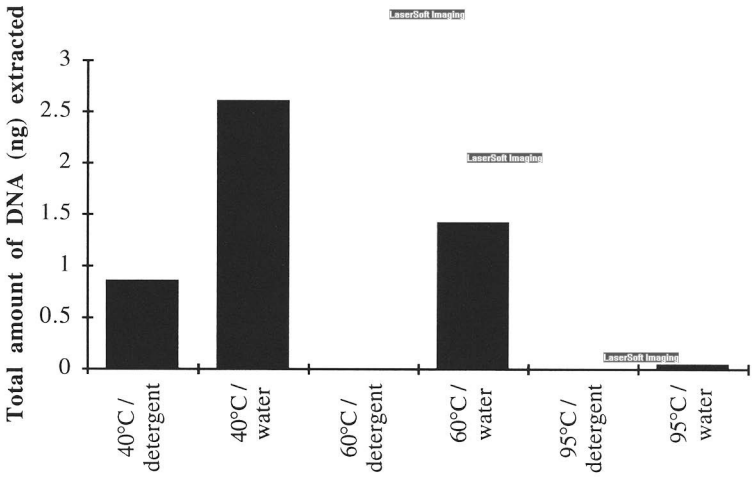

Fig.2 Influence of the washing temperature on the DNA yield from washed 1µl blood stains on cotton

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PCR DNA Typing of Washed Stains

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Introduction

Agressors who get victim's blood on their cloth may try to wash the blood stains, either by superficial cold water rinsing or using a regular washing machine. On the other hand, rape victims sometimes wash their semen-stained underwear to get rid of any contamination related to the offence. In all these situations, it might be hastily concluded that nothing can be recovered from the stains at least if they are no more superficially detectable. In fact, stains submitted to normal washing procedures still contain measurable amounts of DNA. The results mean that the main problem may finally be the simple detection of the presence of the stains.

Material/Methods

Stains : A series of 0.1, 1 and 10µl blood, semen and saliva stains were made onto different substrate materials and allowed to dry. The substrates were:

cotton	- white, 100% cotton
rayon	- pale yellow, 100% rayon
denim	- pale blue, 100% cotton
silk	- orange
"wool"	- green, 50% lambswool, 30% polyacrylic, 20% angora

Washing : Three washing temperatures were used: 40°C, 60°C and 95°C, under two main conditions: washing with water or using a standard biological detergent (Biancomat (Coop Konsumentendienst, Basel, Switzerland) composed of 13% active ionic and non-ionic detergent of which 1.5% is soap; 21% water softener (zeoliths and polycarboxylates); 13% basic components including carbonates, silicates; 16% bleaching agents; 34% neutral salts; 3% specialist substances including enzymes, optical brighteners). All the experiments were made in triplicates and the results present the mean of the three measurements.

DNA extraction : all the stains were extracted using a Chelex extraction protocol [2], amputated of its preliminary water wash step. For semen stains, the extraction was made in the presence of 90mM DTT and 180 µg/ml proteinase K.

DNA quantification : The DNA yield was estimated by slot-blot hybridization using the "Human DNA Quantitation System" (GIBCO-BRL, Gaithersburg, MD, USA), which makes use of the D17Z1 probe [3].

PCR amplification of the D1S80 locus : The D1S80 locus was amplified according to the method of Kasai [1] with minor modifications. The PCR products were analysed on 2% agarose gel followed by staining with ethidium bromide.

Results

Surprisingly large amounts of DNA can be recovered from semen, blood and even saliva stains after washing. For semen stains, the yield is in the nanogram range with stains corresponding to less than 1µl. The yield is about one order of magnitude lower for blood stains and two orders of magnitude lower for saliva stains (Fig.1). The influence of a few parameters on the yield was examined ; their effects correspond to what could be expected:

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of DNA strand, which are different from those of HLA-DQB1. The cloning (TA cloning kit; Invitrogen Corp.USA) of these strands and successive sequencing (Sequenase Version Ⅱ;United States Biochemical Corp.) revealed that they are identical with those of DQB2*1.1,DQB2*1.2 and DQB2*2.1. Fig.3 represents the SSCP images of the DQB2*2.1 allele belong to HLA-DQB2.

It is evident from this result that complete dissolution of DQB1 and DQB2, can be achieved , simultaneously, without any devices.

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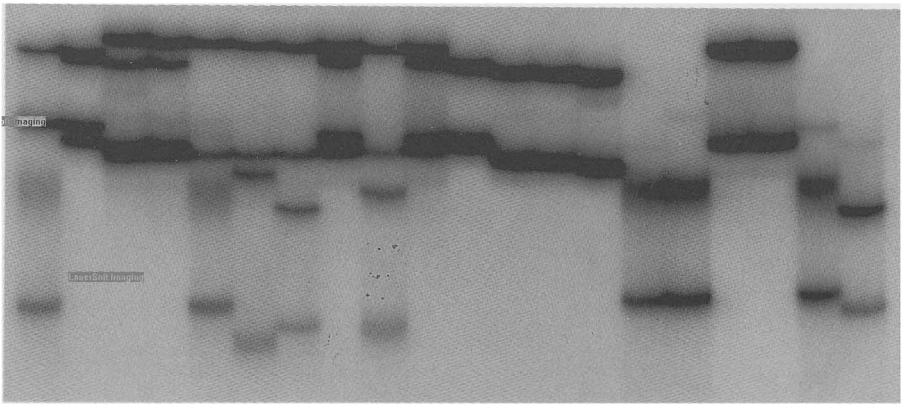

Fig.1. PCR-SSCP analysis of HLA-DQA1 genotypes. Each of the genotypes from left to right;1)0101/0501,2)0101/0102, 3)0103/3011,4)0103/3011,5)3011/0501,6)3011/0601,7)3011/0201,8)3011/0101,9)3011/0401,10)3011/0101.11)0101,12)0102,13)0102,14)0103,15)0501,16)0501,17)3011,18)3011.

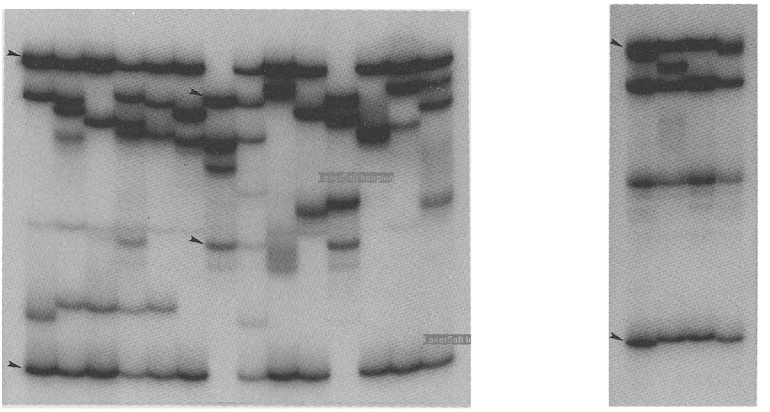

Fig.2.PCR-SSCP analysis of HLA-DQB genotypes using a comformation solution containing 10% glycerol followed by electrophoresis using a gel containing 10% glycerol. An arrowheads are DQB2 of DQB subregion.
Each of the genotypes from left to right;1)B1*0501,B2*1.2. 2)B1*0502/0601,B2*1.2. 3)B1*5031,B2*1.2.4)B1*3032/5031, B2*1.1/1.2. 5)B1*3032/5032,B2*1.2. 6)B1*0601,B2*1.2. 7)B1*0602,B2*1.1. 8)B1*0604/0501,B2*1.1/1.2. 9)B1*0201, B2*1.2. 10)B1*0301,B2*1.2. 11)B1*0302,B2*1.1. 12)B1*3032, B2*1.2. 13)B1*0401,B2*1.2. 14)B1*0402/0301,B2*1.2.
Fig.3. The HLA-DQB2*2.1 genotype of DQB1 subregion. 1) DQB1*3031, DQB2*2.1. 2)DQB1*0301/0402, DQB2*1.2. 3)DQB1*0301, DQB2*1.2. 4)DQB1*0301, DQB2*2.1.

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and 2 units of Taq polymerase(Biotech) and brought to 100 μℓ by adding distilled water. Temperature cycling was carried out in a Program Temperature Control System PC-700(Astec) as follows: 30 cycles of 1 min at 94℃, 1 min at 55 ℃, and 2 min at 72 ℃.

3) The amplified DNA sample after PCR was diluted to the optical concentration (100 ng/μℓ) by adding TE solution using electrophoresis of a 2% agarose gel.

4) Isotope labelling of the amplified DNA for SSCP analysis was performed by mixing 1 μℓ of DNA, 2μℓ of 2 mM dNTPs, 2μℓ of the same primer set for DQA1 and DQB1, 2 μℓ of α^{32}P-dCTP, (9.25 Mbq/ 25 μℓ:Amersham Co., Ltd.,England), 2μℓ of 25 mM $MgCl_2$, 2μℓ of 10X reaction buffer and 1.5units of Taq Polymerase to a final volume of 20μℓ. The PCR was carried out with 15 of the same temperature cycles described the PCR section.

5) The DNA fragments after isotope labelling for SSCP were diluted 1:20 by adding conformation solution containing 10% glycerol, 0.05% bromophenolblue and 0.05% xylenecyanol. The conformation was obtained by heating at 94 ℃ for 2 min, and then placing the mixture on ice.

6) Electrophoresis was performed under the a condition of 12-18 mAmp (1700 V) for 3 hours at 4℃ on a 30X40-cm gel plate (Nihon Eido Co.,Ltd., Japan) containing 7.5% bisacrylamide-0.5XTBE(Tris-borate buffer: 0.04 M boric acid, and 0.002 M EDTA) with glycerol in a final concentration of 10%.

Results and Discussion

From the results, it is possible to make a distinct determination of the HLA-DQA1 and DQB1 types, when the standards including DNA fragments from various common DQA1 and DQB1 alleles are inserted on the same gel plate. Figure 1 is DQA1 genotypes by the SSCP analysis. As shown in Fig.2, it could also be shown arrows in the same figure, that the SSCP bands of amplipfied DNA include another types

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Forensic application of SSCP in the analysis of HLA-DQ, which was amplified by PCR.

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Introduction

Although various method have been developed for the confirmation of various alleles belonging to HLA class II genetic loci, SSO bot hybridization (1), RFLP (2), SSCP (3), SSCP/ARMS (4), some difficults are encountered always in forensic practice. Resently, we have succeeded in establishing method for diagnosis of HLA-DQA1 alleles by appling PCR-amplification and simple SSCP using a covenient primer set, which is recommend by International Histocompatibility Workshop(1991)(5). Based on the experience, we have examined the DNA analysis of alleles belonging to HLA- DQB and found the experimental results give comformity diagnosis of alleles belonging, not only to HLA-DQB1, but also -DQB2. This method can be used for the routine forensic test in determining identical identification.

Materials and methods

The experimental conditions were as follows: 1) A primer set for HLA-DQB1 amplification and isotope labeling (DQAAMP-A:5'-ATGGTGTAAACTTGTACCAGT-3', and DQAAMP-B: 5'-TTGGTAGCAGCGGTAGAGTTG-3') and (DQBAMP-A:5'-CATGTGCTACTTCACCAACGG-3', and DQBAMP-B: 5'-CTGGTAGTTGTGTCTGCACAC-3') recommended by the 11th International Histocompatibility Workshop (1991) was used in this experiment.

2)DNA samples(1μg) were mixed with 10 $\mu\ell$ of the primer set (25 pmol of the respective primers), 2 mM dNTP, 10 $\mu\ell$ of 25 mM $MgCl_2$(15 mM for DQA), 10X PCR reaction buffer (Biotech.International Ltd., Australia),

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Determination of the success rates showed that, with the exception of HumD21S11, an increase in the amount of hair shafts is in general paralleled with an increase in the success rate (Fig. 4). For HumTH01 it increases from only 15% for 1 hair shaft up to approximately 50% for 15 hair shafts. HumVWA and HumMBP have comparable success rates which increased from about 20% for 5 hair shafts up to approximately 40% for 15 hair shafts.

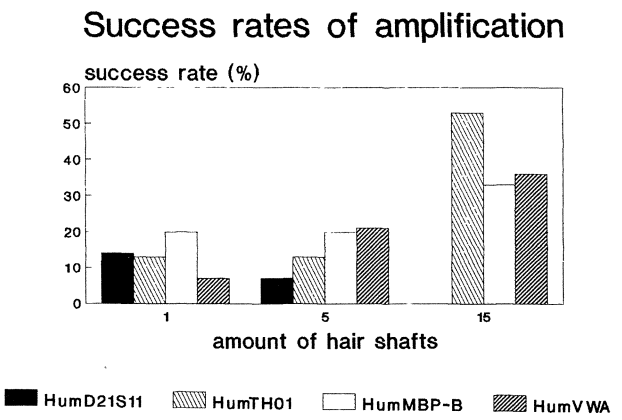

Fig. 4: Success rates of PCR amplification for different amounts of hair shafts using the STR systems HumD21S11, HumTH01, HumMBP-B and HumVWA

Successful DNA typing was dependent on the quantity and quality of hair shafts and the system applied. Due to the low amount of template DNA one has to consider that artefacts such as allelic drop out, additional bands and incorrect amplification patterns could occur. For the future, the aim must be to eliminate these artefacts and to increase the success rate by applying further modifications.

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Results and discussion

Reamplification was the most effective modification for HumD21S11 and HumMBP.
After 31 cycles no PCR product could be obtained for HumMBP with DNA extracted from 5 hair shafts. After reamplification with 10 and 15 cycles DNA fragments within the allelic range could be demonstrated for locus A and locus B (Fig.1). Increasing the cycle number from 10 to 15 produced ladder bands caused by polymerase slippage and/or heteroduplex formation (Fig. 1).

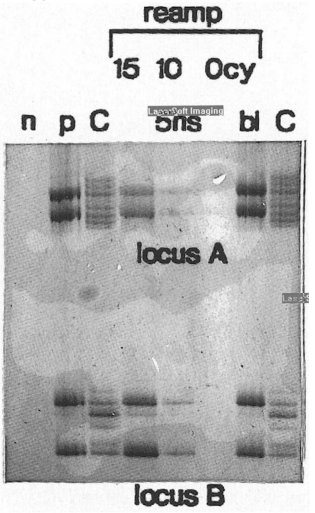

Fig. 1: HumMBP: Amplification patterns of DNA extracted from 5 hair shafts after 31 cycles (0 cycles of reamplification) and after reamplification with 10 and 15 cycles
C = allelic ladder
bl = blood DNA
p = positive control sample
n = negative control sample

An increase from 30 to 32 cycles improved the results for HumVWA. The pattern of 1 and 15 hair shafts matched with the correspondending root pattern whereas 5 hair shafts gave ladder bands (Fig. 2).

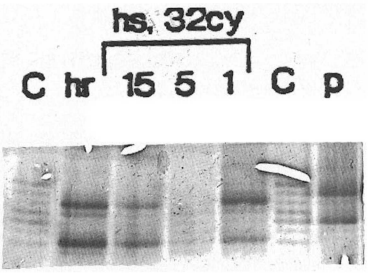

Fig. 2: HumVWA: Amplification patterns of DNA extracted from 1, 5 and 15 hair shafts after 32 cycles
C = allelic ladder
hr = DNA extracted from 1 hair root
p = positive control sample

Using the fluorescence detection system and semi-nested PCR HumTH01 was the most successful STR system and highly sensitive. Clear amplification patterns could be demonstrated with DNA extracted from 1 hair shaft (Fig. 3). The peak heights of blood DNA after standard PCR and 1 hair shaft after semi-nested PCR were similar indicating that the application of semi-nested PCR is a powerful tool to maximize the sensitivity.

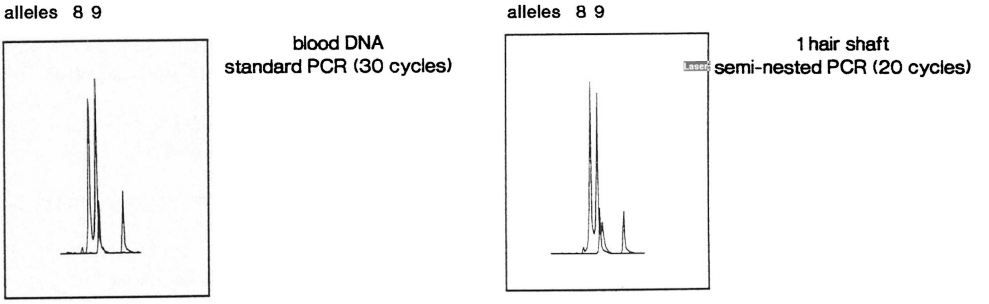

Fig. 3: HumTH01: Amplification patterns of blood DNA after standard PCR (30 cycles) and DNA extracted from 1 hair shaft after semi-nested PCR (20 cycles)

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Extraction and PCR amplification of DNA from hair shafts

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Introduction

The increasing occurrence of single hair shafts without recognizable roots in forensic cases requires the optimization of extraction and amplification parameters. The aim of this study was therefore the determination of the success rate of amplification dependent on the amount of hair shafts and the STR (short tandem repeat) system applied.

Materials and methods

Blood samples were extracted as described previously (Brinkmann et al. 1991). The studies were carried out on freshly plucked hair from the back of the head from 13 different persons. The first 2 mm at the root were removed and the hair shafts divided into 5 cm pieces each. 1, 5 and 15 standard hair shafts were investigated.

Extraction protocol:
Hair shafts were cleaned with 100% ethanol (30 min, rt) followed by treatment with proteinase K (250 ng/µl) and 0,188 M DTT (50°C, overnight), phenol-chloroform extraction and centricon 100 purification.

Amplification protocol:
1. HumD21S11 (Sharma and Litt 1992)
5 µl centricon eluate, 1.5 mM $MgCl_2$, 2 µl 10xPCR buffer (Promega, USA), 1 µM of each primer, 100 µM each dNTP, 1.5 U Taq polymerase (Promega, USA) diluted with water to a final volume of 25 µl.

94°C-15s, 62°C-30s, 72°C-75s; 31 cycles
(Biometra Triothermoblock, Germany)
Reamplification of 1 µl of the PCR assay was carried out for 10 and 15 cycles as described above.

2. For HumVWA (Kimpton et al. 1992) and HumMBP (Polymeropoulos et al. 1992)
PCR amplification was carried out with 5 µl centricon eluate according to a previous publication (Möller et al. 1992, in press) except that the cycle number was increased from 30 to 32 for HumVWA. Reamplification was carried out with 10 and 15 cycles for HumMBP.
The electrophoretical separation of the amplified fragments was carried out according to Möller et al. (1992, in press). Visualization of the bands was performed by silver staining (Budowle et al. 1991).

3.HumTH01 (Edwards et al. 1992)
-first amplification: 5 µl centricon eluate, 1.5 mM $MgCl_2$, 2 µl 10xPCR buffer (Promega, USA), 0.24 µM of each primer (Edwards et al. 1992, primer 1 fluorescence-labeled with FAM), 100 µM each dNTP, 2.5 U Taq polymerase in a final volume of 25 µl.

95°C-45s, 60°C-30s, 72°C-30s, 30 cycles (Perkin Elmer 9600)

Semi-nested PCR was carried out with 20 cycles, 2 µl of the PCR assay and the primers developed by Gill et al. (1992, primer 1 fluorescence-labeled with FAM) using the PCR conditions as described above. PCR fragments were separated on a 6% denaturing gel and analysed on the ABI 373A Sequencer using the Genscan software 672 and the internal standard Genscan 2500 labeled with ROX.

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These data permits us to affirm that the Single Tube extraction together with Hot-Start give us a high-success rate. However, a relatively low percentage (35%) of these amplified DNA is useful for identification, considering the high probability of non-specific dots.
Taking a high number of samples with non-specific results into consideration, we tried to transfer our experience reached on HLA DQα to other more frequently utilized loci in forensic biology (D1S80 and Apo B) to confirm the results obtained.
This was necessary so as to resolve some real cases, where the discrimination capability of HLA was not enough to show what exactly had occurred.
Starting from telogen hair and always using Single Tube protocol extraction together with Hot Start, we obtained a positive typing in each case either for D1S80 locus or for Apo B.
Although our data are not complete, we can certainly declare that an aimed amplification protocol allows us to obtain genotypes useful in identification, even if the evidence consists of only a shed human hair in the telogen phase.

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The following steps of extraction proceeded according to the standard phenol-chloroform extraction protocol and the instructions given by Biotecx Bullettin NO. 8,1990. The method allows to limit the presence of inhibitors mainly represented by digested proteins.

AMPLIFICATION

Amplification and typing of the HLA-DQ alpha locus.

DNA was amplified by PCR using the Amplitype HLA-DQ alfa Forensic DNA Amplification and Typing Kit (Cetus Corporation,Emeryville, CA).

Amplification and typing of the VNTR's loci D1S80 and Apo B

Amplification of the VNTR's loci D1S80 and Apo B were achieved using the methods described previously by Budowle et al. and Boerwinkle et al. respectively.
The electrophoretical separation of the PCR products was performed using a high resolution horizontal rehydratable polyacrylamide gel as described by Budowle et al.. The gel was exposed through silver stain.

RESULTS AND DISCUSSION

Our experiment starts from some data taken from researches which investigate the possibility of extracting DNA from a telogen hair root. In particular, the Criminal Laboratory Department of Illinois presented a very interesting case history from Chelex extracts.
Considering the very good results obtained through Single Tube extraction on various types of biological traces, our aim was to test its effectiveness from telogen hair.
In the last six months, our lab has analyzed about 100 telogen hair, always using a Single Tube extraction.
The first twenty attempts to amplify the HLA-DQα locus, according to the P.E. protocol, either produced negative results or they were slightly visible dots. Then, we tried to re-amplify the DNA amplification fragments with a yield of two amplified products out of ten.
Both, however, showed weak non-specific dots after the hybridization.
Further experimental tests showed that this method was not the correct one, since the probability of obtaining not specific products proved to be quite high, especially if during the first amplification the maximum extract allowed was loaded.
Changing both the quantity of amplified DNA to be loaded on the reamplification and the number of cycles of re-amplification, no considerable improvements were obtained.
We tried to improve the results by adding BSA to the amplification mix.
The addition of BSA quantities equal to eight or sixteen micrograms in 50 microliters of final volume of mix did not produce satisfactory results (8 tests were performed). Moreover, if the amplification was performed without BSA simultaneously, the amplified DNA was undoubtedly better.
We obtained a higher quality in the typing of the HLA-DQα locus for hair in the telogen phase, when we carried out a Hot-Start on the amplification mix (25 µl mix, 25 µl $MgCl_2$, 20 µl of DNA extract, 95°C for 3 min., 2.5 U Taq Polymerase, amplification according to P.E. protocol).
From the 63 Single Tube extractions carried out on telogen hair,the majority resulting from forensic cases (ski hats, sweaters, foam rubber mattresses, etc.) only eight were unsuccessful, even if in thirty-three cases we obtained weak non-specific results in reverse Dot-Blot strips.

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DNA TYPING ON SINGLE HAIR - RECENT POSSIBILITIES BASED ON NEW EXTRACTION METHOD

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Centro Carabinieri Investigazioni Scientifiche - Rome - Italy

INTRODUCTION

The analysis of hair is becoming increasingly important in the field of forensic science. It often represents the only element connecting the assailant to the victim, sometimes the only signature of crime, especially in cases of brutal crimes like kidnappings, murders, rapes. Usually, these evidences are collected on the crime scene or are found stuck on various surfaces (hood-caps, cushions, nails, etc.) but they are very limited in number, often only one sample.
The use of PCR and VNTR's as well as the most recent STR's analysis, have considerably increased the possibilities of success to the extent that in a matter of a few years we have gone from the limited methods based exclusively on the morphological study of hair, to the possible identification of the suspect, even starting with just one fiber.
The research in this field is very rich and interesting, but in most of the cases, freshly plucked hair samples were analyzed without taking into account that hair coming from crime scenes are usually shed hair in the telogen phase and, as is known, hardly allow to achieve DNA typing results. This is due to the fact that most of the DNA wich is located in the root and surrounding sheath cells is very poor in quantity and is usually less than 10 ng. Hence, among the various steps characterizing the analysis of DNA polymorphisms, the extraction method is certainly the most important, owing to the success of a test. The use of chelex ion-exchange resin, though very pratical, quick and particulary effective in the case of bloodstains, does not seem to be suited for DNA extraction from shed hair, as confirmed by the huge amount of scientific literature and by our experience.

MATERIALS AND METHODS

DNA EXTRACTION

Chelex Extraction

Telogen hair were separated into root portions and placed into a 1.5 ml microcentrifuge tube with 1 ml of sterile water. After an overnight incubation and centrifugation in a microcentrifuge 157.MP (OLE DICH), 200 µl of chelex solution (Chelex 100. Biorad Richmond, CA: 5% w/v in sterile water) were added. The following steps proceeded the indications of chelex protocols.

Single Tube Extraction

Telogen hair were separated into root portion and placed into a 2 ml microcentrifuge tube where an aliquot of a yellow resin portion (DNA SINGLE TUBE - BIOTECX Houston, Texas cat.NO.BL5500) had previously been added. The DNA sample was digested with proteinase K and then treated with phenol-chloroform mixture in the same tube during centrifugation, the polymer gel moves between the organic and acqueous phases to form an impenetrable barrier.

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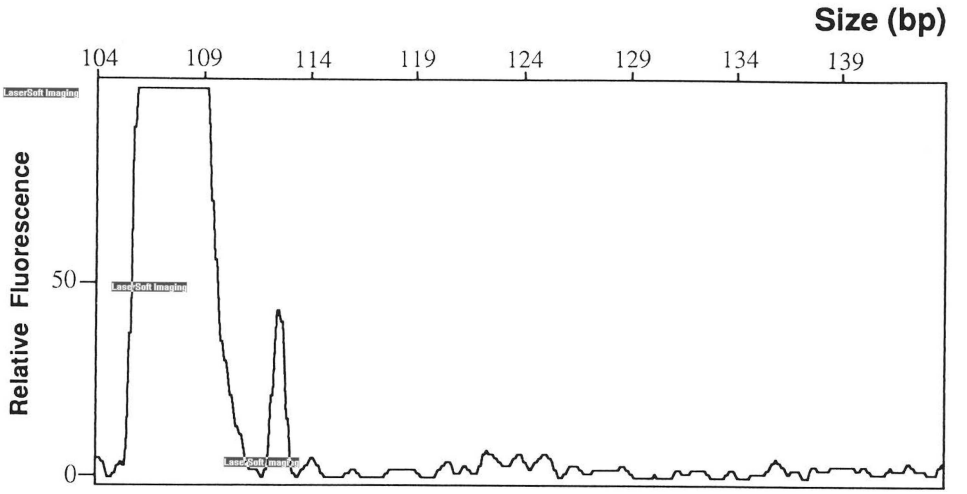

Peak / Lane	Min.	Size	Peak Height	Peak Area	Scan #
1B, 11	209	107.20	7122	46374	1047
2B, 11	216	112.87	54	203	1084

Figure 2. PCR product electophoretogram of male/female mixed DNA, generated by an Applied Biosystems 373 A GeneSequencer. Peaks from PCR product. The male specific peak (112bp) is clearly distinguishable from the larger female peak (106bp). Size in bp and peak height and area in arbitrary units are determined for each fragment. Time (min) and scan number of band detection from the start of electrophoresis are also given. Male/female DNA template ratio 1/100.

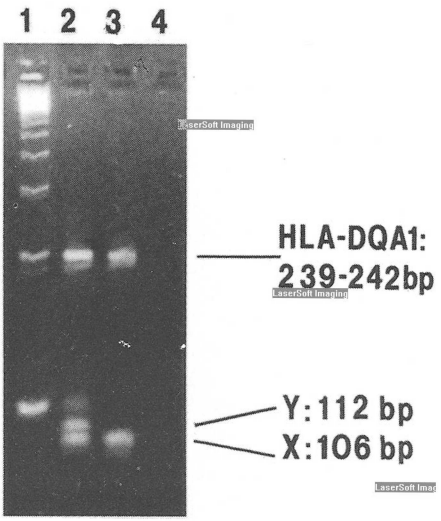

Figure 3. Amelogenin-HLA-DQA1 coamplification product evaluation gel. Lane 1, 123 bp ladder size standard, lanes 2 and 3, DNA samples; lane 4, negative control.

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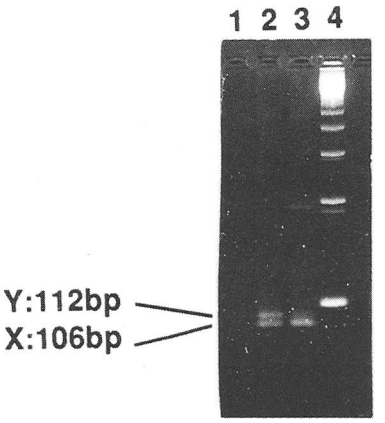

Figure 1. Sexing of seventy years buried bones. Lane 1, negative control water blank; lanes 2 and 3, PCR products from bone specimens, identified as male and female respectively; lane 4, 123 bp ladder size standard.

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denaturing acrylamide gel for 4 hrs at 1360 V on a ABI 373A Sequencer in conjunction with 672 software, combined with 2.5μl formamide and 6fmol internal size standard for automatic sizing, generated electrophoretograms in which the DNA segments were depicted as coloured peaks and peak area was automatically estimated as a measure of product yield. Such a high sensitivity and quantitation potential implicit to the test in conjunction with advantages of automated fluorescence dye-detection technology supplied by ABI 373 A GeneSequencer, provided a means to successfully type difficult mixed samples, e.g. detecting trace amounts of male DNA in a vast excess of female DNA template. Clear detection of Y specific product in a particular mixture that contained 100 ng of female DNA and 1 ng of male DNA, was obtained. The expected ratio of peak areas is 201:1 compares with the observed ratio of 228:1. This method could be potentially a useful tool to detect the presence of sperm (or white cells in case of azospermy) on vaginal swabs. Fluorescence detection also allowed to quantitate the relative amplification yelds: using normal males, peak area measurement generated 1:1 ratio of X and Y products while DNA from an XYY individual gave a 1:1.8 ratio and from XXY gave 1.8:1 ratio.

The versatility of this sex test was further demostrated by co-amplifing with 4 STRs loci (HUMTHO1, HUMVW31/A, HUMFES, F13A1) and the HLA-DQA1 locus, thereby providing a combined gender/identity DNA test. Co-amplification with 4 microsatellite with 130-240bp lenght products provides a combined probability of random match between unrelated caucasian of $1.06x10^{-4}$ [7].

A major advantage of the test described in this paper is that amelogenin single copy X-Y homologous regions are detected together, in the same reaction using a single primer pair. Our test greatly simplifies interpretation of results, especially when only the X-specific product has been generated, because this serves as an in-built positive control for the amplification reaction. Interpretation of results is further facilitated by the X and Y specific products being only 6bp different in size: even severely degraded samples can be typed without the risk of amplifying only the smaller of the two alleles. This "allelic drop-out" problem can afflict amplification and typing of loci with wider ranges of allelic sizes such as VNTRs [8]. The test may also have medical application in characterising sex chromsome abnormalities such as Klinefelter' s syndrome (XXY), monitoring sex chromosome dosage in cell lines and typing histopathological samples in which DNA is severely degraded.

In conclusion, this sex test is rapid sensitive and robust: PCR products are readily visible on agarose gel after ethidium bromide staining. Moreover, fluorescent technology allows a quantitative extimation of PCR products and results in useful application in both forensic and medical purposes for characterising mixtures of DNA, co-amplification with STRs and determinig sex chromosome aneuploidy.

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DNA SEX TEST: A NEW RAPID AND QUANTITATIVE FORENSIC APPROACH USING AMELOGENIN GENE BASED FLUORESCENT PCR.

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For forensic PCR applications, it is recommended that the investigation of X and Y sequences should be carried out in parallel or simultaneously, and the distinction of male and female DNA cannot be made based solely on the absence of a band [1]. Several PCR-based tests have been developed for gender identification, among them multicopy repeat sequences on the X and Y chromosomes can be amplified together and provide a highly sensitive assay but quantitation of the relative X/Y product is not possible because of significant differences in repeat copy number [2-4]. Alternatively, single copy X-Y homologous regions such as amelogenin offer the advantage of requiring only one pair of primers and both X and Y sequences are of equal copy number [5]. Primers described by Sullivan and coll. flank a 6bp deletion within intron 1 of the X homologue resulting in 106bp and 112bp PCR products from the X and Y chromosomes respectively [6]. Dye labelled PCR products were generated using one primer coupled with fluorescent dye 'FAM' via a 5' aminolinker. These primers allowed DNA samples ranging from 10pg to 100ng to be amplified through 35 cycles comprising 1 minute at 94^0C, 1 minute at 60^0C and 1 minute at 72^0C in a Perkin Elmer 480 thermal cycler. PCR products (20 μl) were visualised simply after electrophoresis in a 4% agarose gel for 90 mins at 100V and by ethidium bromide staining.

With this test we were enabled to sex a total of 50 male and 50 female DNA samples extracted from blood: all gave the expected results of a single band (106bp) in females and a doublet (106 / 112bp) in males. PCR products generated from as little as 1 ng of male and 1 ng of female template were readily visible. Unconfirmed origin DNA, extracted from muscle samples, that had been recovered from the scene of a mass disaster, gave clear-cut results meanwhile control "blind" analysed samples of known sex from the same investigation, were typed correctly. Approximately 20 pg of two severely degraded DNA samples, coming from seventy years buried bone, were amplified through 39 cycles. The products were clearly visible following electrophoresis in an ethidium bromide-stained agarose gel and confirmed conclusions regarding their sex drawn from physical examination of the bones. This also demonstrated the ability of this test to analyse samples of a very degraded nature. Electrophoresis of 1μl in 6%

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Table 1

Observed and expected COL2A1 phenotypes in a small Pan troglodytes population. A provisional nomenclature has been employed (from A to D for major alleles, from highest to lowest molecular weights).

TYPE		OBSERVED	EXPECTED	CHI SQUARE
B	B	5	4.23	0.14
C	C	1	1.88	0.41
C2	C2	1	0.18	3.62
D	D	1	0.73	0.10
D1	D1	1	0.06	13.18
A	B	2	1.41	0.25
A	B1	1	0.17	3.84
A	C	1	0.94	0.00
B	B1	1	1.05	0.00
B	C	8	5.64	0.98
B	C1	1	0.35	1.19
B	D	2	3.53	0.66
B1	C2	1	0.22	2.75
B2	C2	1	0.07	11.67
C	C2	1	1.17	0.03
C	D	4	2.35	1.15
D	D1	1	0.44	0.71
D	D2	1	0.14	4.95
sum		34		

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size compared with humans. Four major and six rare alleles were identified in 40 individuals, accounting for 18 genotypes. The system has a heterozygosity is 0.74 and the commonest genotype occurs in 20% individuals. A fair agreement between observed and expected genotypes was surprisingly observed. The high level of homology in the repeat unit and the complete identity of the flanking regions suggested that the entire COL locus is probably highly conserved in these primates.
Three cases of disputed maternity - involving as many controversial cases of animal identity - were solved by DNA analysis of Col2A1 hypervariable gene. The protocol employed, besides being highly informative and more effective than other methods so far employed, had the additional advantage of avoiding blood withdrawal to animals. Characterization of several other Pan troglodytes polymorphisms is under way in our laboratory.

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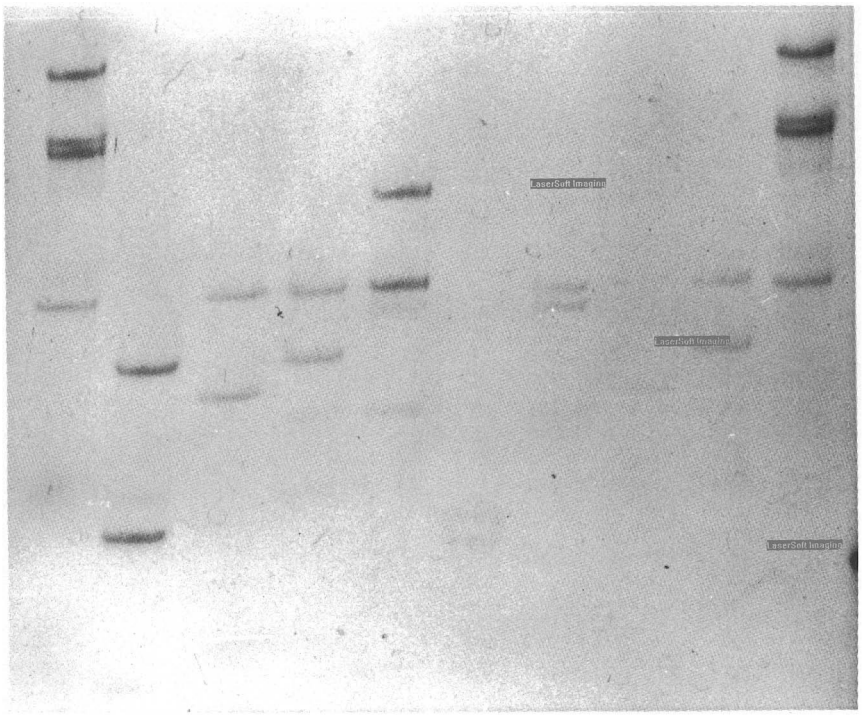
Figure 1
Some alleles and genotypes identified are here shown. At both extremities of the gel a human allelic ladder, obtained by the admixture of the most common human alleles has been run.



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ANALYSIS OF THE COL2A1 POLYMORPHISM IN A SMALL Pan troglodytes POPULATION, AND ITS FORENSIC APPLICATION

E. d'ALOJA, G. DESTRO-BISOL, U. MEREU, M. DOBOSZ, V.L. PASCALI and A. FIORI

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Common chimpanzees (Pan troglodytes) are a protected species, whose capture and trading is forbidden. In spite of international agreements, a high number of such great apes is annually reduced to captivity, then sold to private zoos and circuses. To elude controls, private owners use to declare false births in captivity (true births are rarest events) and exchange animal identities. All means enacted to control such illegal activities have been unsuccessful. We have been committed in a project aimed at developing a biological test to certify controversial animal identities, inspired to procedures for individual identification and parenthood tests used in humans.
An obliged step of this project was the development of hypervariable DNA polymorphisms (AmpFLPs; STRs) in Chimpanzee genome. Given the close relatedness between primate genomes, some of the human DNA markers may be also used in Chimpanzees. We here refer on a human gene - Col2A1 3'HVR (12q14.3) - which is in fact highly polymorphic in Pan troglodytes.

MATERIALS AND METHODS

Genomic DNA was obtained by hair roots using standard extraction procedures (an average of 30 nanograms per root was obtained). Twelve hypervariable loci were investigated using human oligonucleotide primers. We first focused on the 3'HVR COL2A1 locus (1), which showed a singularly high number of allelomorph fragments. Amplification conditions were similar to those used for the corresponding human gene, and separation of the amplified fragments was achieved by horizontal PAGE (8.5% T, 3%C) and silver-staining. An allelic ladder, prepared by mixing equimolar quantities of fragments, helped the typing work. To establish the degree of omology between chimpanzee and human alleles, the most common allele was run on agarose, excised and purified, subcloned in pUC18 vector and sequenced. The resulting basic repeat unit showed very high omology (98%) with man's.

RESULTS AND DISCUSSION

Alleles and genotypes detected in this study are shown in Fig. 1. In Table 1, the distribution of observed and expected genotypes is shown. Chimpanzee alleles have a lower molecular

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We think that DBE system is an easy, low cost and high resolution system which is ideal for separating STR systems like SE33. The gel is reusable and by using other labels (e.g. biotin, fluorescein) it is possible to separate multiplex PCR's with different staining for each label. The DBE system has multitasking capability so it is not restricted to sequencing or sequencing-like purposes. But with minor changes it is also usable for RFLP- and VNTR- analyses.

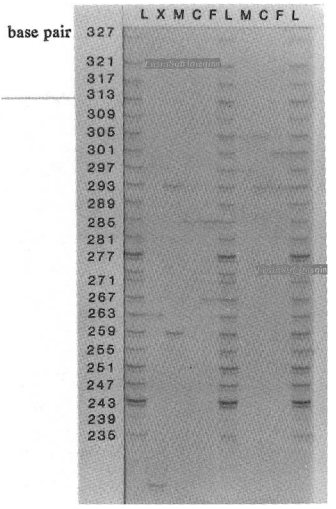

Figure 2

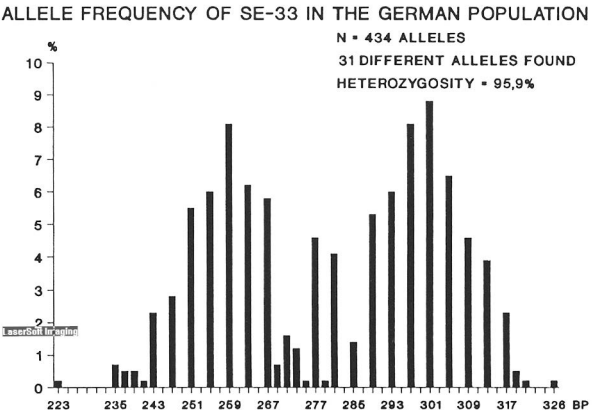

Figure 3

Figure 2: Blotting result of SE33: L = allelic ladder, X = unrelated person with the smallest allel (223 bp) found in our panel, M = mother, C = child, F = Father

Figure 3: Allele frequencies of a series of 217 unrelated German individuals. The allele length is plotted on the x-axis in base pairs (bp). Heterozygosity rate is 95.9%.

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Acknowledgement: The authors are indebted to Ursula and Harry Hanks, San Francisco, for helpful improvement of the English.

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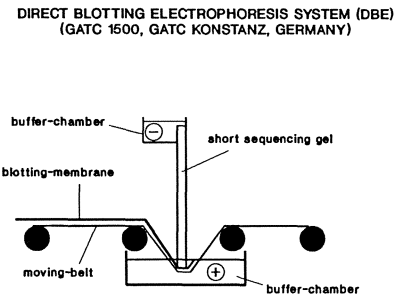

Figure 1: A schematic representation of the direct blotting electrophoresis system. DNA fragments are separated through a 30 cm long denturating 4% sequencing gel. Upon reaching the lower end of the gel the fragments are blotted onto a nylon membrane that is fixed to a moving belt and moved at a constant speed across the lower gel margin.

The total run time was 2h 45 min. After electrophoresis the membrane was dried overnight or for 1h at 80°C and exposed for 2 min to UV light in order to crosslink the DNA to the membrane. Visualization of PCR-products was carried out with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Germany) according to the manufacturer's advice. DNA mixture experiments were carried out with DNA from two persons (X,Y). Initial concentration of the DNA in 1:1 mixtures was 2.5 ng each. Other mixtures tested were 0.5 ng Y (1:5), 0.25 ng Y (1:10), 0.125 ng Y (1:20), 0.05 ng Y (1:50), 0.025 ng Y (1:100) with constant 2.5 ng X. After PCR the samples were diluted 1:250. Two mixes (1:50 and 1:100) were also diluted directly 1:5 in dye mix. 1μl of these samples were loaded onto the gel. Sizes of the alleles were determined by comparing the products with a sequencing reaction ladder of M13mp9. The allele frequencies are the result of analyzing 217 unrelated persons from Northwest Germany.

Results and Discussion

Running under conditions described above the GATC 1500 DBE-system is able to resolve a 1 bp difference. It is therefore very easy to type interalleles of STR-systems. The usage of the DIG detection system enables us to reduce the sample volume and to increase the detection limits. It is possible to dilute a PCR product generated after 30 cycles from 2.5 ng of genomic DNA 1:250 and this could be visualized on the blot after 2 h color development (Fig. 2).

With the DBE system we were able to type more than 300 persons in a short time. Thus, the system is also ideal for population studies. Up to now, we observed 31 different alleles in a group of 217 unrelated German individuals (Fig. 3). Alleles ranged from 223 bp to 326 bp. Alleles showing a higher frequency than 9% could not be observed. The heterozygosity rate is about 96%. Length variation occur at a number of sites within the repeat region. Therefore the allele length could differ by 2 or 4 bp.

Mixtures of two DNA samples could be visualized down to a ratio of 1:20 in a standard 1:250 gel loading dilution. This is similar to other AMPFLP- and STR- systems. However, in contrast to other systems it is possible, by using a lower gel loading dilution of 1:5, to detect DNA in mixtures down to a ratio of 1:100. Higher dilutions could be visualized by increasing the time of color development from 2 h to 24 h. Thus, using the DIG detection system we achieved a very high sensitivity which is very important for case work. Due to increased sensitivity one has to be very careful with postamplification-handling.

A further advantage of the DBE system combined with the DIG detection is that the results can be kept as original hardcopies as opposed to automatically generated product sizes.

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Analysis of the Short Tandem Repeat Polymorphism SE33: A New High Resolution Separation of SE33 Alleles by Means of Direct Blotting Electrophoresis

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Introduction

The analysis of the short tandem repeat (STR) polymorphism SE33 provides very useful information to both forensic casework and parentage testing. The advantage of this PCR based-system (1) is the high number of alleles showing an even distribution. The major problem of this system however is the separation of the alleles with a high resolution for an easy typing of alleles and interalleles. Sequencing gels are useful for such purposes but most laboratories no longer want to work with radioactivity or cannot afford an "automatic sequencer". For these reasons we chose the new technique of direct blotting electrophoresis (DBE) combined with a digoxygenin- detection protocol. This technique was developed by Beck et al. (2) and has the advantage of non-radioactive detection of products. Instead of a computer generated result, a hardcopy is produced.

Materials & Methods

DNA was extracted from whole blood according to standard procedures. PCR was carried out with the primers (1):

SE33/1: 5' - AAT CTG GGC GAC AAG AGT GA -3'
SE33/2: DIG- 5' - ACA TCT CCC CTA CCG CTA TA - 3'

The second primer was digoxygenin labeled.

Amplification conditions were:
50 mM KCl, 10 mM Tris pH 9.0, 0.1 % Triton X-100, 0.2 mM from each dNTP, 0.5 μM each primer, 1mM $MgCl_2$, 0.5 U Taq polymerase, 2.5 ng DNA.

The reaction volume was 12.5 μl. The cycler (Biometra TRIO- Thermoblock) was set to 80°C for 4-5 min for hot start conditions and the reaction started by adding primer SE33-2 and Taq polymerase to the heated reaction mix (DNA, SE33-1, $MgCl_2$). The reaction conditions were 94°C - 1 min, 60°C - 2 min, 70° - 1 min, for 30 cycles.

1μl of the amplification product was diluted 1:40 - 1:50 in H_2O dest. 1μl of this dilution was further diluted 1:5 in dye-mix (Formamid + 0.025% Bromphenolblue + 0.025% Xylenlcyanol). 1μl of the dye sample mix dilution was loaded onto a 0.19 mm thick, 30 cm long 4% denaturating polyacrylamide gel on the GATC 1500 DBE System (3) . The gel runs at constant 30W, about 1700 V and 17mA. After the xylencyanol dye reached the bottom of the gel, the nylon membrane fixed to a moving belt was started with a speed of 19 cm/h (Fig. 1).

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run in adjacent lanes, a procedure which wasted area on the gel that could have been utilized for samples. In order to increase the throughput, we employed a sizing ladder that was labeled with a dye that fluoresces red and placed it in the lanes with the samples. A calibration curve for the internal standard was constructed by the software, and then the size of the bands was calculated based on their position on the curve. Rather than visually estimating the genotype, the analyst obtains the size of the bands.

The variation of the size calls was very low, when compared to the length of the bands under consideration. The ratio of highest standard deviation observed to the lowest band size was 0.1% (0.1 bp), 0.2% (0.2bp), and 0.3% (0.45 bp) for the 24 cm denat., 12 cm denat. and 24 cm native PAGE systems, respectively. The standard deviation for the STRs is given in Table 1. From these data, we see that the typical denaturing gel is indeed the most precise one. However, is such a degree of precision necessary, when one is dealing with alleles that are separated by only 3 to 4 bp?

Table 1. Average Standard Deviation (bp) for Multiplex and SE33 STR Systems

	24 cm denat.	12 cm denat.	native
vWA	0.08	0.32	0.15
THO1	0.07	0.27	0.40
F13A1	0.12	0.15	0.26
FES/FPS	0.10	0.15	0.30
SE33	0.27	0.32	0.54

To measure the resolution of a gel system, one can utilize the familiar graphic procedure, whereby the peak width at one-half peak height is related to the distance between the peak maxima. Another way is to perform an experiment with alleles that vary 1, 2, 3, and 4 bp from each other. We have in our database an allele that runs one bp in size slower than the 10th allele of the THO1 allelic ladder, which I will call 10*. Now, the 10th allele has recently been sequenced and is reported to have one repeat of 3 bp (Puers, C., Hammond, H.A., Jin, L., Caskey, C.T. & Schumm, Am. J. Hum. Genet., in press), explaining why it is 3 bp distant from allele 9 and 5 bp distant from allele 11. These results indicate that it might be difficult to obtain the correct genotype by comparing a band in an adjacent lane to one of an allelic ladder. It is possible to discern the mobility difference between allele 10 and 10* visually in *all* the PAGE systems examined here. However, without using the in-lane sizing ladder, we have no way of knowing whether the slight mobility decrease noted for such an allele is due to differences in the local field strength of the particular lanes, or to length differences in the amplimer size. Another case for which an allelic ladder led to problems was with F13A1 on native gels. These gels are more capable of detecting differences in conformation of the DNAs than denaturing gels. Thus, whilest the repeat between the F13A1 alleles was tight and centered at 4 bp in denaturing gels, we saw what appeared to be 2 bp repeats between some of the alleles run on native gels (Table 2). This extra sensitivity of native gels for conformation of the DNA will lead to problems in inter-laboratory comparisons for the F13A1 locus.

Table 2. F13A1 Genotyping Differences on Native and Denaturing PAGE

	Native PAGE		Denaturing PAGE	
ID	genotype (bp)	Δ (bp)	genotype (bp)	Δ (bp)
2 8	189.3, 192.6	3.3	183.3, 187.3	4.0
3 0	192.8, 198.3	5.5	187.3, 191.1	3.8
3 5	198.4, 200.9	2.5	191.1, 195.0	3.9
3 6	192.6, 198.0	5.4	187.3, 191.1	3.8
7	198.4, 200.8	2.4	191.1, 194.9	3.8

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In this paper, data are reported for five, 4-bp repeat STRs utilizing three types of PAGE systems. The STRs chosen include the polymorphism at the human beta-actin related pseudogene H-beta-Ac-psi-2 (ACTBP2), also known as "SE33" (Polymeropoulos, M.H., Rath, D.S., Xiao, H. & Merril, C.R. Nuc. Acids Res. 20, 1432, 1992), which is popular due to its high number and even distribution of alleles. The other four STRs derive from the "Multiplex 1" system described recently by Kimpton et al. (1993) and presented elsewhere in this volume. It includes HUMVWA31, HUMTHO1, HUMF13A1, and HUMFES/FPS. Since we are interested in adopting automation to STR analysis, we have utilized the Applied Biosystems model 373A DNA Sequencer with fluorescent detection of PCR products generated with fluorescently labeled primers. In this system, all bands traverse the same distance from the well to "reading" (WTR) region of the laser before they are detected. We utilized a WTR of both 12 and 24 cm, the latter distance being the typical one for DNA sequencing. Data for 6% T 5% C denaturing gels with 8M urea (12 and 24 cm WTR, 44V/cm) or 6% T 5% C native gels (24 cm WTR, 30V/cm) are reported here. Five gels of each type were compared. We also analyze the STR systems on the GIBCO BRL/Life Technologies SA32 apparatus with silver stain detection.

The time required for the separation of the STR bands could be greatly reduced when native gels were employed. A 24 cm WTR sequencing type gel required 5.5 h and a bit more than 7 h for separation of the multiplex and SE33 amplimers, respectively. The time necessary for the same degree of separation of the STR-PCR products on native gels was reduced to 3.5 h and 4.5 h, respectively. However, we obtained just as good results with the 12 cm WTR denaturing PAGE system, and this required less than 3 h for the multiplex and 3.5 h for the SE33 one.

Extra bands were sometimes detected in all three PAGE systems. When we utilized the PCR conditions of Kimpton et al. (1993), we found the system extremely robust. We found it advantageous not to include Triton X100 in the PCR buffer, because this led to extra bands in all PAGE systems and to a turbid solution following the PCR. The locus F13A1 often yielded faint extra bands on native gels, which were not present in denaturing gels. We attributed these extraneous bands to heteroduplex formation during the final stage of the PCR. Under different gel crosslinking conditions (10% T, 2.5% C), extra bands for vWF could be distinguished on native gels, which were absent in the standard PAGE crosslinking conditions. When the denatured PCR products were not loaded immediately, there was some degree of re-annealing, giving rise to extra bands on the denaturing gels. Sometimes, for reasons that could not be determined, the PCR samples did not denature, and gave rise to smears. Thus, a 95°C block containing oil in the wells and a careful sample treatment protocol did not always ensure denaturation.

Another problem giving rise to extra bands appeared in some cases and could be attributed to the non-templated addition of a nucleotide to the blunt ends of the amplimers by Taq DNA polymerase (Clark, J.M., Nuc. Acids Res. 9677-9686, 1988). Kimpton et al. (1993) described two enzymatic procedures to eliminate these extra bands. This approach introduces not only an extra step in the procedure, requiring time of the analyst, but provides another quality assurance consideration. We found a simpler procedure: the doublets one observes due to -A/+A in 24 cm WTR denaturing gels are absent on 12 cm WTR gels, and a single band appears. This not only makes the interpretation of the patterns simpler, but it also allows a more accurate measurement of mixed samples, because one is dealing with a single band, rather than a split one.

Obtaining the genotype when allelic ladders were utilized was a facile procedure in most cases. However, this required the use of multiple colors in the case of the multiplex system. For example, since the alleles of F13A1 and FES/FPS overlap, we used green for one and blue for the other. It also meant that the respective allelic ladders had to be

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Evaluation of native and denaturing polyacrylamide gel electrophoresis for short tandem repeat analysis.

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In recent years, one has been exposed to a number of advances in the methods of human identity analysis. Why, in this high-tech age, one may wonder, should time be devoted to another paper on polyacrylamide gel electrophoresis (PAGE)? The answer to this question is intimately associated with the increasing popularity of short tandem repeat (STR) analysis. Many laboratories are using PAGE to both optimize PCR conditions and to obtain databases, and there are almost as many PAGE systems being used as there are loci being analyzed! It is time to standardize the PAGE conditions, so that inter-laboratory variation in results can be minimized. In this communication, three PAGE systems are compared, in order to generate a discussion that may lead to a consensus towards the adoption of a single PAGE system for STR analysis.

First, however, we need to review some features of STRs, that cause one to adopt certain PAGE systems over others. STR-PCR generates amplimers in discrete size ranges between about 100 and 350 bp. The PCR cycling times are short, and the small PCR products do not require long PAGE runs for their separation. One caveat of STRs, however, is that there are few alleles per locus, a fact that makes it necessary to include many loci in the assay, in order to increase the probability that two randomly selected individuals in a population will differ in overall type. Co-amplification of different STR loci is often possible, and this feature allows the analyst to save preparative time. However, this also causes a problem in the analysis of the PCR products, because many of the loci yield amplimers that have similar mobility on PAGE. Thus, one needs to choose loci, whose alleles do not overlap, when silver staining is utilized for detection of the bands. This restricts the analysis to certain loci, and may exclude other loci that are just as informative. Of course, one can run the STRs in many different lanes of the gel, but that technique disallows rapid screening, unless the laboratory is set-up to run 20 or so gels a day. To do that, a very high degree of quality assurance would be necessary.

Since one of the advantages of STR analysis is high throughput, some investigators have concentrated on speed, and chosen to utilize native PAGE, because native gels run much faster than denaturing gels (Frégeau, C.J. & Fourney, R.M., BioTechniques 15, 100-119, 1993). In addition, time is saved by not having to perform the denaturation step. The samples from many PCRs can be combined, but they might need to be desalted before denaturing PAGE, since DNA in solutions of high salt may be refractory towards denaturation. Native gels, on the other hand, are not so sensitive to salt effects, as long as each sample is similar in its composition. Another characteristic of STRs that helped make them popular was the stability and reproducibility of their 3 and 4 basepair (bp) repeats. Nevertheless, some investigators have chosen sequencing-type denaturing gels, which, of course, provide single-base resolution (Kimpton, C.P., Gill, P., Walton, A., Urquhart, A., Millican, E.S. & Adams, M., PCR Methods and Applications 3, 13-22, 1993). These gels offer a high degree of accuracy and precision, but they require long run times, too. Thus, we have two things to consider in the adoption of a PAGE system: a low degree of variability on one hand, and speed, or throughput, on the other.

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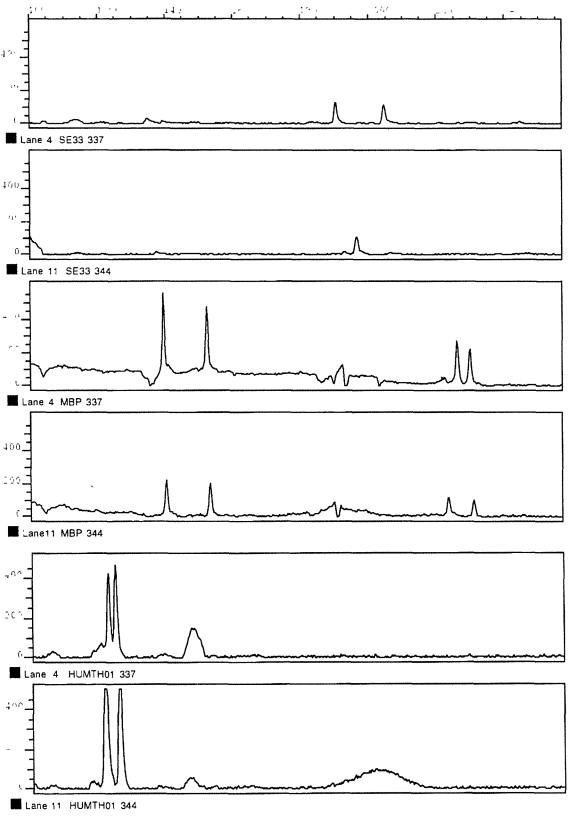

Fig. 2: Electrophoretogram display of PCR products of three STRs run in the same gel lane and distinguished by three different dyes.

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RESULTS:

Comparisons of allelic profiles obtained from conventional electrophoresis (3) with results from GENESCAN 672 Software have indicated no significant difference. Indeed, when we compare allele lenghts derivated from technique using allelic ladder standard and results from automated fluorescent analysis some difference has been found on allele designation.

Our prelyminary results (Fig. 1) indicate that high accurancy and precision can be obtained with GENESCAN 672 Software. The size of PCR products is determinated automatically by the software using the labelled size standard in each lane. It is the best way to provide lane to lane reproducibility and a precise size calling. For forensic area to obtain valid and reliable results from a variety of biological sample is an important parameter. The amplification from old bloodstains and a gel separation with standard method can displayed differences in the size of alleles in each STR locus. In this condition we observed an apparently different sized allele which do not match with an allelic ladder. In this experiment an inclusion or exclusion is determinated by profile comparison. The areas in a ladder where there in no an allele for comparision, can be assigned an appropriate allele size. This is significant in microsatellite analysis where alleles can differ in size by as little as one or two base pairs.

Futhermore, this technology increases the number of the markers that can be genotyped in each gel lane. We have multiplex different colors and different size ranges in the same lane (Fig. 2).

Finally, we believe that the advantage to use this technology which is based on three available fluorescent dyes, is the high sensitivity and resolution that should address the realistic amount of DNA in forensic nature.

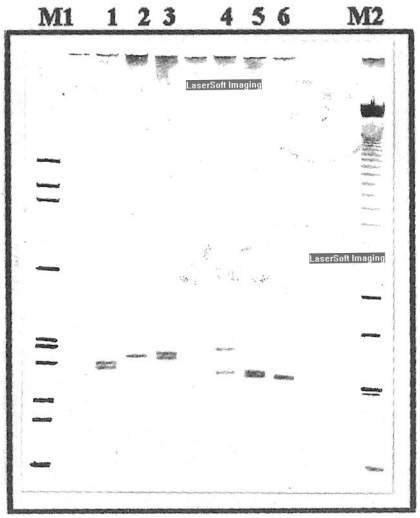

Figure 1. Horizontal gel page,
silver-stained STR (ACTBP2).

M1, M2	Molecular weight standard.
Lane 1	Amplified DNA blood suspect 1.
Lane 2	Amplified DNA bloodstain.
Lane 3	Amplified DNA blood suspect 2.
Lane 4, 5, 6	Mendelian inheritance.

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COMPARISON OF MANUAL AND AUTOMATED DETECTION FOR STRs ANALYSIS.

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Introduction

The short tamdem repeat (STRs) loci are ideal for identification in forensic investigations. Being STRs repeated in a tandem fashion, highly polymorphic, extremely abundant and evenly distributed in the genome (1). PCR amplification has improved the sensitivity of their DNA profiling (2). Here we report comparative results from the standard approch to microsatellite analysis (3) and automated fluorescence based technology (4) to detect three different microsatellite loci.

Materials and Methods

Human genomic DNA was prepared from peripheral blood and dry bloodstains by standard methods involving cell lysis, proteinase K digestion and ethanol precipitation. Three microsatellite loci were used in this study: ACTBP2 (Polymeropoulos, M. M. et al.), MBP (Polimeropoulos, M.M. et al.) and HUMTM01 (Edwards, et al.).

All oligonucleotide primers used were syntesized using a Model 380 DNA Synthesizer (Applied Biosystems, Foster City, CA). For the automated detection a fluorescent dye (JOE, FAM and TAMRA) was attached to the 5'- end of the forward PCR primer used for amplifying each locus using Aminolink 2 (Applied Biosystems, Foster City, CA) and HPLC purified.

We used an internal lane size standards consisted of a PstI digest of Lambda phage, labelled with a fourth red dye (ROX) by a legation method (5). Nonfluorescent primers were synthesized using the same sequences.

PCR were carried out for fluorescence primers for 25 cycles with 25-50 ng of DNA in 50 µl reaction volume. Each locus was initially amplified in a single reaction. Then MBP and ACTBP2 loci were amplified in the same tube with fluorescent primers labelled with JOE (green) and FAM (blue) respectively. HUMTM01 was labelled with TAMRA-dye (yellow) and amplified with single PCR.

For gel analysis with fluorescent amplification products, 0.5µl of the internal lane standards and 2µl of PCR reactions for multiplex or 1µl for simple locus were combined with 3µl of deionized formamide. Were electrophoresed in a 6% Acylamide gel (BIORAD) with 8M urea in 1x TBE. Gels were electrophoresed for 6 hr at 30W constant power on an Applied Biosystems automated DNA sequencer model 373A. The size of PCR products is determined automatically and precisely by GENESCAN 672 software (Applied Biosystems, Foster City, CA) using the labelled size standard in each lane.

Non fluorescent amplified STRs loci were analyzed on horizontal polyacrylamide gels according to the F.B.I. protocol.

Gel separation was performed by horizontal discontinuous PAGE (7% to 10%). The gels were cast on gelbond using the Flap technique. The gels were cross-linked with piperazine diacrylamide (29:1) prepared in 0.12M Tris-Formate buffer, pH 9.0.The trailing buffer consisted in 2x tris Borate, pH 9, 0,28M.Electrophoresis was conducted at 20mA, electrode distance 2 cm.Following electrophoresis, the gels were stained with silver (Allen, 1989).

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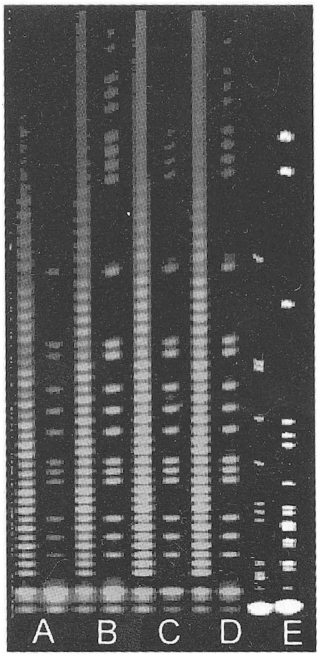
Table 1. STR Allele Number and Empirically Derived Annealing Temperatures

Locus	Primer/Length	%GC Content	Optimal Temperature °C	Product Size	Repeat Unit	Allele #
CD4	32/34	53/54	68	140-170	AAAG	7
vWF	24/28	38/36	64	102-154	TCTA	7
THO1	24/24	50/50	68	179-207	AATG	5
D21S11	18/22	50/36	64	172-264	TCTA/TCTG	12
FABP	25/24	44/50	64	199-223	AAT	6
ACTBP	20/20	50/50	64	231-339	AAAG	44
HPRT	24/24	46/42	60	257-297	AGAT	8
ARA	24/24	54/50	68	255-315	AGC	15

Table 2. Multiplex STR Triplex

STR System	Most Common (2pq*)	Most Rare (2pq*)
D21S11	1/8	1/15,900
FGA	1/17	1/30,700
Amelogenin	1/2	1/2
Combined Triplex	1/272	976 million

*RCMP Canadian Caucasian Database



Fluorescent MVR Analysis Using Tagged dUTP **Figure 2**

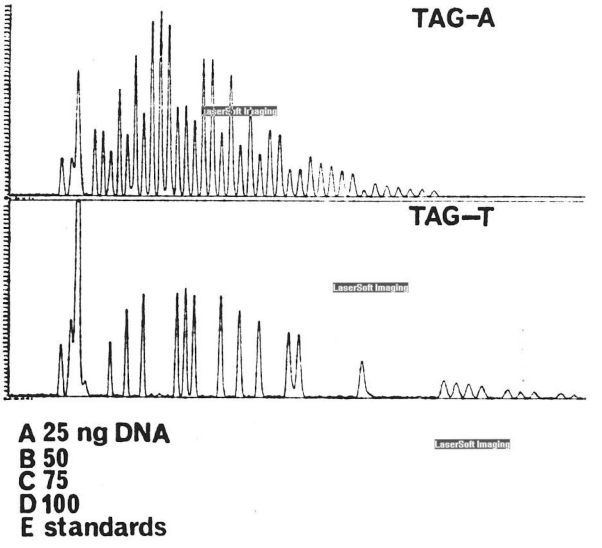


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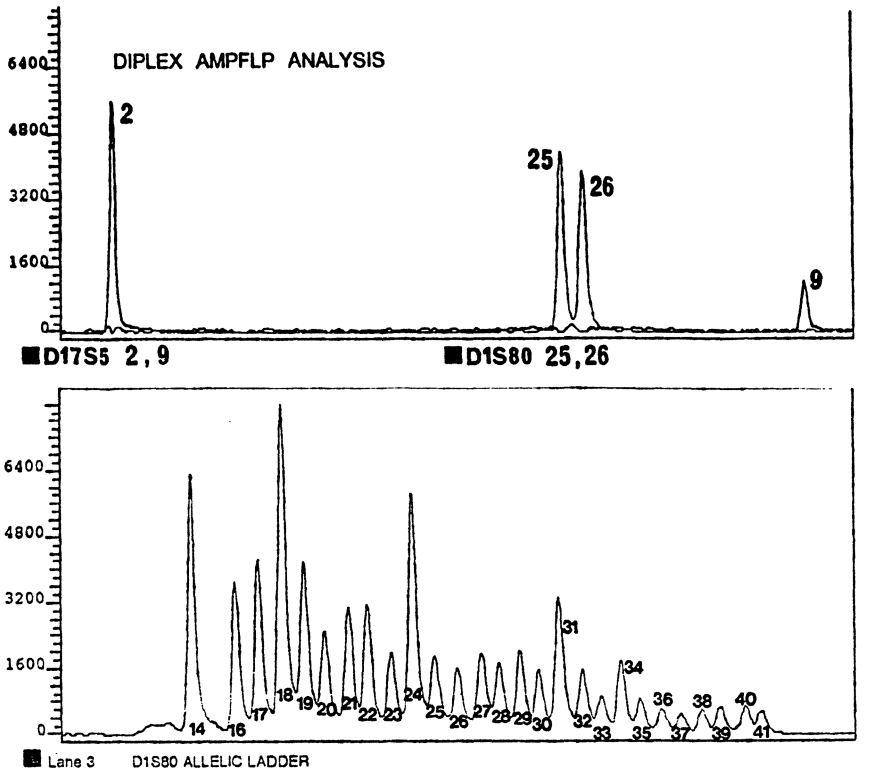
Figure 1. Ampflp Allele Measurements



D1S80 Allelic Ladder Size Comparison Using ABI Internal Size Standards:

Allele	Size bp	Allele	Size bp	Allele	Size bp	Allele	Size bp	Allele	Size
14	356.1	21	468.3	27	549.0	33	643.9	39	742.4
16	388.8	22	479.9	28	560.4	34	660.9	40	760.9
17	406.4	23	493.4	29	577.2	35	678.2	41	772.9
18	424.6	24	506.3	30	592.5	36	697.0		
19	441.6	25	520.0	31	608.8	37	711.2		
20	454.3	26	534.4	32	628.9	38	727.7		

DIPLEX AMPFLP ANALYSIS

Ampflp System	Most Common (2pq*)	Most Rare (2pq*)
D1S80	1/6	1/20,000
D17S5	1/12	1/10,000
Combined Diplex	1/72	200 million

*RCMP Canadian Caucasian Database

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may prove to be more challenging than with conventional PCR-based systems. Ease of interpretation and recording the high number of potential alleles makes automated detection using fluorescent tagged alleles ideal for MVRs. Unlike silver-staining or intercalating detection methods that are based on the mass binding relationship of dyes or stains for detection sensitivity, fluorescent labelling is a simple molar incorporation phenomenon such that a DNA fragment with more fluorochromes attached will be detected with much higher sensitivity. After considerable empirical investigation, it was demonstrated that a fluorochrome dye labelled nucleotide provided the best method for tagging multiple MVR alleles. As noted (Figure 2), 20 ng of template DNA will provide sufficient material for the discrimination of greater than 30 MVR repeats. Optimization of the amplification reaction and methods for eliminating free fluorescent tagged nucleotides will have to be established before a practical and robust routine forensic analysis will become available. The use of internal size standards such as ABI fluorescent tagged Genescan 2500 in each MVR sample lane proved highly effective as an internal calibration standard for the potential adjustment of mobility shifts encountered when comparing several sample lanes. Ideally, the use of automated detection of fluorescent labelled MVR alleles should allow for the rapid generation of databases and the recording of the vast amount of discrimination data obtained from each sample as well as provide an excellent fluorescent quantitation method for unambiguous determination of homozygote repeat patterns.

Acknowledgments: We thank Applied Biosystems Inc. and Dr. Rebecca Reynolds of Roche Molecular Systems, for the opportunity to evaluate the prototype fluorescent D1S80 allelic ladder. References to commercial reagents, supplies or equipment are for identification purposes and are not intended as endorsements by the Royal Canadian Mounted Police.

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STR DNA Analysis

The current scientific literature (3) suggests that the number of potential short tandem repeats (STRs) for polymorphic identification in the human genome could easily surpass 500,000. The more familiar STR systems (Table 1, ref 4,5), composed of trimeric and tetrameric repeats have fewer alleles but may provide high discrimination through the subsequent addition of cumulative STR systems. Ideally, routine forensic analysis of several STR systems could be accomplished by co-amplification in a multiplex procedure, or simply by analysing the products of single amplified reactions in a combined sample lane on a polyacrylamide gel. Both these approaches require complete knowledge of the optimized amplification conditions for each STR system and the resolution characteristics of non-overlapping alleles using silver-stained detection, or alternatively different fluorescent tagged STRs and differential colour detection.

The approach taken in our laboratory has involved the differential fluorescent labelling of each STR system and the utilization of optimized amplification conditions to eliminate potential PCR artifacts. As noted in Table 1, the empirical optimization of amplification conditions clearly delineates different optimal annealing temperatures, and therefore provides the initial criteria for combining several STR systems in a multiplex format. The multiplex analysis of single sample with a potentially limiting amount of target DNA could provide probative information on several STR systems in a timely manner. However, a multiplex reaction involving different primer sets, may not be as robust as a series of single STR systems amplified independently. Consequently, the limitations and advantages of multiplex reactions must be carefully examined and validated using forensically relevant samples with specific recommended reaction conditions and allele detection methodologies. For example, preliminary analysis of multiplex reactions has suggested that 5 ng of target DNA may provide an optimal multiplex template under the amplification conditions specified for many single amplification reactions. Clearly, conditions could be optimized for specific multiplex amplification reactions which would enable detection of multiple alleles with a smaller concentration of target DNA, but these conditions undoubtedly will differ from the "singleplex" reaction conditions. Numerous multiplex STR systems will undoubtedly be described in the future forensic literature, yet the ultimate challenge will be to develop a robust procedure with high discrimination that is easily detected in an automated fluorescent format. One such system under investigation in our laboratory (Table 2) combines the advantage of gender discrimination through the use of the amelogenin sex-typing locus (6) with discrimination at two other STR loci.

Automated Fluorescent Detection of MVRs (minisatellite variant repeats)

There has been considerable interest in the utilization of MVRs (7) for discrete identification. MVRs can potentially provide unlimited discrimination and appear to be immune to many potential problems encountered in other PCR-based systems (e.g. electrophoretic variation, the use of different size or allelic markers). However, deciphering complex mixtures or documenting the vast amount of discrimination data

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An Evaluation of Automated Fluorescent PCR-Based DNA Typing

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Abstract - Introduction

The recent revolution of new technologies in forensic DNA typing has presented several promising PCR-based identification systems. Compared to current RFLP VNTR systems, PCR offers the advantages of enhanced sensitivity, more discrete alleles and the efficacy of working with degraded DNA samples. In order to achieve the equivalent DNA discrimination experienced with RFLP VNTR analysis, the new PCR-based systems will require a greater number of STR systems to be run or additional procedures involving AmpFLPs systems or digital DNA typing (MVR). A concerted approach using several DNA typing technologies represents the future reality in the processing of numerous biological forensic samples. PCR-based DNA typing with automated real-time analysis of fluorescent amplification products is currently a major thrust of the RCMP DNA program.

AmpFLP Detection Procedures

Currently many laboratories have adopted the use of AmpFLP (amplified fragment length polymorphism) PCR identification for forensic applications (1,2), with specific interest in the loci D1S80, D17S5, Col 2A1, and Apo B. The use of fluorescent labelled primers allows the potential of simultaneous multilocus discrimination. In our own studies, we have found that the simultaneous analysis of the two AmpFLP systems, D1S80 and D17S5, offers excellent discrimination potential in a "diplex" reaction (Figure 1). Allele dropout encountered for the upper alleles in the D17S5 locus is generally a detection sensitivity issue that in the majority of cases is circumvented by using labelled fluoroprimers for both the forward and reverse reaction. In addition, although most alleles are easily designated by precise size identification using internal molecular weight size standards (ABI Genescan 1000 or 2500 size standards),variant and normal alleles are easily compared using fluorescent labelled allelic ladders (Figure 1). Several considerations, such as the great number of alleles found for each system, less problematic discrimination of variants due to gel resolution limitations, and the precedent for acceptance of these systems using silver-stained detection procedures, make AmpFLP systems excellent introductory automated fluorescent identification systems for PCR-based identification.

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HYPERVARIABLE LOCI

Certain highly polymorphic STR loci (eg. ACTBP2, APOAI1 and D11S554) contain complex compound repeat regions and may display alleles which differ by just 1bp (Adams et al 1993; Urquhart et al 1993). Although single base differences can be resolved on denaturing polyacrylamide gels, the consistency of automatic sizing employing the Lambda *Pst*I sizing standard is not sufficient to allow accurate allele designation. We have therefore constructed allelic ladder sizing standards for these hypervariable loci. By labelling these allelic ladder standards with a different dye marker to the individual samples we are able to co-electrophorese the ladder and sample in the same gel lane. The 672 Genescan analysis software then allows us to programme the sizes of the ladder bands into the computer thus allowing the automatic sizing of sample products to a high degree of precision. Using this approach we have developed a highly informative duplex amplification system containing the hypervariable loci ACTBP2 and D11S554. This system has considerable potential for routine forensic identification.

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MULTIPLEX SYSTEMS

The availability of 4 distinguishable fluorescent dyes facilitates the development of STR multiplex PCR systems by the allowing the labelling of loci which have overlapping allele size ranges with different fluorescent dyes. By combining loci which have similar optimal amplification parameters and adjusting individual primer concentrations we have constructed 3 STR multiplex systems containing a total of 14 different loci. These multiplex systems have been employed to rapidly generate population databases and to assess the usefulness of the loci for the identification of individuals.

A further multiplex system was developed (Table 1, Fig 2) containing the four loci which we felt were most likely to be suitable for routine forensic identification, based on the information generated from the above database construction and the individual STRs amplification and sizing efficiency. All four loci within this quadruplex system have relatively simple repeat regions and display regularly spaced alleles differing by 4 bases - with the exception of one 2 base allele in the HUMF13A1 locus. This quadruplex has undergone detailed evaluation for use in routine forensic case work and found to be both robust and reproducible.

Table 1: Matching Probability (pM) of STR loci in multiplex 1

STR LOCUS	MATCHING PROBABILITY Caucasian	Afro-Carib.	Asian	SIZE RANGE (bp)	DYE LABEL
HUMVWA31/A	0.05	0.06	0.07	135-167	ABI "JOE"
HUMTH01	0.09	0.12	0.08	154 -178	ABI "FAM"
HUMF13A1	0.13	0.05	0.08	182-236	ABI "JOE"
HUMFES/FPS	0.16	0.09	0.14	214-238	ABI "FAM"
COMBINED pM	$1.1x10^{-4}$	$3.0x10^{-5}$	$5.9x10^{-5}$		
MOST COMMON	$8.0x10^{-4}$	$9.0x10^{-5}$	$4.4x10^{-5}$		

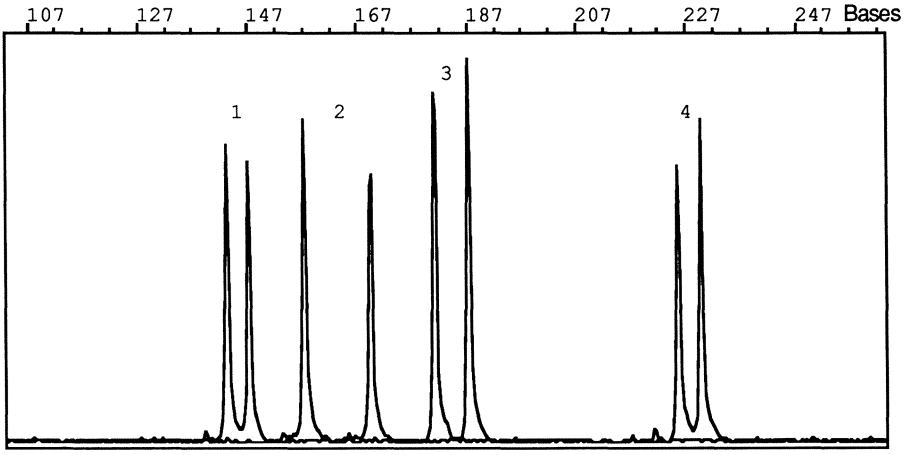

Figure 2: Electrophoretogram of a quadruplex amplification. Locus 1 HUMVWA31/A, locus 2 HUMTH01, locus 3 HUMF13A1, Locus 4 HUMFES/FPS.

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AUTOMATED DNA PROFILING EMPLOYING MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI.

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INTRODUCTION

Short tandem repeat (STR) loci are a class of polymorphic markers which occur throughout the human genome and which consist of simple tandemly repeated sequences 1-6bp in length. Their abundance, hypervariability and amenability to amplification by the polymerase chain reaction (PCR) make them ideal markers for use in the identification of individuals.

DNA profiling based on PCR amplification of STRs has the advantage of being more sensitive than conventional techniques. Furthermore, typing of material containing highly degraded DNA is more likely to be successful when using STR analysis (Hagelberg et al. 1991; Gill et al. 1992; Jeffreys et al. 1992).

Tri-, tetra- and pentameric STR loci appear to be less prone than dimeric STRs to artifactual "stutter" banding caused by enzyme slippage during amplification and such loci are therefore more suitable for routine forensic applications (Edwards et al. 1991; Fregeau and Fourney 1993; Kimpton et al 1993; Wiegand et al; 1993).

We have evaluated the use of automated fluorescence-based technology to automatically detect tri-, tetra- and pentameric STR loci electophoresed on denaturing polyacrylamide gels.

AUTOMATED STR DETECTION.

STR loci were fluorescently tagged during amplification by employment of a dye-labelled PCR primer. Amplified products electrophoresed on 6% denaturing polyacrylamide gels were detected by laser scanning on an Applied Biosystems automated DNA sequencer. An internal size standard consisting of fluorescently labelled Lambda *Pst*I restriction fragments was co-electrophoresd with each sample. This eliminated differences in electrophoretic mobility between gel lanes and allowed the automatic sizing of STR-PCR products with Genescan 672 analysis software.

The consistency of the automatic size calling was evaluated for each locus studied by examination of the distribution of computer-generated bands sizes for a number of samples run on different gels (Figure 1). All the STRs evaluated which contained straight-forward repeat regions were shown to be sized to a high degree of precision allowing unambiguous allele designation.

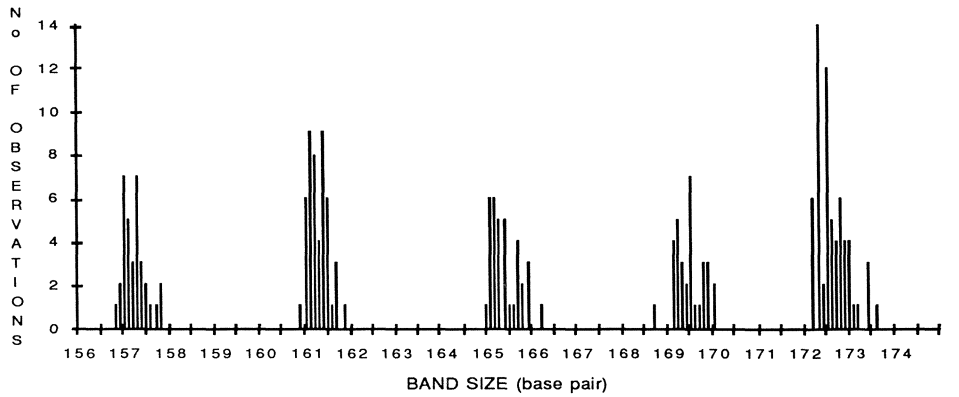

Figure 1: Frequency distribution of automatically generated TH01 PCR product sizes.

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7. PCR methodology

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three systems could be demonstrated. In this case, there was no real mixing-up of samples. According to documentation, the defendant's claim that he had not been at the wheel of his vehicle at the time in question was confirmed. Someone had apparently furnished the defendant's name instead of his own.

Summary

Several reports on the subjects frequency and importance of identity checks concerning stored blood samples, the difficulty of blood group determination in these samples as well as DNA analysis by SLS or MLS (DNA fingerprinting) have been made (literature survey Henke, L. et al, 1990, Kleiber, M. 1987).

As shown by our results, DNA analysis with PCR-systems HLA-DQa, D1S80 and YNZ22 provides adequate results even in cases where prior toxicological examination, putrefaction of the sample or small sample volume have occured. Cases in which classic methods of blood group determination and DNA fingerprinting on samples stored at 4°C may lead to problems.

More and more of the samples taken in the area served by our institute and analysed by us for BAC are subsequently ordered to be examined toxicologically and the results, if positive for drugs, are often doubted; an increasing number of samples which were again analysed toxicologically are then sent to our institute to have their identity checked. On the basis of experiences made by us, we suggest that PCR analysis is the method of choice in cases such as the above.

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mixtures (total volume 50 µl) consisted of 0,2 mM dNTPs, 10 mM Tris-HCl (pH 8,4), 1,5 mM $MgCl_2$, 50 mM KCl, 0,25 µM each primer and 2,5 U Taq DNA polymerase. No paraffin was layered over the reaction mixtures. Amplification was carried out in the GeneAmp PCR System 9600 from Perkin Elmer Cetus. Temperature cycling conditions were as follows: D1S80, denaturation 15s, 95°C, annealing 15s, 66°C, extension 30s, 72°C, 30 cycles, final extension 10 min). YNZ22, denaturation 30s, 94°C, annealing 30s, 63°C, extension 120s, 72°C, 30 cycles, final extension 10 min. PCR products were separated on 2% agarose gels in TBE puffer at 3,5 V/cm for 7 h. The bands were visualized by staining with ethidiumbromid.

Results and discussion

	storage time (months)	notes	blood group systems	frequency	frequency including PCR-analysis
1	5		ABO, Rh	1 : 5	1 : 110000
2	5		ABO, Rh	1 : 5	1 : 37 mio.
3	9	2x tox. analysis	ABO, Rh, Kk, P, PGM, AK, GPT, EsD,	1 : 107	1 : 15 mio.
4	8		ABO, Rh, Kk, P, PGM, AK	1 : 25	1 : 625000
5	14	2x tox. analysis, rem. volume 1 ml	ABO indirect, AE	1 : 2	1 : 213 *
6	9	2x tox. analysis, rem. volume 1,5 ml	ABO indirect, AE	exclusion	exclusion
7	14		ABO, Rh, Kk	diff. A_1/A_2	1 : 100000

*PCR-Systems: HLA-DQα, D1S80

As you can see from the chart, cases 1,2 and 4 provided unsatisfactory statistical results in spite of empty case history and sufficient sample volume, as only between 2 and 6 systems with insufficient phenotype frequency could be typified.

Case 7 was originally typified as A_1,while the blood group ID card, which had accompanied the blood sample, said A_2. The subsequently drawn comparison sample was typified as A_2 in the ABO system. However, PCR analysis proved beyond doubt that original and comparison sample were of the same origin. Apart from this, it could be shown again that changes in blood group properties can occur through storage, which was first reported by Thomsen, O. and Friedenreich, V. in 1926 and now confirmed by us by means of PCR.

Due to complete haemolysis and between only 1 and 1,5 ml remnant sample volume in cases 5 and 6, samples were split and a blood stain was created for ABO typification. Indirect blood group determination was carried out using absorption-elution technique (AE). In doing so, the constellation of case 6 made exclusion possible: blood stain/original sample 0, comparison sample B. The remainder of the sample was used for PCR analysis, where exclusion for all

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Blood Group Typing and PCR-Analysis in Stored Blood Samples

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Checks concerning the identity of stored blood samples may be requested by the court in cases where the blood alcohol concentration (BAC) or the results of toxicological analysis are doubted by the defendant, who may subsequently maintain that blood samples were mixed up during analysis. In most cases, linkage of original and comparison sample provide no difficulties. Sufficiently high statistical probabilities to satisfy even the high-level requirements of criminal law are attained by typifying an adequate number of membrane, serum and enzyme systems. Problems concerning the identity check may arise if:

- a toxicological analysis was performed beforehand, due to the fact that quantitative analysis requires homogenisation by means of Ultraturax, which leads to a virtually complete destruction of erythrocyte membranes and membrane systems. Also, serum can no longer be separated by centrifugation. Our experiences show that, on top of this, enzyme systems are suppressed.
- only few systems can be determined due to hardly adequate sample volumes and/or long storage times. Note that no correlation exists between storage time and preservation (Osterhaus, E., Birkner, P., 1985; Sachs, V., et al. 1988).
- less than 2 ml of sample remains for analysis

The following results will show that a definite statement (exclusion/no exclusion) can be made in problematic cases by means of supplementary PCR analysis.

Materials and methods

Blood samples were stored at 4°C after BAC analysis. Systems which are utilised in routine identity checks could only be successfully typified in very few of the cases mentioned below. AB0, MNSs, Rh, Kk, P, Gm (1,2), Km (1), Hp-sub, Gc-sub, Tf-sub, Pi, a2HS, SEP, PGM, AK, ADA, GPT, 6-PGD, GLO, EsD. If phenotype frequency was too low or exclusion was not definite in this first analysis, DNA-PCR-Analysis was performed.
DNA was isolated from the remnant blood samples of serological examination using standard methods (Manniatis et al. 1982). The amount of DNA was determined by comparison of the ultraviolet fluorescence of an aliquot of each sample with known quantities of lambda DNA. Size was estimated on agarose gels and in some cases southern blotted and hybridized with single locus probes MS43a or MS31 from Zeneca. PCR reactions were carried out using 20 - 50 ng DNA. Amplification and typing of the HLA-DQα locus followed the recommended protocols (Amplitype™ User Guide). Primer sequences for the D1S80 locus were described by Budowle et al. (1991) and for the YNZ22 (D17S30) locus by Horn et al. (1989) . Reaction

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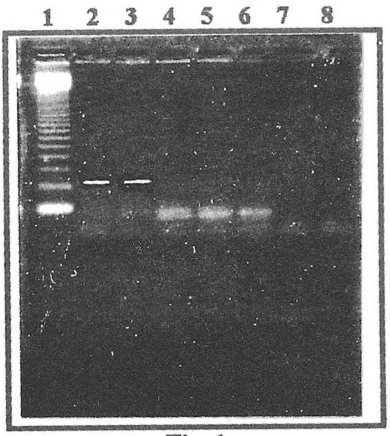

Fig. 1

Lane 1	**Ladder 123**
Lane 2	**PCR Control HLA DQα Locus**
Lane 3,4,5,6,7,8	**PCR products (10 ngr DNA) from bloodstains ten years old without BSA and Hot Start method.**

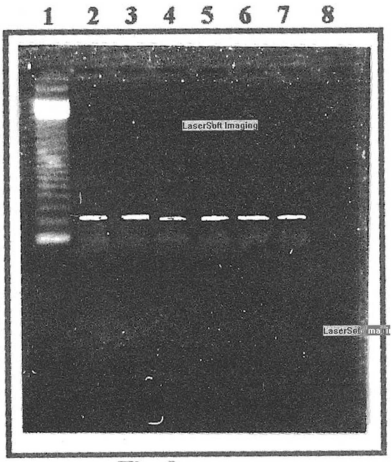

Fig. 2

Lane 1	**Ladder 123**
Lane 2	**PCR Control HLA DQα Locus**
Lane 3,4,5,6,7,8	**Like in Fig.1 but with BSA and Hot Start method.**

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PCR AMPLIFICATION OF DNA FROM OLD BLOOD STAINS

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Introduction

DNA typing from old bloodstains often gives inconclusive results, due to high degradation, as well as several inhibitors and enviromental factors that play a negative role on DNA extraction and amplification.
In order to better the results the Authors have applied the method of "Hot Start"by Mullis and others (1991), according to the technique suggested by Perkin Elmer Cetus.

Materials and Metods

In the manual Hot Start method, all reagents except Amplitaq DNA polymerase are mixed at room temperature though AmpliWax PCR Gems that procedure greatly facilitate a decrease the potential for contaminations.
This procedure involves dispensing one AmpliWax PCR Gems into a tube containing all components of the GeneAmp PCR process with the exception of DNA template, AmpliTaq DNA Polymerase, and buffer, heating and cooling the tube to melt the AmpliWax PCR Gem, thereby creating a solid wax layer above a lower reagent mix.
In the first denaturation step of the GeneAmp PCR process, the wax layer melts, the upper reagent mix thoroughly mixes with the lower reagent mix, and the DNA is denatured (see figure).
The basis principle of hot start PCR is the mixing of DNA and reagents only when both components are at high temperature. This maneuver prevents any interaction of primer and enzyme with DNA templates at low temperatures where specificity of annealing is not stringent. The original protocol has been slightly modified adding BSA (4 μgr/μl) to improve its efficiency.
In this case it was possible to perform the HLA DQα locus amplification after the completely negative results obtained through Chelex extraction.

Results

The characterization of genetic variation at the DNA level has generated significant advances in the forensic identification of individuals. A more flexible approach for individual identification at the DNA level is to use PCR. Specificity PCR provides a technique that is able to analyze small minute amounts of DNA from varios sources (1), including impure or degradated samples (2). DNA analysis from ten years old bloodstains was performed before by standard PCR, and then with HOT START Method (with BSA) (3). Inizially higly degraded human DNA produced negative results (Fig. 1).
In order to increase the likelihood of successfull amplification by PCR and to reduce the risk of contamination, we developed a HOT START Method with BSA. In this case dry and old bloodstains produce positive results.

The result obtained seems very interesting and represent a valid aid which can certainly contribute to the solution of a crime, even if we want to chek them in the near future through other marker and expand them with new samples.

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Predictive value of late onset of mixed chimerism for a relapse: 7 of 36 patients developed a relapse after BMT. 6 of these relapsed patients developed their recurrent disease later than 4 weeks after transplantation and each patient, who developed a relapse, showed host derived hemopoietic cells in his peripheral blood as unstable MC with increasing fraction of host cells. Analysis of the data proved, that **MC with an onset later than 4 weeks after transplantation is highly predictive for a relapse** ($p = 0.017$).

	stable mixed chimerism	relapse
MC < 4 weeks after BMT	4	1[1]
MC < 20% host DNA	3	0
MC > 4 weeks after BMT	1[2]	3
MC > 40% host DNA	0	4

[1]Patient relapsed 2 weeks after BMT, [2]not tested until 4 months after BMT

Correlation of the amount of patient's DNA in MC with the occurrence of relapse: Quantitative determination of host and donor haematopoiesis in 4 out of 6 patients with stable MC always yielded < 20% patient's haemopoiesis. In contrast, 4 of 7 patients, who relapsed, showed more than 20% patient's DNA shortly before their relapse became clinically evident. Thus **MC with more than 20% host DNA is highly correlated with relapse** of the leukaemic disease ($p = 0.008$).

Conclusion

Confirmation of engraftment of HLA-identical allogenic bone marrow after transplantation by detection of donor DNA polymorphism and the differentiation of stable or transient MC from a relapse of the leukaemic disease is important for BMT patients, because the incidence of MC after allogenic bone marrow transplantation is rather high. Patients transplanted with T cell depleted bone marrow developed a stable mixed chimerism in around 25% (7). The detection of host derived DNA often is the only early evidence for reoccurring leukaemic clones. The technique of quantitative RFLP turned out as a **simple and fast method** to distinguish between benign MC and the relapse of leukaemic clones as well as to **confirm the engraftment of the donor stem cells**. The results of this study show also, that **MC occurring early after BMT is not correlated with the later occurrence of a relapse**. Although stable MC could induce immunological tolerance and subsequently lower graft-versus-host (GvH) reaction, a decreased graft-versus-leukaemia (GvL) effect from immunological reasons obviously does not play an important role in the frequency of a later relapse of leukaemic disease. It may be even useful, to establish a stable MC in patients undergoing an allogenic bone marrow transplantation in order to diminish acute graft-versus-host-disease (GvHD).

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Materials and methods

Patients: 36 patients, 4 treated for a CML, 3 for a MDS, 9 for an ALL, 1 for a NHL, 11 for AML, 5 for a SAA, 2 for a MLD and 1 for a Hurler disease were trans-planted between 1990 and 1992 with unpurged bone marrow. Blood and bone marrow samples were analyzed 1 to 3 years after transplantation. Bone marrow biopsies were taken to distinguish between benign mixed chimerism and relapse.

RFLP-Analysis: To evaluate the percentage of donor and recipient haematopoiesis, genetic fingerprints were prepared from peripheral blood and bone marrow of the patient and of the donor before and after transplantation using the minisatellite DNA-probe MZ 1.3 (1, 3). Genomic DNA was prepared after red blood cell-lysis using proteinase K and non-ionic detergent (SDS), removal of proteins with saturated NaCl and DNA-precipitation with pure ethanol. HINF 1 (TAQ 1, HAE 3) was used for restriction enzyme digestion. After gel electrophoresis DNA-fragments were transferred to a positively charged nylon membrane and immobilized, DNA-fragments were hybridized to the digoxigenated DNA-probe MZ 1.3 (2). Hybridized probe was detected by a digoxigenin specific antibody, labelled with alkaline phosphatase and colorimetric staining with BCIP and NBT.

Quantisation of nuclear donor- and recipient cell compartments: Standard curves were established from dilution series of DNA of different size from densitometric scanning of the data and polynomic regression. To measure the recipient nuclear cell compartment, donor and recipient specific bands were evaluated. The ratio between donor- and recipient cell compartment serves as an additional internal standard.

Results

Sensitivity of the method: Recipient specific bands were detectable in mixed dilution experiments down to ratios of 2% recipient-DNA and 98% donor-DNA.

Applications of the method: In each of the 36 cases the RFLP band patterns of the donor and the recipient were sufficiently different (> 2 bands different in size) to distinguish their genotypes (4). This quantitative technique needs the setup of a standard curve only once and enables the user to evaluate fingerprints on different membranes without introducing additional internal standards. We tested the reproducibility of standard curves and sample data by testing the intra-class-correlation-factor. This factor is 0.98 for standard curves considering fragments of sizes between 9.42 kb and 6.56 kb and 0.66 for fragments of sizes between 23.13 kb and 9.42 kb.

The influence of an early mixed chimerism on a later relapse: 6 patients with malignant diseases of the haematopoietic system showed mixed chimerism within the first 4 weeks after transplantation, 1 of them developed an early relapse 10 days after BMT whereas 5 did not show any sign of a relapse during the surveillance period of 1-3 years. We can conclude, that **the appearance of a mixed chimerism is not correlated with an increased incidence of a later relapse**, compared with those patients, who show a complete donor band pattern within the first 6 weeks after transplantation ($p = 1.0$).

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QUANTISATION OF MIXED CHIMERISM IN BLOOD AND BONE MARROW

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Bone marrow transplantation (BMT) in haemato-oncological patients is a recent area of applied DNA analysis. The determination and quantisation of donor cells in samples of blood or bone marrow in allogenic BMT verifies engraftment and detects relapses, especially if there is no specific marker for the leukaemic disease. Quantisation of components in tissues of different genetic origin might also be valuable in mixed lymphoid chimerism (MC) after liver transplantation or in zygosity studies of twins.
We developed a method to quantify MC in BMT patients by density analysis of single bands employing Southern blotting. In detail, we extracted DNA from blood or bone marrow by hypotonic lysis of red blood cells, high salt removal of proteins and ethanol precipitation (2). After digestion of purified DNA by a restriction enzyme (Hinf-I, Hae-III, Taq-I), we separated the DNA fragments by agarose gel electrophoresis, transferred them to a nylon membrane and hybridized with the multilocus probe MZ13. The DNA bands were analysed by automated densitometry (Image Master System, Pharmacia) and the percentage of host cells was calculated by polynomic regression of standard curves, employing common bands of host and donor origin as internal standard. In total, 14 of 36 patients, transplanted with homologous bone marrow for haematological malignancies, developed hemopoietic MC. In 5 patients, MC developed within 4 weeks after BMT, did never exceed 20% host cells and these patients did not relapse during the observation period (5 months to 2 years). 1 patient died of a very early relapse (10 days after BMT) and 7 patients developed "late MC" (> 4 weeks) or MC with > 20% host cells followed by a relapse ($p = 0.0269$). We conclude from these data, that stable "early MC" (< 4 weeks) after BMT does not predict a relapse, whereas "late MC" or MC with > 20% host cell DNA is highly predictive for a leukaemic relapse.

Introduction

Mixed chimerism is the **persistence of recipient blood cells** together with donor cells after **allogenic bone marrow transplantation** (BMT). The reappearance of recipient blood cells after the aplastic period in BMT can either be caused by the coexistence of **benign recipient cells**, resulting in a so-called **stable mixed chimerism**, or by **relapsing malignant recipient blood cells**. Some authors reported, that the occurrence of the mixed chimerism is correlated with a decreased incidence of an acute graft-versus-host-reaction and possibly correlated with a decreased graft-versus-leukaemia-effect (7). Whether a mixed chimerism, appearing early after allogenic bone marrow transplantation is influencing a later relapse, has been discussed controversially (6). The differentiation of a relapse from a stable mixed chimerism using DNA-techniques is still a problem, because often there are no leukaemia specific DNA-sequences available, with the exception of some cases of chronic myelogenic leukaemia (CML) (4, 5). We show in this study, that the **early occurrence of a stable mixed chimerism is not associated with a higher incidence of a relapse**. Taking into account the date of the reappearance and also the percentage of the recipient cells in the patient's blood, it is thus possible to **distinguish between relapse and stable mixed chimerism** using this method.

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RESULTS: PCR showed that several cigarette ends had been smoked by more than one person. One or two persons, besides the victim, were identified. One of them was a man.

CASE N°2: Several sexual assaults were committed in the same area. Circumstances suggested they could be related.
SAMPLES: vaginal swabs, hair, cigarette ends found at the scenes of the crimes, blood from one suspect, cigarette ends known to have been smoked by each of three other suspects.
Genetic typing of the victims was achieved from epithelial cells and/or pubic hair (we did not received blood samples from any of the victims).
RESULTS: Results from semen identification were not clear (scarce remains, only a very small number of spermatozoa heads, poorly preserved, were found in the samples from one of the victims).
According to DQA1 typing of cigarette ends and hairs, one of the suspects was excluded from the scenes of crimes. The other three suspects and two other persons were probably present (TABLE III).

TABLE III

SAMPLES		DQA1
PERSONS INVOLVED:	VICTIMS 1	4, 4
	2	1.1, 1.1
	SUSPECTS 1	1.1, 1.2
	2	4, 4
	3	1.1, 4
	4	1.1, 3
EVIDENCES:	CIGARETTE ENDS	1.1, 1.2
		1.2, 1.1, 4
		1.1, 1.2
		1.2, 2
		1.2(?), 1.1, 4, 2
	HAIRS FOUND IN A CAR	1.2, 2
		4, 4
	PUBIC HAIR: HAIR COMBING VICTIM 1	1.1, 4
	ON VAGINAL SWAB VICTIM 1	1.3, 2

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TABLE I **QUANTIFICATION**

SAMPLE	EXTRACTION METHOD	ADN (ng/μL)
1	PHENOL: NO PK (A.1)	2.0
	PK (B.1)	0.8
	CHELATING RESIN: NO PK (A.2)	1.6
	PK (B.2)	3.8
2	PHENOL: NO PK (A.1)	1.0
	PK (B.1)	0.4
	CHELATING RESIN: NO PK (A.2)	6.4
	PK (B.2)	5.6
3	PHENOL: NO PK (A.1)	0.4
	PK (B.1)	0.4
	CHELATING RESIN: NO PK (A.2)	3.0
	PK (B.2)	2.0
4	PHENOL: NO PK (A.1)	1.2
	PK (B.1)	1.6
	CHELATING RESIN: NO PK (A.2)	3.8
	PK (B.2)	1.8

* The following PROTOCOL was established and used for forensic samples: Pipette 1 mL of sterile destiled water into a 1.5 mL microcentrifuge tube. Add one half of 0.5 cm cigarette end paper and mix gently; Incubate overnight at 4°C; Spin in a microcentrifuge for 2-3 min; Remove supernatant (all but 50μl) and discard; Add sterile distilled water up to 200μL and 2μL of 10 mg/mL proteinase K. Mix gently; Incubate at 37°C for about 2 hours; Add 50 μL of 20% Chelating Resin; Incubate at 37°C for about 1 hour; Vortex at high speed for 5 to 10 seconds; Spin in a microcentrifuge for 10 to 20 seconds; Incubate in a boiling water-bath for 8 minutes; Vortex at high speed for 5 to 10 seconds; Spin in a microcentrifuge for 2 to 3 minutes. Samples ready for amplification. Store and reuse as usual.

* Table II shows the results of individual characterization of experimental samples.

TABLE II **TYPING RESULTS**

	INDIVIDUAL	DQA1	D1S80	DYZ3
1	BLOOD	1.1, 1.2	T24 T36	
	CIGARETTE END	1.1, 1.2	T24 T36	XX
2	BLOOD	1.3, 2	T18 T21	
	CIGARETTE END	1.3, 2	T18 T21	XY
3	BLOOD	1.1, 1.3	T23 T31	
	CIGARETTE END	1.1, 1.3	T23 T31	XX
4	BLOOD	4, 4	T24 T34	
	CIGARETTE END	4, 4	T24 T34	XX

CASE N°1: A woman was found dead in her apartment. The body showed many wounds and bone fractures. No weapon was found. There was no suspect. Besides toxicological and histopathological analysis, the police wanted to know how many people had been in the apartment.
SAMPLES: 24 smoked cigarette ends (tobacco and marihuana) found in different ash-trays.

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USE OF PCR FOR FORENSIC ANALYSIS OF DNA IN CIGARETTE ENDS.

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INTRODUCCION

DNA polymorphic sequences can be amplified up to one million fold by the polymerase chain reaction (Mullis and Faloona, 1987). It is possible to characterise minute samples (a single hair, a diploid cell, or single sperm (Higuchi et al., 1988; Li et al., 1988) as long as the target fragment of DNA remains intact. Good results can also be obtained from very old or deteriorated samples, in which DNA is highly degraded (Beroldingen et al., 1989). Due to the remarkable sensitivity of PCR and in spite of its stringent methodology, use of this technique is rapidly becoming widespread in forensic laboratories, among other reasons because it allows cases to be solved in which sample shortage, age or deterioration impede solution by conventional techniques, often inapplicable. With relative frequency cigarette ends are important biological evidence in forensic casework, sometimes the only clue left by a criminal at the scene of the crime. This work presents the results of identification of the individual from traces of saliva left on a cigarette end, in experimental samples and in two forensic cases, using PCR techniques (DQA1, D1S80, DYZ3 for sex determination).

MATERIAL AND METHODS

- Experimental samples: blood and cigarette ends from 4 individuals (2 cigarettes per person)
- Forensic samples: cigarette ends taken from the scene of crime in criminal or sexual assaults; blood samples from suspect and victim, bloodstains.
- DNA Extraction: Blood and bloodstains: 3 μL blood or 3 mm^2 stain were extracted with chelating resin (Imidoacetic Acid, Sigma) according to Cetus Corporation Technique (Amplitype User Guide Version 2)
- Cigarrete ends: From the proximal end of the cigarette paper, 1/2 cm was cut off, and one half of this was introduced in to a 1.5 mL eppendorf. Four extraction techniques were assayed in experimental samples according to Cetus Corporation protocols: phenol extraction followed by Centricon-100 ultrafiltration and chelating resin extraction were carried out with and without previous proteinkinase digestion.
- Spectrofluorometric DNA quantification: in a DNA fluorometer, TKO, Heofer Scientific Instruments. Fluorescence reagent: Bis-benzimide (Hoescht 33258, Sigma)
Amplification: A Perkin Elmer Cetus DNA Thermal Cycler 480 was used.
- DQA1: Amplitype HLA DQA1 Forensic DNA Amplification and Typing kit (Perkin Elmer). PCR product was detected by hybridisation with allele specific oligonucleotide probes
- D1S80: The D1S80 DNA Amplification Reagent Set (Perkin Elmer) was used, PCR product was detected by flat bed SDS-PAGE followed by silver staining (gel: 8-18% SDS PAA; gel buffer: 0.12 M Tris-Acetate pH 6.4; electrode buffers: anode: 0.3 M Tris-Acetate,4 g/L SDS, pH 6.4, cathode: 0.08 M Tris, 0.8 M Tricine, 4 g/L SDS, pH 7.1; Electrophoretic conditions: 400V, 100 mA, 30 W, 50-55 min).
- DYZ3: According to Witt and Erickson (1989). PCR product was detected by agarose gel (3% Nusieve GTG + 1% low EEO), stained with Ethidium bromide, using as size marker Hinf I digested φ x 174 (Promega)

RESULTS

The EXTRACTION TECHNIQUE of cigarette ends which yielded the best results in quantification (Table I) and amplification of experimental samples was chelating resin extraction of proteinkinase digests.

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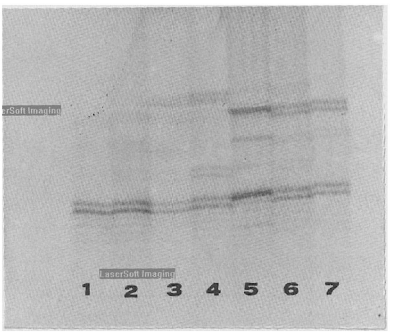

Fig. 1: autoradiogram of the PCR products of the microsatellite locus TC11. In lane 1, 3 and 5 blood control samples typed 183-183, 183-198 and 183-195 respectively In lane 2 DNA from a sample immersed in water (6 months); in lane 4 DNA from a sample buried in soil (6 months); in lane 6 and 7 DNA from the same sample immersed in water and buried in soil respectively. Allele sizes are reported as base pairs (bp)

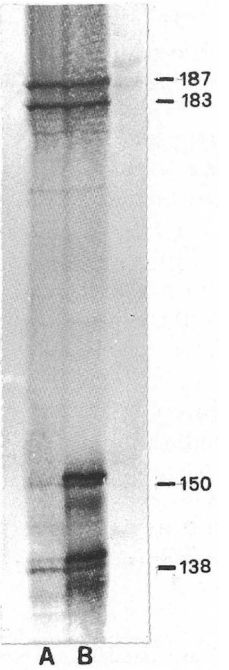

Fig. 2: autoradiogram of the co-electrophoresis of the microsatellite loci TC11 and vWA. DNA from two 50 years old samples: in lane A femur bone sample and in lane B tooth. Allele sizes are in base pairs (bp)

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Results

The results of the storage study are reported in Table I. Human DNA was extracted from all bone samples and , as previously reported by Lee et al. (1991), the yield of DNA /gram of bone was higher in spongy bone than in compact one; in addition DNA from samples buried in soil seemed to be more quickly degraded than that from samples immersed in water. All the samples typed by PCR matched the corresponding blood control sample.

Table I :

	bones immersed in water		bones buried in soil	
	spongy	compact	spongy	compact
Samples	7	7	7	7
Human DNA/gram bone tissue (Averange)	4.5 μg	700 ng	2 μg	75 ng
PCR typing (bone typing matches the corresponding blood control sample)	7/7	7/7	5/7[a]	4/7[a]

[a] PCR analysis failed in five samples , probably because of the presence of uncharacterized PCR inhibitors not removed even in presence of bovine serum albumin.

The results of the characterization of the old bone samples are summarized in table II. No correlation was found between PCR typing, quantity of DNA recovered and the age of the samples. In fact, PCR gave good results in the analysis of a 50 years old femur bone sample from which the quantity of human DNA recovered (30 ng/gram) was higher than in a 15 years old occipital bone sample (20 ng/gram) that could not be typed by PCR. The ability of typing the bone samples by PCR was however in part influenced by the presence of uncharacterized PCR inhibitors coextracted with the DNA. Moreover the good stability of the DNA extracted from teeth was confirmed; the quantity of the DNA recovered from a 50 years old tooth was higher than that recovered from bones with similar age and the sample was successfully typed by PCR for all the genetic markers analyzed.

Table II:

Samples	Human DNA/gram bone	PCR analysis
Temporal bone (7years)	120 ng	+
Tooth (7 years)	80 ng	+
Occipital (15 years)	20 ng	–
Parietal (30 years)	30 ng	–
Femur (30 years)	50 ng	+
Occipital (50 years)	10 ng	–
Vertebra (50 years)	20 ng	–
Femur (50 years)	30 ng	+
Tooth (50 years)	40 ng	+

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APPLICATION OF PCR TECHNIQUE FOR THE CHARACTERIZATION OF HUMAN BONES

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Introduction

One of the sources of more stable DNA and, at times, the only physical evidence of human remains is bone tissue. DNA from bone tissue, even if extensively degraded, can be typed by PCR analysis of loci with a low range of alleles (< 300 bp) such as microsatellites. The aim of this preliminar work is to evaluate the feasibility of genetic identification of human skeletal remains. Moreover we tried to establish a correlation between DNA degradation and storage conditions of bones.
Genetic characterization of bone tissue could be very important for forensic individual identification, expecially if relatives are available for testing.

Materials and methods

Storage study: bone samples were collected in the course of autopsy from 7 cadavers, 24 hours after death. From each one we took a fragment of about 2 grams of spongy (sternum) and compact (femur) bone. These specimens were subjected to different storage conditions: immersed in water or buried in soil for a period of 6 months. Blood samples drawn during autopsy were used as controls for this study.
Old bones: bone samples from unknown skeletal remains were analyzed. In particular the specimens were: 1) fragment of compact bone (temporal) and one tooth (molar) from a partially burnt body recovered in 1986 and stored at room temperature until analyzed (7 years); 2) fragments of compact bone (occipital) from a body that had been recovered in a river and stored at room temperature for 15 years; 3) fragments of compact bone (parietal and femur) recovered in 1964 and stored at room temperature until analysis (30 years); 4) fragments of compact bone (femur,occipital,vertebra,teeth) from different bodies, recovered from a grave, about 50 years old (end of the 2nd World War-1945).
DNA extraction: DNA from bone samples was extracted according to the method described by Hochmeister et al. (1991).
Slot blot analysis: the quantification of human DNA was achieved according to the method of Waye et al. (1989) utilizing an Alu probe.
PCR analysis: the genetic loci analyzed were: HLA-DQ alpha (Helmuth et al.,1990) and microsatellite loci TC11 (Edwards et al.,1992), SE 33 (Polymeropoulos et al.,1992), vWA (Kimpton et al.,1992) and Apo C2 (Weber et al.,1989). The HLA-DQ alpha typing was performed according to the Perkin Elmer Cetus protocol. Microsatellite amplification was carried out including ^{35}S dATP during the reaction. PCR products were fractioned on a 6 % denaturing polyacrilamide gel. Allele sizes were measured relative to DNA sequence ladder derived from a known sequence. In order to prevent PCR inhibition during genetic typing of old bone samples, bovine serum albumin (200 µg/ml) was included in the PCR mix. Finally, positive and negative (no DNA) reactions were routinely included.

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for the PCR-based systems. On the other hand, CM 2 and 6 showed the same YNH-24 genotype. The CM 2 ARA (X chromosmome marker) allele was present in 7. Therefore, biological kinship between CM 2 and 5,6,7 was suggested.

Case 3: TABLE 1 depicts the results obtained. PCR and Southern blot typing systems used showed a clear inclusion of CM 3 as son of 8 and 9.

TABLE 1

	Case 1					Case 2				Case 3		
System	CM1	1	2	3	4	CM2	5	6	7	8	CM3	9
SRY / Y	M	F	M	M	M	M	F	F	F	M	M	F
THO-1	9-10	6-7	9-9	7-9	6-7	6-10	7-10	7-10	6-10	7-9	7-10	10-10
FABP	10-10	10-10	8-13	10-10	10-10	10-11	10-11	10-10	10-11	10-13	13-13	11-13
ARA	-	-	ND	ND	ND	+	-	-	+	ND	+	+
DQ-A	1.3-4	3-4	4-4	1.1-3	1.3-4	1.1-4	1.1-3	1.1-4	1.1-4	2-3	1.2-2	1.2-2
YNH-24/Hinf	ND	ND	ND	ND	ND	2.4-4.4	3.7-4.1	2.4-4.4	3.7-4.1	2.6-3.1	2.6-4.4	2.9-4.4
MS1/Hinf I	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.3-4.4	3.3-3.3	3.3-5.5
PH-30/Hinf	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.5-12.6	7.3-12.6	7.3-7.4

Notes: SRY/Y, F=female M=male. THO-1 and FBAP: numbers indicate the number of repetitions for each allele. ARA: + matching allele, -= no matching allele. YNH-24, MS1 and PH-30 numbers indicate the molecular weight of each allele, expressed in kilobases.

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13 000 x g in a microcentrifuge (or 10 000 rpm in a SS-34 Sorvall rotor) for 10 min. at 4°C. DNA extracted by this procedure is nearly always suitable for restriction assays and PCR amplification ,some cadaveric material may require other options. DNA to be used for Southern blot and PCR analysis were purified by means of Glass Max (BRL) or GeneClean II (Bio101) systems. Samples to be used for PCR amplification were purified with Chelex P-100 (Walsh et al.1991. Sex determination was achieved by means PCR amplifications of: a) SRY of a 299 bp fragment, b) Y specific heterochromatin (Yq_{19-ter}) fragment of 154 bp.**DNA Typing:** *Loci* D2S44, D1S7 and D4S139 were evaluated by means of Southern hybridization assays involving *Hinf I* digests and YNH-24 (ATCC), MS1 (BRL) and PH-30 (BRL) probing.**Short Tandem Repeats (STR)** evaluation: THO-1, FABP and ARA were performed according with Edwards et al.(1991, 1992) **HLA-DQα Typing:** Samples were PCR amplified and detected by means of DQα-Amplitype System,(Cetus-Perkin Elmer).

Case Report 1: The cadaver of a teenager, naturally mummified, whose death may had occured 20 years ago was claimed by potential parents as belonging to a young adult who disappeared last year. In order to establish biological kinship between the cadaveric material (CM 1) and the potential relatives four blood samples (1,2,3,4) were sent to our lab, including two volunteers. Tubes were codified (blind double assay).

Case Report 2: An adult was intentionally burned (CM 2) and three women (5,6,7) claimed to be sisters of the victim.

Case Report 3: A couple (8,9) denounced that immediatelly after the birth of his son the infant had been substituted by a dead one(CM 9).

Results and Discussion

Case 1: DNA obtained from CM 1 was highly degraded, suitable only for PCR based typing. Results are summarized in TABLE 1. Sex determination allowed us to discriminate between the potential mother and the other three male samples. Systems assayed denoted at least one exclusion between CM 1 and samples 1 to 4.

Case 2: Although the cadaver was completely burned, DNA obtained was suitable for PCR and Southern blot typing. Results are summarized in TABLE 1. CM 2 and 7 displayed the same genotype

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Post-Mortem Molecular Identification. Biological Kinship Established by DNA Analysis.

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Introduction:

Molecular characterization allowes to identify individuals when potential relatives are available. Conventional DNA typing complemented with PCR-based forensic analyses such as Short Tandem Repeats (STR) and HLA-DQα evaluation broaden the perspectives in forensic field. The possibility of molecular identification of old cadavers was almost impossible just a few years ago. However, current methodologies made possible the molecular characterization of such remains.

The aim of this presentation is to describe the strategies and methodologies employed in three cases in which from a human body biological kinship evaluation was required. For this report we choose three cases in which cadavers were in different conservational conditions: a- A mummified teenager, b- A burned adult, and c- A semi-putrid newborn.

Based on molecular evaluation, in every case it was possible to establish whether or not biological relatedness existed.

Materials and Methods.

Skin, muscle, teeth and bone were used for DNA extraction in the mummified cadaver, while dermis and muscle were used in the cases of the burned body and the putrid newborn.

DNA extraction was carried out by means of a CTAB extraction protocol (Corach, 1991). Briefly, about 500 mg of tissue are sliced with an scalpel and homogenized in a teflon/glass potter Three volumes of homogenization buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris/HCl, 20mM Na_2EDTA and 0.2% β-mercapto ethanol) were added, following incubation at 60°C for 2-3 hrs with gentle shaking. DNA was extracted with Cl_3CH:isoamyl alcohol (24:1), until the proteic interphase diappeared. Finally, samples are precipitated with 2/3 volumes of isopropyl alcohol, centrifuged

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Quality Assurance and Privacy Protection

The establishment of a national computer system of DNA identification records raises public concerns regarding privacy. The FBI is designing the national DNA identification index and the procedures for its operation to ensure that any intrusion of personal privacy is limited to the greatest extent possible. The DNA records compiled from convicted offenders and crime scenes must remain secure from unauthorized interception and tampering. Some issues related to personal privacy are also operational concerns: CODIS must be both effective and efficient, otherwise false leads that waste limited investigative resources can result.

A detailed description of CODIS, the rules under which CODIS will operate, and the CODIS User Agreement will be made public through publication in the Federal Register. Integrity and confidentiality of the DNA records and the information derived from these records will be maintained through encryption of stored and transmitted data, an access control system, partitioned user authority, backup and restore procedures, agency authentication in transmitted telecommunications (access to the system will be limited to law enforcement crime laboratories), audit logs, and user training. A User Agreement will be developed and enforced through audits and continuous electronic monitoring of controls. This agreement will include the procedures for maintaining CODIS security, analytical testing standards for the acquisition of DNA records, limitations on the information that can be retained, and the procedures for exchanging information between laboratories. Standards will be developed and enforced that ensure DNA records derived from DNA profiling analysis are reliable and comparable.

CODIS potential matches will be confirmed by DNA experts through an exchange of DNA testing data before announcing the match to investigators. In the case of a DNA match that leads to the identification of the perpetrator from the convicted offender index, the match will be confirmed through analysis of a blood sample from the suspect. The DNA analysis of a suspect's blood sample is conducted by the laboratory that processed the case evidence.

The removal of a DNA record in CODIS will be accomplished by electronically overwriting the DNA record, not by marking it as deleted. Furthermore, the confirmation of removal of the DNA record will be forwarded to the laboratory only after the DNA record has been removed from the on-line indexes. Procedures that ensure deleted DNA records are removed from any other medium that may also contain the deleted DNA record, including backup electronic medium, will be followed. The laboratory is therefore assured that even an on-line failure of the system resulting in its restoration by employing backup tapes, will not result in the inadvertent inclusion of the DNA record in the CODIS indexes.

Notes

1. The CODIS pilot laboratories are: Arizona Department of Public Safety, Phoenix, AZ; Broward County Sheriff's Office, Fort Lauderdale, FL; California Department of Justice, Berkeley, CA; Florida Department of Law Enforcement, Tallahassee, FL; Illinois State Police, Springfield, IL; Kansas Bureau of Investigation, Topeka, KS; Metro-Dade County Crime Laboratory, Miami, FL; Minnesota Bureau of Criminal Apprehension, St. Paul, MN; Orange County Sheriff's Office, Santa Ana, CA; Oregon State Police, Portland, OR; Virginia Division of Forensic Sciences, Richmond, VA; Washington State Patrol, Seattle, WA; Washoe County Sheriff's Office, Reno, NV; and the FBI DNA Analysis Unit, Washington, DC.

2. States with enacted legislation establishing files of convicted offender genetic records for law enforcement purposes: Arizona, California, Colorado, Florida, Georgia, Hawaii, Illinois, Iowa, Kansas, Kentucky, Michigan, Minnesota, Missouri, Nevada, North Carolina, Oklahoma, Oregon, South Dakota, Tennessee, Virginia, Washington, and Wisconsin. Additional states are considering enactment of similar legislation.

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will be contained in the forensic index. The local, state, and national forensic indexes will contain the unaccounted for DNA profile(s) from unknown suspect cases as well as DNA profiles derived from the crime scene or other otherwise lawfully obtained evidence in cases where a DNA match has been made and the investigators have identified a suspect. The forensic index will not contain DNA profiles derived from blood exemplars from victims, suspects, or other individuals.

The forensic index is the key to the effectiveness of CODIS. CODIS will conduct regular searches of the forensic index at all three levels (national, state and local) for DNA matches. A verified link between a case for which there is no suspect, and another case, whether or not it has a named suspect, facilitates coordination of investigative activities in a way that may lead to the identification of a serial offender. A match among two or more cases, all of which have the same suspect, can provide additional evidence that will lead to stronger prosecutions. In some cases, the courts may sentence the serial offender using different sentencing standards than if the offender was considered a first-time/one-time offender.

The Convicted Offender Index

The convicted offender index will exist at the national level and in states that have enacted legislation enabling them to maintain such an index. Twenty-two states[2] have enacted legislation that requires individuals convicted of certain crimes, generally including homicide and/or sexual assaults, to provide a sample of their blood for DNA profiling. After the DNA record from crime scene evidence in an unknown suspect case is forwarded to state and national CODIS forensic files, it will be searched against the convicted offender indexes. A match in the convicted offender index will result in a potentially significant new lead for the police investigating the unknown suspect case.

The Population File

The national population file includes DNA records derived from population studies conducted by the CODIS subscribers. These anonymous population DNA records are the basis for the statistical assessment of the significance of a DNA match in case work. The local laboratory has access to national population DNA records in two forms: as raw population data and as statistically processed results. The national CODIS system will perform fixed bin, floating bin, and direct count statistical methods using population groupings which have been previously tested for Hardy-Weinberg proportions and linkage equilibrium. In this way, nationally standardized and validated methods of assessing the statistical significance of a DNA match are available to the local laboratory on a larger number of population DNA records.

The Missing Person Index

The missing person index will exist at the local, state, and national CODIS levels. It will contain the DNA profiles of recovered persons or body parts whose identities are sought by investigators. The missing person index will also contain DNA records of close relatives of missing persons, provided voluntarily or under court order. Searches will be conducted by applying genetic principles of inheritance. Upon resolution of a missing person investigation with a DNA match or through other investigative means, the DNA profiles of living missing persons and their relatives will be expunged from the CODIS indexes. The DNA records from living missing persons and the relatives of missing persons will not be used in the search for criminal suspects.

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CODIS: A NATIONAL INDEX OF DNA IDENTIFICATION RECORDS

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Introduction

In 1990 the FBI began development of a national DNA identification index, CODIS. The concept driving this development is the rarity of a DNA profile obtained from the successful analysis of body fluid stains left at crime scenes. Given the recidivistic nature of rape and other violent crimes, and the fact that body fluids of the perpetrator are left at crime scenes, a national computer-based system of storing and comparing DNA records can result in the successful application of DNA profiling in the fight to prosecute and deter violent criminal behavior. There are now several examples of forensic DNA testing generating previously unavailable investigative leads. Also, this powerful forensic technique has been used to successfully prosecute/adjudicate cases involving the same subject after these cases have been linked together by DNA evidence.

CODIS, a fully integrated local/state/national law enforcement system of DNA records, will establish four files of DNA records: the forensic index, the convicted offender index, the missing persons index, and the population file. DNA records will be forwarded electronically among the indexes. These files will exist at local, state, and national levels, and will be used in the generation of investigative leads by identifying DNA associations among records in the indexes to the DNA record obtained during an investigation of a violent crime or missing person case. The population file, consisting of anonymous DNA profiles, will assist in the statistical interpretation of DNA profiles from case work. The local laboratory retains ownership of its DNA records, and therefore, in addition to determining which forensic DNA profiles are forwarded to the state and national indexes, it also controls which of its submitted DNA records are subsequently removed. The DNA records in each index include only the specimen identification number, the DNA profile and information related to the analytical conditions of the DNA analysis. No personally identifying information or other information unrelated to the identification purposes of the index are included with the DNA record.

The FBI is collaborating with thirteen pilot state and local crime laboratories in ten states[1] which are implementing their respective states' convicted offender laws. Periodic meetings report CODIS development progress and ensure that the needs of the forensic community are met in establishing and maintaining a national DNA identification index. The CODIS pilot laboratories receive development releases of the software for testing. Upon successful completion of testing by the FBI and the CODIS pilot laboratories, the software is implemented in case work and convicted offender DNA profiling.

The Forensic Index

Only the DNA profiles derived from crime scene evidence that cannot be attributed to either the victim or another innocent individual (like the consensual sex partner of a victim)

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In this case the 'single parent' calculation is given by:

$$LR = \frac{1}{4 f_{parent}}$$

The likelihood ratio was calculated to be in the order of 100. **We concluded that body 10 was highly likely to be that of the victim designated V_3 male.**

STAGE 3

Using this information we searched the victims profiles for any bodies which could be related to body 10. Only one body, 99, fitted the criteria:

Again, using the 'single parent' calculation the likelihood ratio was calculated to be approximately 300.

We concluded that the body 99 was very highly likely to be that of the child V_4 female.

STAGE 4

Using the information from the bodies 99 (V_4), 10 (V_3) and K101 (V_1 or V_2), the profiles were inspected to determine whether victim K101 was the mother or aunt of the child V_4. If body K101 represented the mother of body 99 I would have expected to see 5 maternal bands present. However only 3 matching bands were seen and the results were consistent with the body K101 being that of the female V_1, ie the child's aunt.

STAGE 5

Finally, one body, (designated 79), which had given only a very limited DNA profile was found to be consistent with being a 'child' of parents 189 and 'X', and also a parent of the child V_4.

Conclusion

The results of the DNA profiling tests showed that this technique is extremely valuable in assisting in the identification of the victims of mass disasters, provided the appropriate relatives supply blood samples for comparison.

I would like to thank Rachel Atkinson, Mary-Anne Younie and Jonathan Whittaker for their assistance with the preparation of the DNA profiles and discussions concerning the interpretation of the profiles.

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This calculation was employed in the following case.

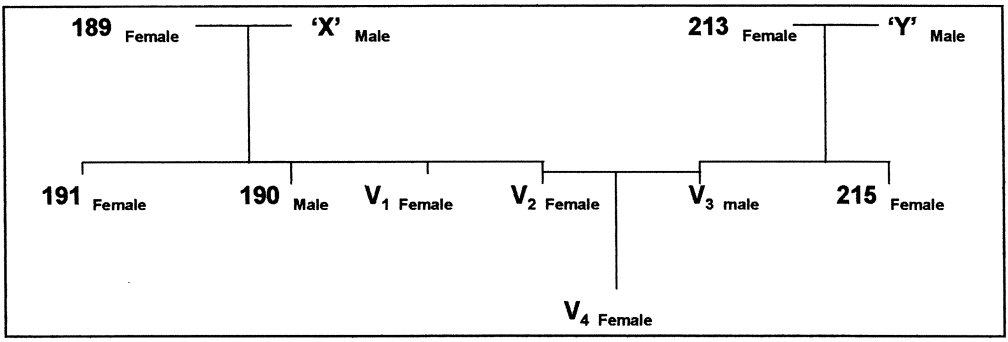
Blood samples were obtained from the mother (206) and father (205) of a female victim.

One body, designated 60, seemed to have a familial connection as for each probe used one band in the DNA profile from the tissue sample matched a band in the profile of the putative father, and the other tissue band matched a band in the profile of the putative mother.

In this particular case the likelihood ratio was calculated to be approximately 140,000 and we concluded that this victim was the child of the mother/father pair 205/206.

Case Study 3

This was the most complex of the analyses tackled. In this case the victims were four members of an extended family, the mother, father and child, and the mothers sister. Blood samples were received from other family members. Blood samples were not received from either of the 'grandfathers'. The extended family tree is shown below:



The problem was broken down into several stages:

STAGE 1 Generation of the profile of 'grandfather X'.

From information contained in the DNA profiles of family members 189, 190 and 191 the profile of 'grandfather X' could be almost completely be constructed. A comparison was then made between the results obtained from all of the tissue samples and the profile from 189 and the constructed profile of 'grandfather X'.

Only one body sample designated K101 fitted the criteria.

The likelihood ratio was calculated to be of the order of 200,000. This was taken to be extremely strong evidence that the body K101 was one of the two offspring of 189 and 'grandfather X'.

At this stage we were unable to determine whether this was the child's (V_4) mother or aunt.

STAGE 2

By inspection of the profiles of the family members 213 and 215 it was possible to construct at least part of the profile of the 'father Y'. Using this constructed profile and the profile of sample 213 we searched the profiles obtained from the body samples. only body 10 fitted the criteria.

Digitized by the Arts & Humanities Research Council (AHRC) funding the Digitization Project

Case Study 1

One of the victims of the aircrash had an identical twin brother who supplied a blood sample for comparison. An exact match was found between the DNA profiles of the relatives blood sample and one body, (designated 72), using four SLP probes.

In this case the likelihood ratio is given by the expression:

$$LR = \frac{1}{2 f_a f_b}$$

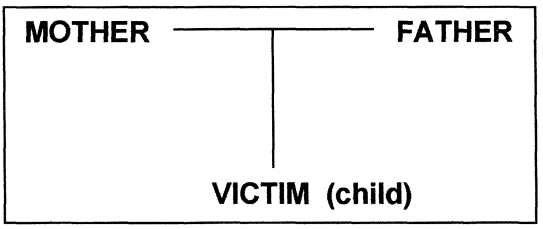
The likelihood ratio was calculated to be approximately 80,000,000.

We concluded that the body 72 was that of the identical twin of the relative submitting the blood sample.

Case Study 2

The majority of the identifications obtained fell into this case type. The victims parents supplied blood samples for comparison:

ie



Where appropriate matches were observed between a victim and a mother/father pair the likelihood ratio was calculated in the following way:

$$p(C \mid F) = \frac{1}{4}$$

ie The probability of obtaining the child's DNA profile if he/she is related to the family in question, and

$$p(C \mid \overline{F}) = \frac{1}{2 f_a f_b}$$

The probability of the profile if the victim is related to another family. Therefore the likelihood ratio is given by the expression:

$$LR = \frac{\frac{1}{4}}{\frac{1}{(2 f_a f_b)}} = \frac{1}{(8 f_a f_b)}$$

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THE USE OF DNA PROFILING IN THE IDENTIFICATION OF VICTIMS OF AN AIRCRASH

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Dr V E Tate Kenyon Emergency Services

On the 28th of September 1992, the Pakistan International Airways flight PK268 crashed into the mountains as it approached Katmandu Airport. 164 passengers and crew were killed. The bodies of the victims were badly damaged by the force of the impact and a subsequent fire.

Kenyon Emergency Services, a UK company specialising in the management of disaster scenes, was contracted to organise the post-mortem examinations, and where possible identify the victims and re-patriate the bodies if the relatives so desired.

The body identifications were carried out by the analysis of documents, odontology the identification of clothing and other personal effects by relatives and by the use of DNA profiling.

In December 1992 we received post-mortem tissue samples taken from 85 of the bodies or body parts at the FSS laboratory in Wetherby. With our assistance Kenyon Emergency Services made arrangements to collect blood samples from direct relatives of the aircrash victims. Between the 18th of December 1992 and the 18th of January 1993 approximately 100 blood samples arrived at the laboratory. Almost all of the relatives who supplied blood samples were European; very few blood samples were received from the relatives of the Pakistani or Nepalese victims.

DNA profiles were prepared from the tissue samples and the blood samples, using standard FSS methods. High molecular weight DNA was obtained from 61 of the tissue samples. The remainder of the tissue samples were in a poor condition and the DNA obtaned from these samples was very degraded. No DNA profiles were obtained from these degraded samples.

The DNA profiles obtained from the tissue samples were compared one to another and in several cases the DNA profiling results indicated that the tissue samples had been taken from a number of parts of the same body. These findings were corroborated by the pathological data.

A total of 57 different DNA profiles were obtained from the tissue samples. Thirty of the tissue samples gave sufficient high molecular weight DNA to enable four or five SLP tests to be carried out. We used the following DNA probes routinely in this investigation; MS43a, MS31, YNH24, pMLJ14 and TBQ7.

The DNA profiles obtained from the tissue samples were compared with the profiles obtained from the blood samples of the relatives. The profiles were inspected for familial relationships. A total of 13 bodies were identified. The DNA profiling tests also gave information to enable us to eliminate many of the families from the identification enquiry.

We used a Baysian type statistical analysis to calculate likelihood ratios for the familial relationships. The complexity of the analysis leading to the identifications ranged from a comparison of the DNA profiles from the tissue samples with the DNA profile of a monozygotic twin brother of one of the victims, to the identification of four members of an extended Spanish family. Examples of the statistical analysis and details of three case types are presented.

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Figure 1
VNTR allele frequencies in the Dutch Caucasian population

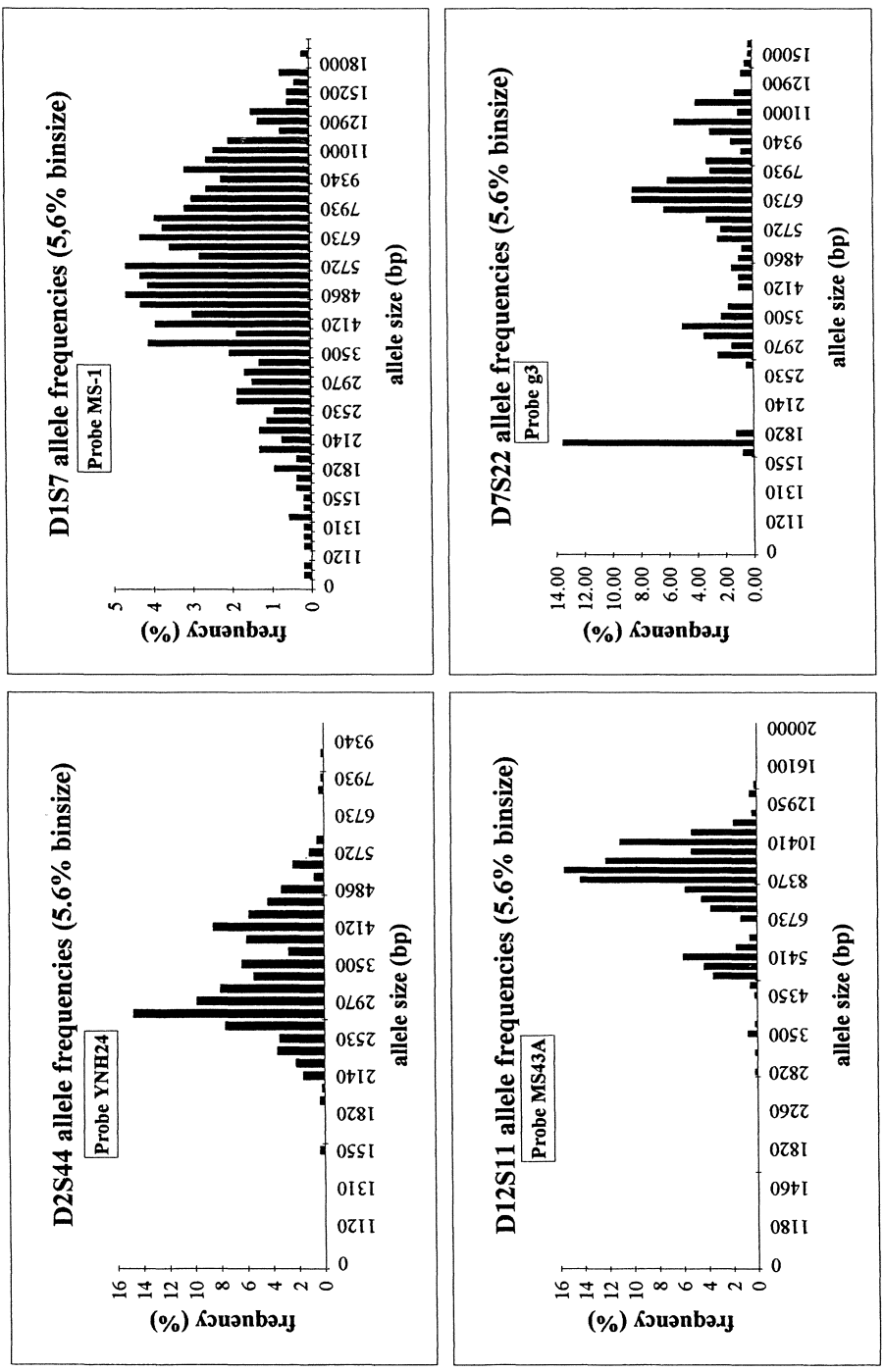

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Table 1 K562 allele length data for four VNTR's.

VNTR-locus	D1S7		D2S44		D7S22		D12S11	
DNA-probe	MS-1		YNH-24		g3		MS43A	
K562 base-pair	allele size (bp)		allele size (bp)		allele size (bp)		allele size (bp)	
measurements	long	short	long	short	long	short	long	short
Average (bp)	4820	4450	4000	2890	7050	1970	13410	5260
Maximum (bp)	4870	4510	4030	2920	7100	1990	13780	5300
Minimum (bp)	4780	4400	3980	2870	6990	1960	13150	5220
1 SD	18	20	12	10	26	8	128	18
CV	0.36	0.46	0.30	0.35	0.37	0.40	0.95	0.34

Table 2 Homozygosity, maximal allele frequencies and maximal genotype frequencies of 4 VNTR loci in the Dutch Caucasian population.

VNTR-locus	D1S7	D2S44	D7S22	D12S11
DNA-probe	MS-1	YNH-24	g3	MS43A
N database	268	275	200	267
% Homozygosity	3.36	7.64	6.00	6.37
bp of max allele frequency	4860 5720	2820	1720	8840
max allele freq (%)	4.66	14.73	13.50	15.54
max genotype freq (%) *	0.43	2.88	2.29	4.42

*: expected maximum genotype frequency (2pq rule)

Table 3 P-values of the quantile chi²-test, using 4 classes

VNTR-locus	P-value
D1S7	0.52
D2S44	0.11
D7S22	0.54
D12S11	0.76

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Results

Matching criteria

The quantitative aspects of our matching criteria were derived from repetitive measurements of human DNA-samples, repetitive measurements of the K562 cell line DNA which runs as a control on every analytical gel and from within-autolumigraph comparisons of DNA from stains from different sources (blood, semen and vaginal fluid) and DNA from liquid blood of the victim c.q. the suspect.

Table 1 shows the relevant data on the K562 DNA for four VNTR loci. The K562 allele sizes are monitored contineously as a QC measure. Except for the 13410 bp fragment for locus MS43A (CV=0.95%) the coefficients of variation (CV) for the K562 alleles were below 0.5%.

The average difference between DNA extracted from stains and the reference DNA comes to -0.26% which indicates that samples which contain degraded DNA tend to migrate faster than undegraded DNA-samples.

About 90% of the band comparisons were within 1.0%, 8% of the band measurements exceeded ± 1.0% and 2% of the comparisons exceeded ±2%. No comparison exceeded ± 2.5%.

These results show that we can safely compare and identify our DNA profiles on the ±2.8% matching guideline which has been proposed by Berry and Evett [5].

Population Genetics

The predefined conservative matching guide line (±2.8%) was used to generate the allele frequency histograms for the VNTR loci. The bin sizes in the histograms are defined by: bin-size = {0.056*fragment length}.

Allele frequency profiles of each system are reported in figures 1a-1d.

From the genotype distributions (not shown) it was concluded that there is little if any correlation between the paternal and maternal fragment lengths in the 4 VNTR loci. We actually tested for deviations from Hardy-Weinberg equilibrium using the quantile chi²-test [4]. Our test results did not indicate deviations from H-W equilibrium for either of the four loci.

Table 2 shows the observed homozygosity, the most common alleles and the most common genotype-frequencies of the four loci.

Exact comparisons with other Caucasian population samples could not be made. However the shapes of the frequency distributions found in this study are comparable with those reported for other Caucasian population samples.

Conclusion

SLP DNA Profiling has been successfully applied over the last three years at the Netherlands Forensic Science Laboratory at providing strong evidence from seminal stains and bloodstains.

Combined with other extensive validation studies this population genetic study will allow the typing of VNTR-loci to be used in forensic identity testing in the Netherlands.

The inter- and intragel variations of fragment length measurements in terms of basepairs were investigated. The variation in terms of % basepairs between duplicate measurements of control DNA and between DNA obtained from blood and stain-material was constant in the region 2-8 Kbp but increased slightly in the region of fragment lengths over 10 Kbp. Although the set window of ±2.8% is highly conservative, especially in the region of 2-8 Kbp it is highly convenient to use a window of constant width in terms of % basepairs.

We have found no evidence for the relationship between the paternal and maternal fragment lengths in any of the four loci. These results allow the use of the 2pq rule to calculate genotype frequencies where p and q are the respective allele frequencies.

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Forensic DNA-typing in the Netherlands using VNTR Single Locus Probes

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Introduction

Variable number of repeat (VNTR) base sequences are one of the most informative genetic DNA-polymorphisms for identification purposes. We have implemented the analysis of VNTR-loci at the Netherlands Forensic Science Institute using the Single Locus Probe hybridization technique. This technique is essentially the same one as agreed upon by the European DNA Profiling (EDNAP) group *[3]*. The forensic use of this technique is regarded as valid and reliable *[1,2]*. However, interpretation of the test results is highly dependent on the population genetics of the various DNA-markers and the forensic validation of this DNA-test in the Netherlands requires:

1. the implementation of match criteria for the comparison of DNA-profiles.
2. the collection of allele frequency data from the relevant population(s).

To this end allele frequency distributions of four different VNTR loci (D1S7, D2S44, D12S11 and D7S22) were collected in a population sample of 275 Dutch Caucasians. The criteria for the evaluation of matching samples were obtained from the interlab variations in allele size measurements of matching samples and K-562 genomic control DNA.

We have also evaluated the actual independence of VNTR allele-frequency data within the four different loci. With the results of this study an allele frequency of an estimated fragment is calculated by counting the number of alleles from the population database within the 2 extremes of the set window.

Methods

Population sample

Blood was obtained from unrelated male and female Dutch Caucasian donors.

Electrophoretic system and DNA-probes

Hinf I digested DNA was electrophoresed according to the EDNAP protocol *[3]* and Southern blotted onto Nylon membranes. Membranes were hybridized according to the manufactures instructions with synthetic oligonucleotide VNTR-probes which were already labeled with alkaline phosphatase.

The DNA-fragments were detected by chemiluminescence.

Analysis of band positions

The length of the DNA-fragments was calculated by reference to the DNA-analysis system (BRL) using the Pharmacia Gelscan XL laserscan with the Gelscan XL (version 2.1) software. HinfI restricted K562 genomic control DNA was included on every analytical gel.

Statistics

Allele-frequencies were estimated for each VNTR-locus by counting the number of fragments within a given interval and dividing by the total number of alleles in the database. The estimation of the allele frequencies was highly facilitated by using the Excel 4.0 frequency function. The homozygote frequency for each locus was obtained by counting the number of individuals with a one fragment pattern.

We tested for deviations from Hardy Weinberg equilibrium using the quantile chi-test proposed by Geisser and Johnson *[4]*.

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DISCUSSION

Besides confirming the results achieved in our previous experiments, this case stresses once more that in order to solve crime cases, it is very important to have a proper analytical strategy which enables to type more possible loci and, among them, especially those which are highly polymorphic. It also confirms that the use of Luminol is practical, effective and extremely sensitive and suited for aged and/or latent blood stains.It also indicates that choosing an appropriate method for DNA extraction is of basic importance.
Finally this case shows the high potentials of forensic biology. Therefore it is necessary and appropriate to analyse evidence that may appear traceless and could be discarded after a superficial analysis because they are considered useless for investigation purposes.

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Luminol Test

The stains were subsequently assayed by the Luminol Test. Three compounds were used in order to perform the test:1) 0.5 gr. of Luminol (3-Aminophtalhydrazide); 2) 25 gr. of sodium carbonate; 3) 3.5 gr. of sodium perborate in 500ml. of distilled water. The stains were sprayed with a 2% hydrochloric acid solution and then with a mixture of the previous three compounds mixed together.

DNA EXTRACTION

The stains which were positive to the presumptive tests were cut into small pieces and placed into a 2 ml microcentrifuge tube where an aliquot of a yellow resin portion (DNA SINGLE TUBE - BIOTECX Houston, Texas cat.NO.BL5500) had previously been added. The following steps of extraction proceeded according to the standard phenol-chloroform extraction protocol and the instructions given by Biotecx Bullettin NO. 8,1990.

AMPLIFICATION

Amplification and typing of the HLA-DQ alpha locus.

DNA was amplified by PCR using the Amplitype HLA-DQ alfa Forensic DNA Amplification and Typing Kit (Cetus Corporation,Emeryville, CA).

Amplification and typing of the VNTR's loci D1S80 and Apo B.

Amplification of the VNTR's loci D1S80 and Apo B was carried out by the methods previously described by Budowle et al. and Boerwinkle et al., respectively. The electrophoretical separation of the P.C.R. products was perfomed using a high resolution horizontal rehydratable polyacrylamide gel as described by Budowle et al.. The gel was exposed through silver stain.

RESULTS

The preliminary blood tests using both the Sangur strips and the Benzidine reaction produced negative results,while the treatment with Luminol was positive and it showed the areas originally stained with blood by producing a characteristic bluish-white luminescence. As to amplification and further typing of the three loci taken into account, HLA-DQ alpha provided results which could not be interpreted since they were probably due to contaminants and inhibitors connected to the substances the evidence got in contact with in the river.Very good results, on the other hand, were obtained both with D1S80 and with APO B with well-defined bands which proved to match with the victim's blood.

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ABOUT A CASE OF DNA ANALYSIS FROM SOME LATENT BLOOD STAINS ON OVERALLS FOUND IN A RIVER.

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INTRODUCTION

A major problem in forensic cases concerns the identification of the nature of blood samples and the extraction of a maximum amount of DNA suitable for PCR reaction. In the most serious criminal cases it is quite frequent to find out that surfaces which were probably stained with the blood of the victims (clothes, car tapestry, etc.), underwent careful washing in order to remove traces of the crime.As we reported earlier, DNA was extracted from 5 ul experimental blood stains, hand-dried and machine washed,then amplified by PCR and finally typed by horizontal acrylamide gel for the three most common loci (HLA, D1S80 andAPO B), with a yield of 74% positive results.In this paper we report a real case linked to a murder where the evidence to be analysed was a mechanic's overalls collected from a river. Some suspected halos of probable blood were first singled out by means of catalytic tests then amplified and typed by PCR for the three VNTR's, showing a match with the victim's blood.

MATERIAL AND METHODS

The sample was a mechanic's overalls thrown away in a river by the suspect, just after the murder, hoping to eliminate the evidence of the crime. It was found by the police, in a container where all the trash collected from the river was deposited, within a river water draining plant. An accomplice suggested the police to go there, and look for the overalls. He was the author of another murder commited on that same day and had provided investigators with precise indications to prove he did not commit the first murder. The overalls were brought to the lab still damp and besides the many holes and rips, probably caused by its being thrown into the river, the front part had some small suspect areas, though nothing would make us think that it was blood. Just the same, we decided to test these suspect areas to see if there was some blood.

BLOOD IDENTIFICATION

Sangur test

The suspect areas underwent the Sangur Test strips (Boehringer Mannheim, Sangur Test Strips Cat. No.126233) which were previously dipped in a physiological solution (0.93 % NaCl).

Benzidine Test

Small cloth portions scraped from the same areas were put on a filter paper and treated with 1-2 drops of the Benzidine reagent (glacial acetic acid saturated solution of benzidine base) followed by 1-2- drops of 3% hydrogen peroxide.

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Panel a shows example results from liquid blood.
1 week - Satisfactory detection of bands.

4 weeks - Expected bands detected when stored at room temperature and outdoors but no detectable bands from material stored at 37°C.

Panel b Example results from liquid semen

1 week - Expected bands are clearly detectable.

4 weeks - No bands detected in material stored at 37°C. Expected bands detected from material stored at room temperature but additional bands also seen.

Panel c Example results from stains stored at room temperature.

Semen stains show additional band after 1 week and no bands after 4 weeks.

CONCLUSIONS

These results indicate DNA may be more labile than previously observed.

DNA from liquid semen shows some degradation even within 1 week.

Material extracted from liquid blood and semen stored at 37°C shows weak band patterns after one week and no bands at all were detected after 4 weeks.

DNA in bloodstains is considerably more stable and typing possible after 4 weeks.

The observation in some instances in material from liquid semen and semen stains, both stored at room temperature, of bands additional to those expected gives cause for concern and warrants further investigation. In this limited study although such bands were seen in association with the bands which were expected to be present in the sample they could appear stronger than the expected bands.

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Analysis of the extracted DNA by mini gel agarose electrophoresis with ethidium bromide provided information on the quality of the DNA extracted.

Figure 1a and 1b show example results from liquid blood and semen samples from one individual stored for up to 4 weeks under three different temperatures.

DNA from the semen shows degradation after one week.

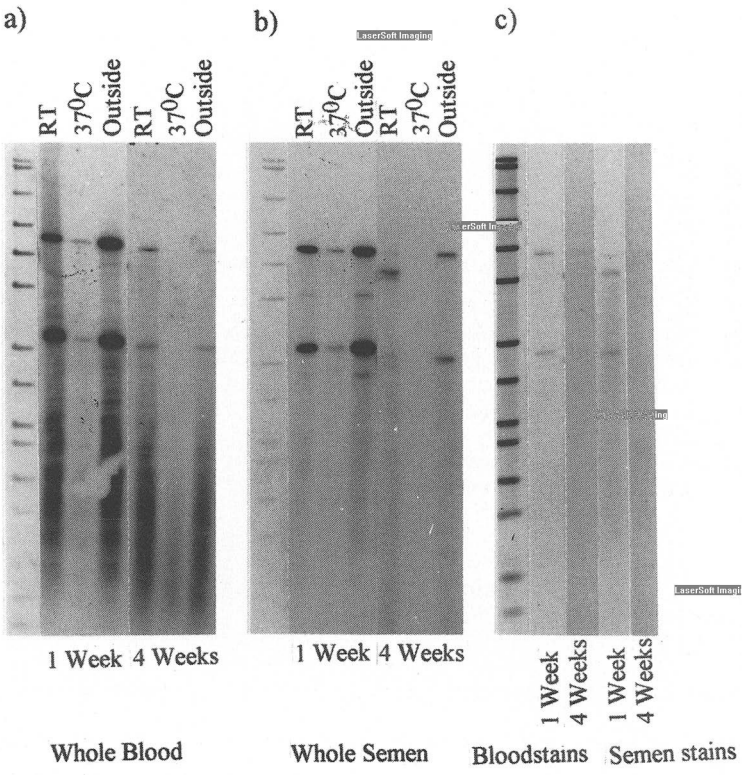
DNA from blood shows a discrete band of high mol. weight DNA after four weeks, even when stored at 37°C.

Figures 1c and 1d show example results from stain material.

DNA from the bloodstain gives clean, high mol. weight DNA after four weeks.

DNA from the semen stains show some degradation even after 1 week.

Figure 2 shows examples of MS43 probe test results



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Stains of the blood and semen were made on cotton cloth, air dried and stored at room temperature for up to four weeks.

DNA was extracted from 200ul of liquid blood and semen samples by a conventional phenol/chloroform method except that with the semen samples the addition of 20ul of DTT (10mg/ml stock) was included in the proteinase K digestion step.

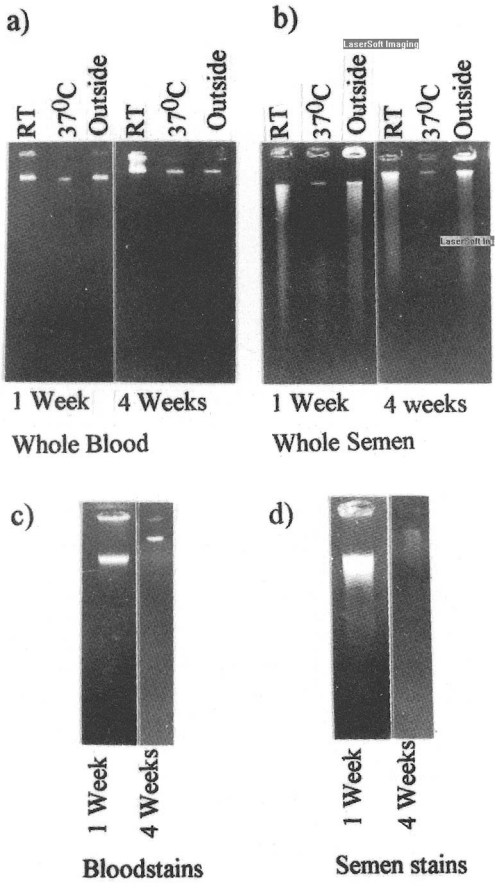
Stain material measuring 1 cm by 1 cm was extracted overnight at 37C in 400ul extraction buffer* plus 40ul each of proteinase K (10mg/ml) and DTT. Subsequent extraction was as for liquid blood and semen.

All samples were subsequently digested with Alu 1, analysed by mini-gel electrophoresis and then subjected to Southern blot analysis using the MS43 probe.

*100mM Tris, 10mM Na2 EDTA, 100mM NaCl, 2% SDS

RESULTS

Figure 1 Quality of DNA recovered



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The degradation of a VNTR polymorphic site in various biological specimens when subjected to different temperature conditions

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SUMMARY

Liquid blood and semen samples were left at room temperature, 37°C and outdoors temperature for four weeks.

Stains of blood and semen were left for up to four weeks at room temperature.

After digestion with Alu1, extracted DNA samples were analysed by mini gel electrophoresis for degradation and then the polymorphic VNTR locus MS43 was investigated by Southern blot electrophoresis.

In addition to the finding of evidence of degradation of the DNA, the appearance of bands additional to and stronger than the expected bands after probing with MS43, was an unexpected observation.

INTRODUCTION

Both discrimination power and stability are major criteria relating to the usefulness of polymorphic genetic markers in forensic problems.

There is some evidence for extreme stability of DNA from workers who have extracted small fragments of intact DNA from mummified material (Paabo, 1989).

High molecular weight DNA is required for the single locus polymorphisms currently widely used and whilst some workers have reported successful storage of extracted DNA for up to 20 years at -70°C (Madisen et.al. 1987) and others produced profiles from blood and semen stains up to four years old (Gill et.al. 1985), case work material can be subjected to various unfavourable conditions.

The experiments described here investigate the effect of one condition, temperature, over a period of four weeks.

MATERIALS AND METHODS

Paired EDTA blood and semen samples from 3 individuals were aliquoted into tubes and stored at room temperature, 37°C and outdoors for up to four weeks (in September/October).

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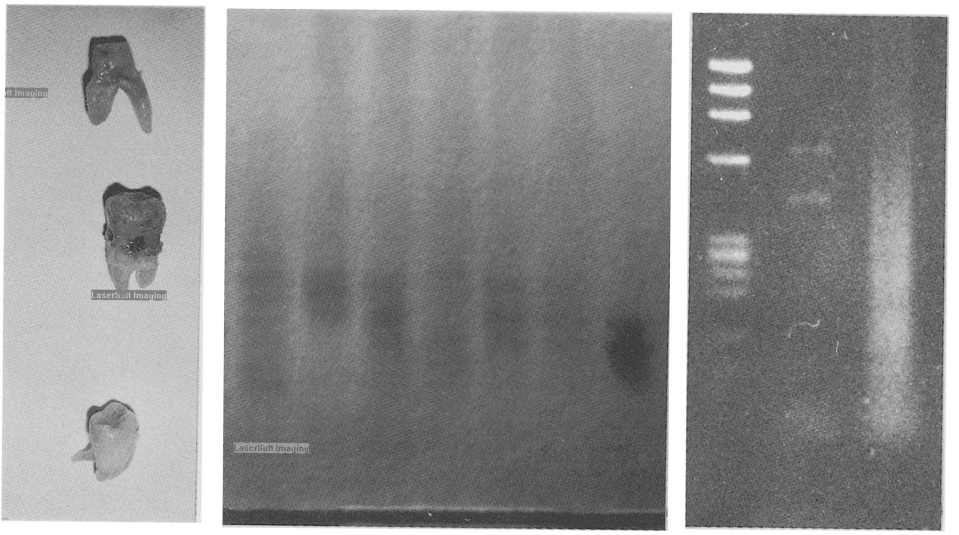

Fig. 1 : THE TEETH USED FOR THE EXTRACTIONS
Fig. 2 : STUDY OF HumTH01
Fig. 3 : STUDY OF THE D1S80 LOCUS (CHELEX EXTRACTION)

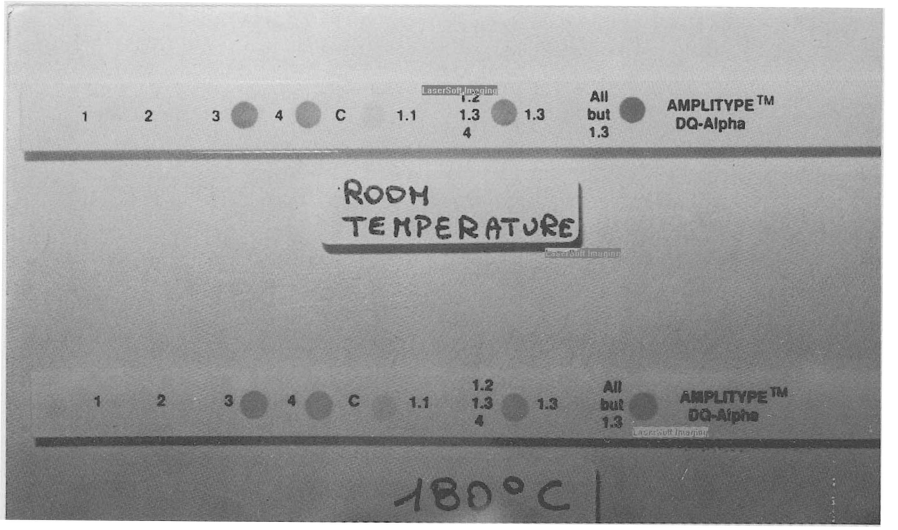

Fig. 4 : STUDY OF THE DQα LOCUS

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crilamide gel (T 8% ; C 5%) followed by silver staining.

Condition of exposure
We exposed teeth to standard maintenance or aggressive conditions. The first group of conditions were: room temperature, refrigerator (4-8 'C), freezer (-20'C) whether immediatly after extraction or within a period of conservation (less than three months) both under various humidity conditions (60-80-100%) and dry environment.The aggressive conditions were both chemical (Hydrogen Peroxide, Hydrocloric acid 0,01 M, Sodium Hydroxide solution 0,01 M, Sodium-Cloride and water) and physical (180'C, 250 'C 800 'C); the specimens were exposed for a short period (30 minutes) only in one half of sectionated teeth (the other half of each tooth was under standard mantenance conditions)

RESULTS AND DISCUSSION
Good results with Chelex extraction protocol were obtained with
DQalpha and TH01 locus studies, instead smear results followed the application of Chelex protocol with D1S80, but for this locus study good results were obtained with phenol-chloroform extraction protocol.DQalpha gave for all conditions good results with exception of the entire set of blue spot indicating the detection of DNA (but degradated and untypified) in the specimen exposed directly at 800' C for 30 minutes. The test executed for comparative reasons on an half of the tooth or on blood indicated always the precision of the technique.TH01 was carried out in the most interesting aggressive conditions giving similar results to DQalpha.D1S80 was supplying favourable answers only in standard maintenance conditions, and in exposition at 180' C too, but only with phenol-chloroform protocol; no results were obtained with the specimen submitted to 800' C.
Significative differencies were found in all situations where a too small amount of substance (usefull and no atrophic matter) was disponible depending both on age of the patients (from 15 to 70 years) and on type of tooth (from incisive to molars), and on integrity of pulp material (from healthy to seriously decayed and infected teeth).

CONCLUSIONS
The positive results obtained in this study encourage us to further application on different loci and in experimental aggressive conditions to simulate true cases of seriously damaged human remains analysis.

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GENETIC IDENTIFICATION FROM DENTAL PULP BY USING DNA AMPLIFICATION (PCR)

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INTRODUCTION

The particular dental envelope resistance to environmental factors and putrefactive phenomena allows to obtain idoneus DNA for amplification by PCR and to use these human remains for forensic purposes so, the dental pulp permits to acquire genetic profiles and to compare these with familiar patterns.In forensic science is also important to determine how environmental factors can effect the human DNA. This kind of study is not very represented in literature. Infact the recent introduction of PCR method allows to carry out these analysis using a small amount of substance and exceed the limitations of Southern Blot.To evaluate the possibility offered by dental pulp and the applicability of different genetic markers, the authors have typified DNA obtained from teeth subjected to various environmental conditions and they have studied three different markers: the DQalpha locus, a VNTR system (D1S80 locus) and a STR system (HumTH01).

MATERIAL AND METHODS

Samples

The teeth were obtained from extraction performed on patients of the Dental Clinic of our University. The specimens were whether healthy or decayed, extracted for therapeutic (orthodontic) or pathologic (periodontology) reasons, from different teeth of patients variously aged. For comparison reasons some peripheral blood samples were prelevated.

DNA Extraction

DNA extraction was carried out using two different protocols: modified Chelex according to Walsh (1991) and the classical method of phenol/chloroform after an incubation with proteinase K (20 mg/ml).

Analysed system

DQalpha Locus: HLA DQalpha genotyping was performed with Amplitype HLA-DQaplha Forensic DNA amplification and typing kit. PCR was carried out at 94'C - 65'C and 72 'C for 30 cycles and the amplification products were identified by the dot blot tecnique according to Perkin Elmer protocol.

D1S80 Locus: The samples were amplified by using D1S80 Forensic Set by Perkin Elmer. PCR was carried out at 94'C - 65'C and 72'C for 30 cycles and the amplification products have been separated by agarose gel electrophoresis followed by staining with ethidium bromide

HumTH01 System: The amplification was carried out according to Edwards et al. (1992) with some modifications. The amplification fragments were separated by electrophoresis using polya-

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C. DNA Quality and Quantity

High molecular weight (HMW) DNA was isolated from 12 of the 15 recovered bloodstains (stains 1,5, 7, 8, 9, 16, 17, 19, 23, 24, 25, and 26). DNA isolated from three stains (4, 18, 21) exhibited partial degradation. Two of these stains (4 and 21) were found in contact with the soil. For test bloodstains yielding HMW DNA, recoveries varied from 5 to 80% when compared to surface control samples.

D. DNA Typing Analysis

The feasibility of obtaining reliable DNA typing profiles from the 15 recovered bloodstains was determined using single locus RFLP analysis. DNA typing results using VNTR probes D4S139 and D17S79, and alpha satellite probes D7Z2 and DYZ1 are summarized below:

1. Eleven bloodstains (1,5,16,17,18,19,21,23,24,25 and 26) matched[4] the DNA profile of donor control samples. Two stains (18 and 21) had very weak DNA band intensities due to the degraded nature of these samples.
2. Two bloodstains (7 and 8) showed partial *Hae* III digestion patterns. One of these stains (8) had been found exposed to the soil.
3. One bloodstain (4) exhibited altered electrophoretic migration. This stain was on a mattress surface which was found soaked with water and exposed to the soil.
4. One bloodstain sample (9) produced no VNTR profile. This stain was prepared on a hammer which was found exposed to the soil.

All DNA typing profile matches were within 1.8 % of the band size of donor control samples. All surface control samples yielded DNA profiles consistent with blood donor control samples. One surface control sample (23) displayed a partial *Hae* III digestion pattern. This was not observed with the test bloodstain sample.

CONCLUSIONS

1. Due to the destructive nature of a natural gas explosion, only a portion of the original bloodstains were recovered (15 of 26).
2. All recovered bloodstains exhibited surface charring, but tested positive for blood using the Hemastix and hemochromogen assays.
3. The majority of bloodstains (11 of 15) yielded DNA typing profiles consistent with the donor control samples. Partial digestion, altered migration, and weak intensity bands, were encountered with the remaining four samples. Three of these stains were found exposed to the soil.
4. This study indicates that bloodstains exposed to a high temperature natural gas explosion, can yield reliable RFLP DNA typing results in the absence of additional environmental stresses.

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4. For a match to occur between two DNA profiles, all corresponding DNA bands must visually match, and their estimated lengths must be within a match window of ± 2.6% of their mean size (RCMP DNA Analysis Protocol).

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Bloodstains were prepared from fresh liquid blood collected in EDTA-containing blood collection tubes. A total of twenty five 200 ul stains were prepared one day prior to the explosion in all 6 rooms of the house. One stain (#17) was produced during the explosion, by dispersal of a fresh pool of blood placed on the chest surface of a mannequin. In general, all surfaces were in poor condition and no attempt was made to pre-clean any surface prior to application of blood. Surface controls were obtained either by placing an equivalent test volume of blood on a fragment of the surface, or by removing an equivalent test volume of blood from an adjacent test area with a cotton swab.

The house was filled with natural gas to a final 10% mixture with air and ignited by an electrical spark placed in the living room. Spot fires were extinguished by Fire Department personnel using water shortly after the explosion. Bloodstains were exposed to the elements for approximately 3 hours prior to their collection. Those found on cloth surfaces were removed by cutting out a fragment. Those present on solid surfaces were removed using a moistened cotton swab. A total of 7 hours elapsed between the explosion and storage at -20°C. The weather for the experiment was calm and sunny with an air temperature of approximately 7°C.

DNA was extracted from control and test bloodstain samples using standard proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation methods [2]. DNA quality was evaluated by agarose gel electrophoresis and ethidium bromide fluorescence staining. The quantity of human DNA isolated was evaluated using a human specific alpha-satellite probe, D17Z1 [3]. Purified DNA was analyzed for restriction fragment length polymorphisms (RFLP) using *Hae* III restriction digestion and Southern blot hybridization techniques. Radioactively labelled, single locus, variable number of tandem repeat (VNTR) probes D17S79 and D4S139 were used to detect DNA alleles. Alpha satellite probes D7Z2 and DYZ1 were used as control probes to evaluate electrophoretic migration and *Hae* III digestion.

RESULTS AND DISCUSSION

A. Explosion and Bloodstain Recovery

The house was completely destroyed by the explosion, with the majority of the debris falling over a 50 m radius. Only 15 of the 26 stained surfaces were recovered from the explosion debris (stains 1, 4, 5, 7, 8, 9, 16, 17, 18, 19, 21, 23, 24, 25, and 26), representing all but one room of the original house. All recovered bloodstains exhibited surface charring, caused either by the heat of the explosion or by exposure to the resulting fireball. Several mattresses were destroyed by fire. Other bloodstains were either destroyed by fire, disintegrated, or remained buried in the basement rubble. Bloodstains on large durable surfaces survived the explosion and were readily located in the explosion debris. Several bloodstains were exposed to additional environmental stresses including water damage (stain 4) and contact with soil/mud (stains 4, 8, 9, and 21)

B. Blood Identification Tests

All recovered stains tested positive for blood using the Hemastix (Miles Laboratory, Rexdale, Ontario) presumptive test, and by the hemochromogen (Takayama) crystal test.

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DNA ANALYSIS OF HUMAN BLOOD RECOVERED FROM EXPLOSION DEBRIS

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A detailed study was initiated into natural gas migration in a residence and the effects of a natural gas explosion on both physical and biological trace evidence. Personnel from several Provincial and Federal agencies participated in this study [1]. The biological portion consisted of two experiments: 1. To determine if a simulated homicide scene could be recreated from blood spatter evidence recovered from the explosion debris. 2. To determine the effects of a brief, but high temperature, natural gas explosion on the ability to obtain reliable DNA typing results from exposed bloodstains.

This paper summarizes the results obtained from DNA analysis of bloodstains subjected to the destructive forces of a natural gas explosion in a residence. The experimental conditions were chosen to be as realistic as possible, with a fully furnished three bedroom house used as a test model. The following experimental parameters were examined.

1. To determine if bloodstain location relative to the ignition point and the nature of the surface material used, is important for both recovery and reliability of DNA typing results.
2. To compare and contrast DNA typing results obtained from dried bloodstains and stains made during the actual explosion from a pool of liquid blood.
3. To determine the quality and quantity of DNA isolated from exposed bloodstains.

MATERIALS AND METHODS

A fully furnished bungalow of approximately 80 sq m was used to study the effects of a natural gas explosion on bloodstain evidence. The surfaces tested and their composition are listed in TABLE 1.

TABLE 1: EXPERIMENTAL BLOODSTAINED SURFACES

1. Bathtub (enamel)	14. Bed Linen, BedRm #3 (cloth)
2. Bathroom sink (ceramic)	15. Mattress-7, BedRm #3 (cloth)
3. Bathroom tile (synthetic)	16. Pants, BedRm #3 (polyester)
4. Mattress-1, BedRm #1 (cloth)	17. Sweater, BedRm #3 (synthetic)
5. Crib frame, BedRm #1 (wood)	18. Kitchen Counter (synthetic)
6. Mattress-2, BedRm #1 (cloth)	19. Kitchen Fridge (enamel)
7. Mattress-3, BedRm #1 (cloth)	20. Kitchen Wall Paper (cellulose)
8. Hammer handle, BedRm #1 (wood)	21. Kitchen Sink (stainless steel)
9. Hammer head, BedRm #1 (metal)	22. Blouse (synthetic)
10. Mattress-4, BedRm #2 (cloth)	23. Sofa arm-1, LvgRm (cloth)
11. Mattress-5, BedRm #2 (cloth)	24. Sofa arm rest-2, LvgRm (cloth)
12. Window Frame, BedRm #2 (wood)	25. Stereo cabinet, LvgRm (wood)
13. Mattress-6, BedRm #3 (cloth)	26. Floor tile, LvgRm (synthetic)

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BODY IDENTIFICATIONS

Other important application of the single locus DNA profiling technique carried out in our laboratory has been the identification of cadaveric remains (carbonized bodies, dismembered corpses...) by comparision of the DNA profile obtained from the evidence to the DNA profile of family members.

The following cases and samples has been analyzed:

CASE TYPE	NUMBER	SAMPLE ANALYZED	SUCCESSFUL RATE
Carbonized bodies	12	Blood and muscle	75 %
Dismembered corpses	4	Blood, muscle, skin, hair, liver...	100 %
Other unidentified cadavers	12	Blood and muscle	100 %

We have obtained a DNA profile in 23 cases and in 7 cases, the persons have been identified by paternity testing.

FETAL REMAINS

The analysis of fetal tissues in rape cases and deserted newborn in cases of ilegal abortions and infanticides has been another application of the RFLP technology carried out in our laboratory.

The following table shows the type of sample and ssucceful rate:

SAMPLE ANALYZED	NUMBER	SUCCESSFUL RATE
Fetal remains	12	83 %
Newborns	3	100 %

ACKNOWLEDGEMENTS.

We are grateful to Cristina Agüero for her colaboration in the microscopical analysis of semenstains.

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RESULTS

SEMEN STAINS

The analysis of seminal stains is the most common type of case analyzed in our laboratory. In these cases, the preferential extraction procedure for separating spermatic DNA from the DNA of ephitelial cells is normally used [3]. The phenol/Chloroform extract was washed and concentrated by ultrafiltration (Centricon-100 concetrator) instead of ethanol precipitation. This procedure has two main advantages over ethanol precipitation:

-The DNA is always maintained in solution and the redisolution problems are abolished.

-The ultrafiltration method allows microsoluble removal which can be of importance to prepare the DNA extract for PCR amplification.

Table 2 shows the range of exhibits examined in our laboratoty. As can be seen, the vaginal samples and the knickers are the most common items submitted for DNA profiling.

EXHIBIT	NUMBER	SUCCESFUL RATE (%)
Vaginal swabs or washing	44	66
Knickers	30	77
Other clothing	39	54
Other semen remains	5	100
Anal swabs	4	0
Condoms	2	50

Table 2. Exhibit types examined at the INT of Madrid.

Sperm concentration on the exhibits was assesed microscopically. The relationship between sperm concentration and successful rate is shows in Fig 1.

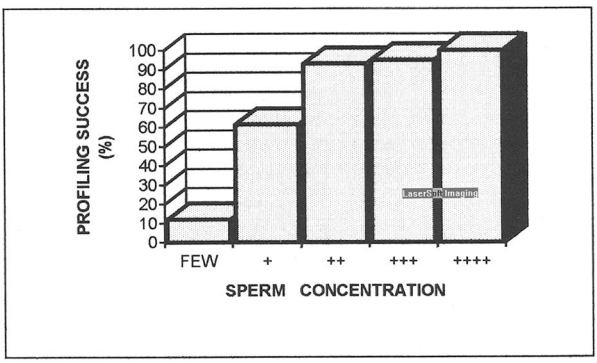

Fig. 1. Relationship between sperm concentration and profiling sucess with single locus probes. Scale ranges from "few" when there are very low numbers of visible espermatozoa on the whole slide to "++++" when there are many visible in every field of view. (This clasification has been stablished according to the criteria used by the Metropolitan Police Laboratory [4]).

In cases where DNA profile were obtained (73.4%), one or more suspects were excluded in 25% and 75% gave a profile matching the suspect or suspects. However, in 26.6% cases where no results were obtained for the RFLP technique (due to insufficient or totally degraded DNA), the PCR technology allowed to obtain a 47 % of success rate. Therefore, the sucessful rate with both techniques was 86.2%.

CASEWORK EXPERIENCE WITH SINGLE LOCUS DNA PROBES AND CHEMILUMINESCENT DETECTION IN SPAIN

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INTRODUCTION

DNA profiling using oligonucleotide coupled with alkaline phosphatase single locus probes (MS1, MS31, MS43a, MS8 and YNH24) has been in use in casework at the forensic biology laboratory of the National Institute of Toxicology since 1991.
Here, we present our DNA profiling experience in cases of sexual assaults, body identifications and paternity testing from fetal remains.

MATERIAL AND METHODS

Types of cases examined

DNA profiling has been employed in over 200 cases. Of these, the mayority have been sexual assaults, body identifications and paternity testing on fetal tissues (Table 1).

CASE TYPE	NUMBER
Sexual assaults	110
Body identifications	28
Paternity testing on fetal tissues	15

Table 1. Case types examined at the INT of Madrid.

DNA profiling procedure

In summary, DNA was extracted by the standard phenol/chloroform protocol [1] with some modifications depending on the sample (see below). DNA was restricted with Hinf I. Separation of the DNA fragments was performed by agarose gel electrophoresis in TBE buffer according to the EDNAP group protocol. Gels contained a control DNA size marker (Hind III/lambda), three DNA control ladders (BRL) and two genomic controls (K562). Gels were depurinated, denatured and Southern blotted onto nylon membranes. Membranes were hybridised sequentially with the probes MS1, MS31, MS43a, MS8 (Cellmark Diagnostic) and YNH24 (Promega Corporation) according to the Non-Isotopic Chemiluminiscent Enhancement protocol (Cellmark Diagnostic). Autoradiography was carried out for 2-5 days. All autoradiographs were analysed using the Bioimage equipment (Millipore) using the method Elder and Southern [2] for band size calculation with reference to the control DNA ladder at three differing positions on the autoradiographs.

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discriminative potential, they are also highly sensitive to exogenous contaminations. On this basis we suggest some general outlines to be followed in case of disaster:

1- All people involved in rescue operations should wear two pairs of disposable gloves; the external one should be changed after contact with a human remains.

2- Any kind of Human remains should be placed separately in an appropiate eindividual disposable, sterile plastic bag.

3- In the morgue all samples should be washed with tap and distilled water and after blotting, should be frozen at -70°C or, at least, at -20°C.

4- All remains should be sampled for molecular analysis, even those recognized cadavers, wether mutilated or not. An adequate and efficient sampling procedure will allow to know the exact number of victims based on the molecular characterization of the remains.

Aknowledgements

We wish thank to Dr. Mary-Claire King for her generous help during our research, to Dr. Charles Leroy Ginther and Dr. Janete Just for their efficient and kind technical support. This research was supported in part by grant FA-018 UBACYT (91/93), Argentina

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the catastrophe. Sampling were taken between one and seven days, and the remains were kept in the morgue at 4°C. Small pieces of tissue of approximatelly 5 ml in volume were stored in polypropylene tubes and inmediately sent to our lab, and be frozen at -70°C. All samples were essayed for radioactivity with a Geiger Müller qualitative counter. DNA was extracted in sample of about 1 ml by means of the proteinase-k/SDS procedure followed by organic solvent extraction, and alcohol precipitation. The molecular characterization of the samples included sex determination by means of Y 3.4 probing *Hae III* digests and by SRY PCR amplification, YNH-24 (D2S44 Loccus) minisatellite typing (Nakamura et al., 1987), ARA, THO-1, FABP RENA4 and CD-4 short tandem repeat typing (Edwards et al., 1991, 1992a, 1992b), HLA DQα oligotyping (Tsuji et al.,1992) and D-Loop mitochondrial DNA (mtDNA) amplification and sequencing (Guinther et al., 1992).

Results and Discussion.

Most samples were burned putrid or both. Some of them exhibited variable degrees of radioactive contamination, but unfortunately we were unable to establish the radiation emitter. Almost complete molecular characterization was attained in 6 samples, 3 were poorly characterized and one impossible to valuate Two samples sayed of taken from the same body exhibited clear differences that were reflected in THO-1 microsatellites and mtDNA D-Loop region I sequence. These results obligated to review different sample collection levels after the catastrophe. The evaluation of photojournalist reports showed that: a) lots of volunteers helped in the rescue procedures. wearing rough non-disposable leather gloves, and b) human remains were placed in inadequate plastic bags. In addition, only ten samples were received for characterization, although more than ten cadavers were recognized. Did the samples we received belong to other cadavers?, did they belong to the already identified ones? These questions will never be responded, nor will the exact number of victims ever be known. Since the current molecular forensic methodologies are highly powerful in terms of their

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Dealing With Human Remains Sampled in Disaster Areas.The Case of the Israelian Embassy Explosion Ocurred in Buenos Aires.

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Introduction:

With the advent of the molecular techniques that allowed the comparison of individual-specific hypervariable DNA sequences (Jeffreys et al., 1985), introducing a dramatical conceptual change in forensic sciencse and so paternity testing, forensic identification of biological stains and human remains have become one of the frequent requirements made by the Justice to DNA typing laboratories in an increasing number of countries all around the world.The evolution of molecular identification techniques paralleled other advances in molecular biology. The Southern blotting approach (Southern, 1975) that allowed minisatellite analysis was more recently complemented by PCR technology (Mullis et al. 1988) which at its time permitted efficient evaluation of HLA alleles, microsatellite variants and mitochondrial DNA sequencing.

The combination of these techniques have made it possible to inditeficate even highly decayed or sever burned human remains at the molecular level. However, the extreme high sensivity of these methodologies require particular care during the collection and handling of samples to avoid all possible exogenous DNA contamination.

The present report evaluates possible causes of discrepancies that arose during the molecular analysis of human remains emerging after the explosion ocurred in the Israelian embassy of Buenos Aires, Argentina, on March 17th, 1992. The aim of this contribution is to propose criteria for evidence manipulation after catastrophes.

Materials and Methods.

Ten samples belonging to not certainly known number of human bodies (possible 8) were sent to our laboratory, two weeks after

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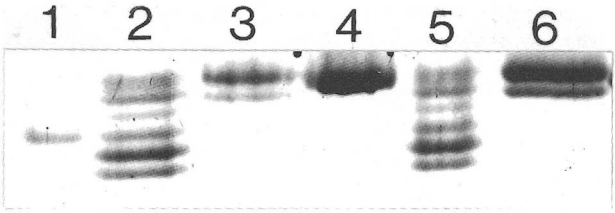

Fig. 2: TH01 amplification (semi-nested PCR) from a casework slide containing a vaginal cell/sperm mixture. Four sperm amplification (lane 4) led to the detection of only 1 allele.
1 = blood DNA pattern of the victim
2,5 = allelic ladder
3 = 25 spermatozoa
4 = 4 spermatozoa
6 = blood DNA pattern of the suspect

The 3 STR systems which were used for sperm DNA investigation showed an improvement in the successful typing using micromanipulation in preparations with very low sperm numbers. TH01 was the most successful STR system and highly sensitive using the semi-nested approach. With VWA a slightly lower sensitivity was found than for TH01 when the cycle number was increased. HumACTBP2 showed also a lower sensitivity and led to the problem of additional bands which may depend on the variable repeat structure of this system. This investigations will now be extended with further STR systems such as HumFES/-FPS and HumF13A1 to test their usefulness for this approach.

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PCR-protocols, primer sequences and electrophoretical conditions used were according to previous publications (Wiegand et al. 1993a, 1993b) with the exception that the number of cycles was increased. In TH01 a semi-nested PCR was additionally used:
The first amplification was carried out with 30 cycles and the primers published by Edwards et al. 1992. The second amplification with 15 cycles and the primers published by Gill et al. 1992. We called this semi-nested PCR because only the second primer was changed, which annealed 25 bp closer to the repeat sequence.

Results and discussion
In the first part of the study experimental microscopical preparations with defined vaginal-sperm cell numbers were investigated. The detection limit was 150 spermatozoa (Fig. 1).

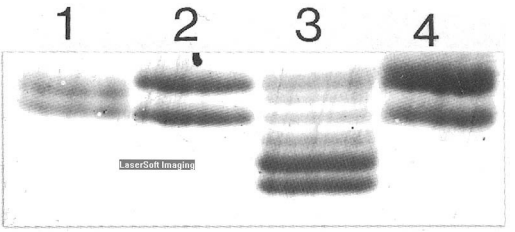

Fig. 1: TH01 amplification (32 cycles) of DNA from a mixture of approx. 150 spermatozoa (lane 2) and 450 vaginal cells (lane 1) extracted from a microscopical silde.
1 = vaginal DNA
2 = sperm DNA
3 = allelic ladder containing 6 alleles
4 = blood DNA pattern which corresponds to the sperm DNA pattern

In the second part of the study micromanipulation was carried out with mixtures containing up to 100 spermatozoa. Amplification with 32 cycles could be successfully carried out using 15 spermatozoa.
To enhance the sensitivity a semi-nested PCR was used. Amplification was successful down to the level of 1 spermatozoa. The semi-nested PCR enhanced the sensitivity but also led to a higher risk of ladder bands.
Semi-nested PCR was also used to amplify DNA from 25 spermatozoa and 4 spermatozoa from a casework sample. Using 25 spermatozoa the complete heterozygote pattern of the suspect could be obtained, while amplification of 4 spermatozoa led to the detection of only 1 allele (Fig. 2).

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Investigations on vaginal cell/sperm mixtures from microscopical slides

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Introduction

In rape cases the situation sometimes occurs that only a very small number of spermatozoa can be found on the microscopical slide. In some former cases previous analysis was therefore not successful and only the microscopical slides are now available for future analysis.
The availability of the highly sensitive PCR-STR systems has improved the chance for typing such stains.
The aim of this study was to investigate which methods are useful for PCR analysis of microscopical preparations. Three highly sensitive STR systems were selected: HumTH01 (TH01-Edwards et al. 1992), HumVWA (VWA - Kimpton et al. 1992), HumACTBP2 (SE33 - Polymeropoulos et al. 1992).

Previous analyses with dilutions of the cell line K562 gave reliable results with 1 ng template DNA using 30 amplification cycles. Using more cycles the sensitivity could be enhanced to 50 pg template DNA.

Materials and methods

I. Extraction procedure for microscopical slides:

* Removal of the cover glass with xylol followed by 2 ethanol washing steps
* proteinase K lysis performed directly on the slide for 20 minutes at room temperature and then pipetted into a reaction tube. Extraction was continued using differential lysis.
* the whole aliquot of DNA was used for PCR analysis.

II. Micromanipulation and spermatozoa extraction:

The micromanipulation was carried out using a microcapillary with a tip diameter of 10 µm. Before starting the procedure the capillary tube was filled with sterilized aqua bidest to avoid passive uptake into the capillary. Fluid contamination into the tube could be avoided by placing a piece of glass fibre tip in the capillary. At the end of the tube we used a sterile filter tip to avoid contamination with saliva. The preparations were overlayed with a drop of aqua bidest. The fixed sperm were first freed by gently scratching with the capillary tube and than sucked in. Magnification was 320 fold. After blowing the sperms out of the capillary, lysis was carried out in a mixture of proteinase K, PCR buffer and DTT. The total volume was approximately 20 µl.
After inactivation of proteinase K the whole sample was used for direct PCR analysis.

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Case 2: Both the suspect and victim shared an HLA DQα type 4, 4. Using D1S80 analysis, the victim was a type 26, 28 and the suspect was a D1S80 type 23, 29 consistent with the evidence in the male fraction.

Case 3: This case involved a sexual assault of one victim with four named suspects. HLA DQα testing produced multiple types in the male fraction of the evidentiary specimen. D1S80 analysis showed the presence of four alleles in the male fraction of the evidentiary specimen. D1S80 alleles 18 and 29 were consistent with the types obtained from the victim. The additional two D1S80 alleles observed (22, 23) were different from the results of all four suspects.

Amplification and gel conditions were optimized for the remaining three AmpFLP markers (YNZ22, Apolipoprotein B, and Collagen 2A1) and population data was accumulated; however, no additional validation work with forensic specimens has been completed because of the introduction of more promising genetic markers. These new markers are classified as short tandem repeats (STRs). To date, two of these markers have been studied in our laboratories (SE-33 and CYP-19). Validation work in our laboratory demonstrated the critical nature of the annealing temperature for SE-33. Annealing at 64°C resulted in secondary high molecular weight banding and annealing at 66°C resulted in the loss of some PCR product. Annealing at 65°C produced optimal results. In addition, experiments regarding the concentration of target DNA demonstrated that optimal conditions were achieved with 5 to 8 nanograms of target DNA. Amplification products with SE-33 were analyzed utilizing precast discontinuous Tris/Sulfate gels (LTI) electrophoresed in traditional 1X TBE buffer at 500 volts, 22mAmps, and 35 watts for 25cm. Amplification products were stained with silver and photographed. The second STR marker preliminary validated in our laboratory is CYP-19. This marker utilizes the same amplification and gel conditions as SE-33 with the exception annealing temperatures are optimized at 64°C. Population data has been collected for these two STR markers and preliminary validation with forensic specimens indicate promising results for use in forensic casework.

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specimens, and hair specimens as well as a variety of mixed stain sexual assault evidence. Extraction of DNA from these specimens was accomplished utilizing both conventional organic-based extraction protocols as well as a modified chelex extraction. Although chelex extractions have been useful for amplifying DNA specimens for the HLA DQα amplitype system, it has been our experience that DNAs isolated via chelex extraction methodologies have not been suitable for amplification with these additional PCR based AmpFLP markers.

Amplification with D1S80 was accomplished as previously described by Kasai, et.al., Journal of Forensic Sciences, vol. 35, pp. 1196-1200 (1990), in a 50 μl reaction volume Amplifications were carried out in a Perkin Elmer 480 Thermal Cycler with the following thermocycling parameters: Denaturation, 95°C for 1 minute; annealing, 67°C for 1 minute; and extension, 70°C for 4 minutes for a total of 27 cycles. Amplified products were resolved on ultra thin polyacrylamide gels (7½%T/2%C) utilizing piperazine diacrylamide as a cross linker. Electrophoresis of specimens was carried out by discontinuous gel electrophoresis in a Tris/Formate (60mM) buffer system at 550 volts, 20 mAmps, and 10 watts for 15cm at 18°C. Size markers included the 100bp (LTI) ladder and 10bp ladder (LTI) as well as an allelic marker constructed at Genetic Design comprised of each D1S80 allele beginning with the 14 repeat allele through the 39 repeat allele. Gels were stained with silver, allowed to dry, and photographed. Population survey show the 24 repeat unit allele to be the most frequently occurring in both North American Caucasians and North American Blacks, (N=424 Caucasians and N=400 for Blacks). Additional population studies were conducted for East Coast North American Hispanics (N=190), West Coast North American Hispanics (N=314), North American Indian (N=216), and North American Orientals (N=210).

The following three case summaries show several types of cases where results obtained from HLA DQα may not be conclusive due to the limited number of alleles detected, especially where mixed stains or cases involving multiple parties are involved. The subsequent use of D1S80 has substantially improved our ability to resolve such cases.

Case 1: Both suspects share the HLA DQα type 3, 4 which was detected in the male fraction of the evidentiary material. D1S80 analysis included the first suspect (homozygous 18) and excluded the second suspect (heterozygous 28, 31).

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EVALUATION OF AMP-FLP MARKERS AND SUMMARY OF PCR-BASED FORENSIC CASEWORK

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Polymerase Chain Reaction (PCR) based testing in forensic casework has been validated in numerous courts with the use of HLA DQα testing for forensic specimens. HLA DQα has been used in thousands of cases throughout the world over the past several years, where the evidence yielded DNA of too limited quantity or too degraded for Restriction Fragment Length Polymorphism (RFLP) analysis. HLA DQα PCR based testing has enabled our laboratory to analyze hundreds of cases which previously would have proven unsuitable for RFLP DNA analysis. Items of evidence from these cases include specimens such as microscopic slides from sexual assault evidence, cigarette butts, fingernail scrapings, swabbings from gun barrels as well as a variety of evidentiary specimens where DNA recovered from the specimen was too highly degraded to produce results with RFLP analysis. HLA DQα, however, represented only a single genetic marker available for PCR based testing in forensic analysis. The purpose of this study was to characterize and validate several additional amplification fragment length polymorphism (AmpFLP) markers for potential use in forensic casework. The use of AmpFLPs with forensic specimens will extend the application of VNTR polymorphisms to specimens which were either too limited in quantity or too degraded for traditional RFLP analysis. Initially, we evaluated the following four AmpFLP markers for their potential use with forensic specimens: D1S80, YNZ22, Apolipoprotein-B, and Collagen 2A1. The results of evaluation with forensic specimens included the optimization of extraction, amplification, and gel resolution conditions for each genetic marker as well as standard methods for interpretation of results. Population distributions for allele frequencies were analyzed for each AmpFLP marker within major North American racial groups.

DNA extraction methodologies were compared for a variety of tissue specific extraction protocols including extractions from liquid blood, blood stains, muscle specimens, bone

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system. It can be envisioned that for those cases where the quantity of DNA is insufficient for non-isotopic RFLP analyses, a simple strategy can be developed to accommodate characterization of the evidentiary materials.

Once the DNA has been extracted, the quantity can be estimated using a chemiluminescent-based slot blot system (such as ACES 2.0 Human Quantitation System, BRL, Gaithersburg, MD). If, for example, the amount of human genomic DNA were greater than 100 ng, then it could be decided to proceed with analysis using the non-isotopic RFLP approach; however, if the total recovered human DNA were less than 100 ng, then the sample can be analyzed with any of a number of PCR-based assays, such as HLA-DQα (AmpliType HLA DQα kit), AmpliType PM, D1S80, or short tandem repeat loci. The actual quantity of human DNA necessary to proceed with either RFLP or PCR-based typing will have to be determined, once the chemiluminescent-based RFLP system is optimized. Regardless, this strategy of employing chemiluminescent-based RFLP assays (or PCR-based testing when the DNA is limiting) enables the forensic laboratory to characterize evidentiary samples with a high degree of discrimination in an efficient, effective manner without compromising quality and without exposure to some potentially hazardous chemicals.

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similarly in that larger alleles (i.e., those containing more repeat sequences) were easier to detect than smaller alleles (Fig. 4).

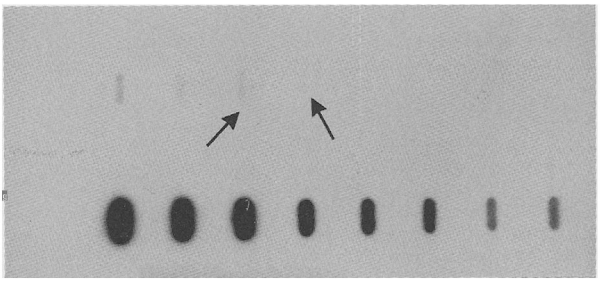

Figure 3. A two-hour exposure chemiluminescent slot blot quantitation of a dilution series of human genomic DNA displaying detection of as little as 10-20 pg of DNA (arrows).

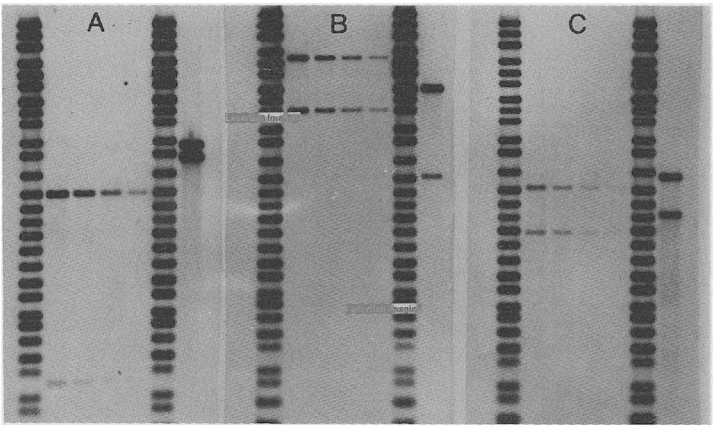

Figure 4. Lumigrams for three VNTR loci: D1S7 (A), D4S139 (B), and D5S110 (C). Each lumigram contains a dilution series of Hae III digested human genomic DNA. The quantities of DNA from left to right are: 400 ng, 200 ng, 100 ng, and 50 ng.

We also have observed that the membranes can be stripped at least six times with no apparent loss of bound DNA. Therefore, the analysis of forensic samples is similar to the ^{32}P-based system in that one membrane can be analyzed sequentially for several loci. This reduces the total amount of DNA required for a forensic analysis.

Chemiluminescence detection of alkaline phosphatase-labeled VNTR probes provides a rapid, sensitive technique for visualization of small quantities of DNA under conditions relevant for forensic analyses. An ancillary benefit of directly-labeled probes is their inherent stability. A batch of probe can be quality checked and used over a long period of time (unlike isotopically-labeled probes). Thus, quality control for RFLP typing can be improved.

While it may be that on average ^{32}P labeled probes provide greater sensitivity than non-isotopic systems, this should not present a problem for the majority of DNA human identification analyses. For paternity testing, the limitation of the quantity of DNA is rarely an issue. For forensic cases that yield conclusive results, there generally is sufficient DNA to be detected using a chemiluminescence-based detection

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An alternate chemiluminescent system which has found application in DNA detection protocols is the enzyme catalyzed oxidation of luminol in the presence of enhancers (Mathews, et al. (1985). Luminol is modified by complexes formed between oxidants and horseradish peroxidase to form luminol radicals which decompose through an endoperoxide intermediate to form the excited state 3-aminophthalate dianion (Thorpe and Kricka 1986). When the excited state decays into its ground state, it emits light. This detection system has been used with Southern blot analysis (Polliard-Knight, et al. 1990: 84-89) and a human quantitation slot blot method (Walsh, et al 1992). However, it appears that alkaline phosphatase, with its ability to turn-over more substrate for a longer period of time, may be a better enzyme for a chemiluminescent assay (Polliard-Knight, et al. 1990:353-358). Furthermore, while luminol-based systems have faster ramp times than dioxetane-based systems, the signal from luminol also tends to decay more rapidly. These two factors make the use of alkaline phosphatase/dioxetane systems more desirable than horseradish peroxidase/luminol systems for RFLP typing.

Forensic Applications of Chemiluminescent DNA Detection

Giles, et al. (1989) were the first to apply alkaline phosphatase-labeled probes and lumigen as a detection substrate for human DNA identity typing. They found that the technology provided sensitivity with sharp bands, enabled sequential probing, and was relatively simple. Our laboratories are developing an assay system, using Lumigen PPD because of its demonstrated effectiveness in various research applications, for detection and analysis of DNA samples under conditions relevant to forensic use of chemiluminescent technology. Our results are similar to those of Giles, et al. (1989).

Dioxetane-based chemiluminescent detection was evaluated by us for quantitation of human DNA with the alphoid probe D17Z1 by a slot blot hybridization assay and for detection of human genomic DNA in a Southern blot format using oligonucleotide probes to the VNTR loci D1S7, D2S44, D4S139, D5S110, D10S28, and D17S79. The conditions for hybridization and detection of alkaline phosphatase-labeled probes have been optimized in our laboratories to facilitate use, speed, and sensitivity. A protocol using ACES 2.0 (Advanced Chemiluminescent Enhancement System) (BRL, Gaithersburg, MD) has been evaluated by us. This protocol has been developed for use with the non-charged nylon membrane Biodyne® A which (after UV-crosslinking of the target DNA to the filter) yields good results with low background staining. The same buffer (0.5M $NaPO_4$, pH7.2, 1% casein, 0.5% Tween 20) is used for prehybridization (20 min. at 55°C) and hybridization (20 min. at 55°C) of the membrane. This is followed by two 10 min. washes at 55°C in a solution containing 0.05M $NaPO_4$, pH7.2, 0.5% Tween 20 and two final washes (5 min. each) at ambient temperature in a solution containing 0.01M Tris-HCl, pH 7.5, and 0.15M NaCl (for human quantitation by slot blotting the temperature is at 50°C). Approximately 0.01 ml/cm^2 Lumi-Phos 530 is added to the membrane and the solution is incubated to allow the gradual increase in light intensity (termed "ramping") before exposure to XAR film (Kodak) at room temperature. The ramp-up of light intensity is often allowed to proceed for 3 hours or overnight. Kinetic studies have shown that 70% of the maximum light intensity output of the reaction is achieved within 3 hours of incubation and maximum light intensity generally is reached in 5 hours. The light intensity remains stable for at least 21 hours after ramping (Carlson et al. 1990). Multiple exposures with X-ray film may then be taken over a 24 hour period. Typical exposure times range from 30 minutes to 5 hours.

Klevan, et al. (1992, 1993) demonstrated that this detection system, employing an enzyme-triggerable dioxetane and chemiluminescence enhancers, provides a high degree of sensitivity in forensic protocols with a significant savings in overall filter processing time and development. The sensitivity of detection for human genomic DNA quantitation can be as little as 10-20 pg with the alphoid probe D17Z1 (Fig. 3). Moreover, they found that three probes for the VNTR loci D2S44, D4S139 and D1S7 enabled detection of DNA bands from 50-100 ng genomic DNA with a high degree of consistency and reproducibility. Furthermore, the nonisotopic chemiluminescent assay and the isotopic ^{32}P detection procedure behaved

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CHEMILUMINESCENT DETECTION BY STABILIZED DIOXETANES

Chemiluminescence is the production of electromagnetic radiation as a result of a chemical process. Although many systems have been developed which produce light through a variety of chemical reactions (for a review see Campbell 1988), only a few have found application in DNA detection protocols. Many chemiluminescent assays were designed so that, upon activation, they emit radiation very rapidly; however, specialized instrumentation was required to detect the single "flash" of light emitted. Application of chemiluminescent detection to Southern blotting procedures used by the forensic scientist requires a system with continuous light output so that signal can be collected over time (for increased sensitivity) and multiple detections are possible. The most sensitive chemiluminescent system which emits a continuous "glow" and has found widespread application in genetic research and biomedical analysis involves the selective cleavage of stabilized 1,2-dioxetanes.

1,2-Dioxetanes are 4-member ring peroxides which decompose to form products which may be in an excited electronic state. These species then decompose further and emit light. The most familiar demonstration of dioxetane decomposition is the yellow flash of light observed from the firefly, *Photinus pyralis*. In this multistep bioluminescent reaction, the molecule luciferin reacts with ATP and molecular oxygen to form a substituted dioxetane intermediate which undergoes further rearrangement and light emission. The dioxetane is a short-lived unstable intermediate in the process, the half-life of emission for the reaction is approximately 40 msec (Handley et al. 1990). The first example of a dioxetane reagent which was stable at room temperature was the compound bisadamantyl 1,2-dioxetane (Wieringa et al. 1972). The molecule consisted of a dioxetane ring surrounded by two adamantyl groups and was found to be stable up to 240 degrees. More importantly, the reaction of this compound provided further insight into the mechanism for dioxetane decomposition. The mechanism involves stretching of the oxygen-oxygen bond followed by ring cleavage to produce two carbonyl species, one of which may be in an electronic excited state. The decomposition of the peroxide ring is sterically constrained by the presence of the bulky adamantyl groups which confer stability to the compound. This observation led to the development of the first enzyme-triggerable substrate.

Schaap, et al. (1987) developed a stable phosphate-substituted dioxetane, which was the first chemiluminescent substrate compatible with alkaline phosphatase. Upon removal of the phosphate stabilizing group, a reactive aryl oxide was generated which decomposed with low overall efficiency of light output. Dioxetane chemistry was advanced further when Schaap (1988) synthesized the phenylphosphate substituted dioxetane: 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane]. This compound, termed Lumigen® PPD, has a calculated half-life at 25 degrees of 19 years and is extremely stable in aqueous solutions (Schaap, et al. 1987). Schaap et al. (1989) also reported on the development of system for chemiluminescent energy transfer which greatly increased the quantum efficiency of the light producing reaction with Lumigen PPD. Removal of the phosphate group from PPD by alkaline phosphatase at high pH generates a highly reactive phenoxide dioxetane which associates with a surfactant preparation composed of cetyltrimethylammonium bromide (CTAB) and 5-(N-tetradecanoylaminofluorescein). The phenoxide derivative decomposes in the hydrophobic environment of the micelle to give an activated ester product which transfers its energy to the fluorescein acceptor so light can be emitted. The rate of dephosphorylation of PPD is not effected by the presence of the CTAB preparation and the observed efficiency of the light producing reaction is increased 400 fold over that observed when no modified surfactant is present. Further details on the chemical nature of dioxetane stabilization and activation have been described by Beck and Koster (1990). The PPD and surfactant in 2-amino-2-methyl-1-propanol buffered solution at pH 9.6 is the reagent termed Lumi-Phos® 530. Alternatively, Voyta, et al. (1988) reported the synthesis of a similar substrate termed AMPPD. AMPPD also has been demonstrated to be very stable with a half-life in water of 142 hours at 30°C and an indefinite shelf-life in solid form at 4°C (Bronstein, et al. 1989a, 1989b).

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laboratories employ RFLP methodologies which vary to a degree (e.g., organic vs. non-organic extractions, ME vs. LE agarose, ethidium bromide vs. no ethidium bromide in the resolving gel, and different size standards) the D5S110 allele distribution appear similar for the same populations between the two laboratories (χ^2 p=0.053 and G_{stat} p=0.062 for rebinned Caucasian data; χ^2 p=0.083 and G_{stat} p=0.090 for Black data; and χ^2 p=0.110 and G_{stat} p=0.108 for southwestern Hispanic data).

Adams, et al. (in preparation) recently have evaluated the forensic utility of D5S110 for analyzing environmentally-insulted biological samples. The results were similar to that observed for other forensically-validated VNTR loci (Adams, et al. 1991). Based on these data, it is recommended that laboratories consider D5S110 as a useful locus for the forensic characterization of human biological samples.

CHEMILUMINESCENCE

PROBE LABELING

Hybridization of a DNA target sequence with its complementary DNA probe is a rapid, sensitive, and highly specific method for identifying a specific DNA component amongst a myriad of unrelated DNA sequences. Typically, a labeled nucleic acid probe is used to identify the position of a target DNA molecule which has been immobilized on a solid support. The strong affinity of the DNA probe for its target sequence results in a high degree of selectivity and precision in the hybridization reaction. The level of sensitivity of detection of target DNA is dependent upon the quantity of probe, hybridization conditions, and the procedure used to visualize the annealed probe/target hybrid, as well as the sequence of the probe. The quantity of probe and hybridization conditions required for augmenting sensitivity of detection are well-described and are used routinely in forensic RFLP analyses (Budowle and Baechtel 1990).

The ease of incorporation into DNA probes and the sensitivity of autoradiographic procedures have made radioisotopes (specifically ^{32}P) the most widely used detection component of hybridization systems. However,

there are inherent limitations associated with the use of radioisotopes, which include the instability of ^{32}P-labeled probes, disposal problems, and health concerns. The latter two can impact negatively on the transfer of RFLP typing to some application-oriented laboratories.

An alternative to incorporation of radioisotopes into probes is the attachment of enzymes to DNA probes. Enzymes provide catalytic activity that amplifies the primary hybridization signal, and a wide variety of substrates for subsequent detection are available. The enzymes most often employed in DNA detection strategies are alkaline phosphatase and horseradish peroxidase.

Alkaline phosphatase and horseradish peroxidase can be attached to a DNA probe either after (i.e., indirect assay) or before the hybridization reaction (i.e., direct assay). In indirect protocols, a probe is labeled with a ligand, such as biotin or digoxigenin; following hybridization, the probe-target DNA complex is recognized by a streptavidin or anti-hapten conjugated enzyme (Klevan and Gebeyehu 1990). This method is often employed for DNA probes obtained from cloned inserts from which little sequence information is available. Probes from several hundred to several thousand nucleotides in size can be labeled indirectly, and hybridization times ranging from several hours to overnight are common. In contrast, a direct assay employs synthesized oligonucleotides covalently linked to an enzyme. Several methods to prepare probes in this manner have been described (Jablonski et al. 1986, Mackey et al. 1992), and commercial probes and labeling kits are now available. The use of direct labeled probes reduces the number of development steps, as well as the overall time required to process the filter prior to the detection procedure. The annealed probe-target hybrid can be detected using a variety of reagents, such as chemiluminescence substrates.

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provides a sensitivity of detection similar to pH30/D4S139 (approximately 10-20 ng of human genomic DNA may be typeable) (Fig. 2).

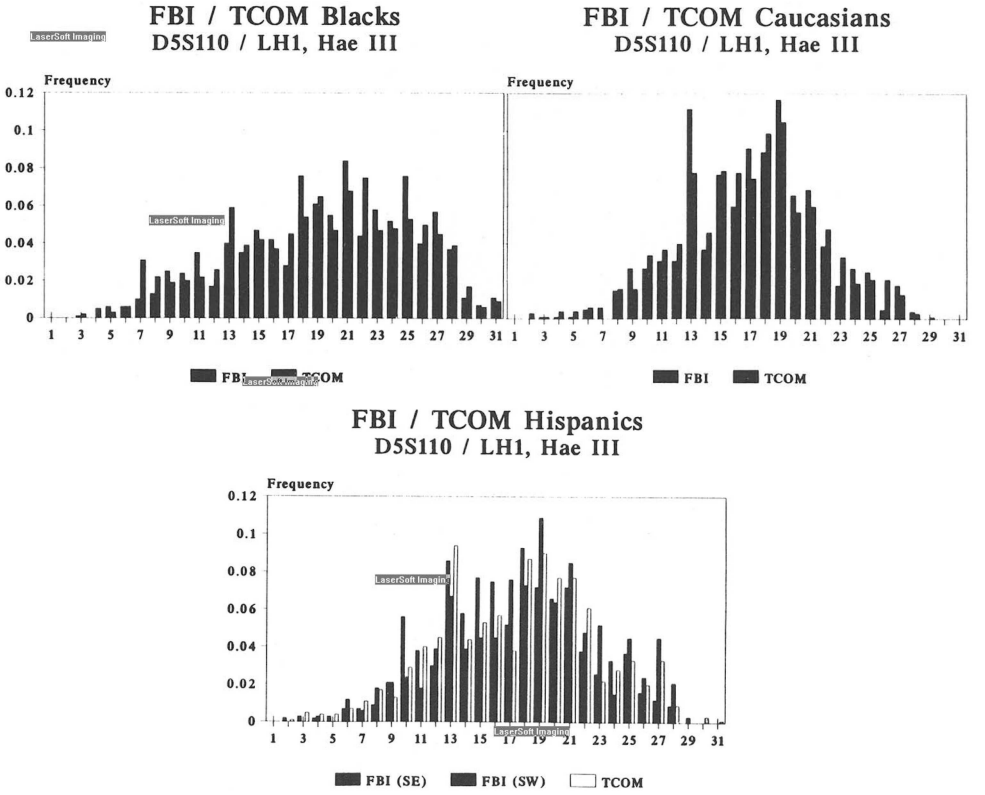

Figure 1. Histograms of binned allele distributions for Caucasians, American Blacks, and Hispanics from the FBI and TCOM.

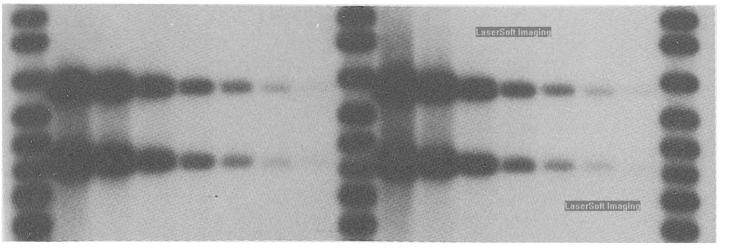

Figure 2. An Autoradiogram displaying a dilution series of Hae III digested human genomic DNA probed for the D5S110 locus. The quantities of DNA from left to right are: 1000 ng, 500 ng, 250 ng, 100 ng, 25 ng, and 10 ng.

The Caucasian, Black, southeastern Hispanic, and southwestern Hispanic population samples (FBI databases) were tested for independence using the exact test described by Guo and Thompson (1992); thus, all satisfy Hardy-Weinberg expectations for D5S110 (p=0.554; p=0.850; p=0.475; and p=0.163, respectively). In addition, D5S110 allele distributions from the FBI were compared with those obtained by TCOM. Although the FBI and TCOM

RFLP TYPING: A NEW HIGHLY POLYMORPHIC VNTR LOCUS AND CHEMILUMINESCENT DETECTION

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INTRODUCTION

There are two basic DNA typing methodologies available to the forensic scientist for characterizing biological evidence. The first technology to gain wide use in the forensic arena was typing of DNA for variable number of tandem repeat (VNTR) loci by restriction fragment length polymorphism (RFLP) analysis. RFLP typing is well-defined, provides a high degree of discrimination, and can be accomplished, at times, with less than 50 ng of high molecular weight genomic DNA. This methodology has been validated for forensic applications (Budowle and Baechtel 1990). The other strategy for typing DNA is based on increasing the number of copies of a target sequence of DNA by amplification using the polymerase chain reaction (PCR) (Saiki, et al. 1985). Since the number of target sequences of interest is increased dramatically by PCR, simplified typing methods can be used for determining DNA polymorphisms in a sample. The advantages a PCR-based technology offers, compared with the currently employed RFLP approach, are: 1) augmented sensitivity and specificity, 2) decreased assay time and labor, 3) absence of an isotopic label, and 4) many degraded DNA samples can be amplified by PCR and subsequently typed because alleles amenable to PCR are much smaller in size compared with alleles detected by RFLP analysis. These qualities combine to make PCR-based technologies extremely useful tools for analyzing biological material found at crime scenes.

While the application of PCR-based technology to the genetic characterization of forensic evidence is coming to fruition, the RFLP method currently in use provides a higher degree of discrimination, and the molecular biology aspects of the RFLP technique are better understood. Therefore, research to facilitate RFLP typing of DNA still should be continued. This paper discusses two such aspects: additional VNTR loci to augment the discrimination power of RFLP typing of forensic biological evidence and the use of chemiluminescence, instead of ^{32}P, as a detection component of the probe-target DNA sequence hybridization.

D5S110 - A HIGHLY POLYMORPHIC VNTR LOCUS

Of the genetic markers used for forensic analyses, the most informative for discrimination among individuals are the highly polymorphic VNTR loci. By using a panel of DNA probes, sufficient data can be obtained to produce a composite profile that is unique to an individual (excluding monozygotic twins). More importantly, typing VNTR loci currently provides the forensic scientist the best avenue to exclude a suspect who has been falsely associated with an evidentiary sample.

Two factors which influence the effectiveness of RFLP analysis are the availability of well-characterized VNTR loci and the quantity and quality of DNA in evidentiary material. RFLP analysis can be made more effective by identifying other VNTR loci, compatible with the restriction enzyme utilized for RFLP analysis (for our laboratories that would be HAE III), whose alleles generally fall in a size range that is greater than 1000 base pairs and less than 10,000 base pairs, are highly polymorphic, and have a high degree of sensitivity of detection. The VNTR locus D5S110 (Amour, et al. 1990) meets these criteria.

The histograms displaying allele frequencies in 31 fixed bin formats for Caucasians, Blacks, southeastern Hispanics, and southwestern Hispanics are shown in Figure 1 for locus D5S110. The D5S110 locus is highly polymorphic in all sample populations studied, with the Black population sample demonstrating the highest degree of variation. Most alleles are less than 10,000 base pairs in length. Furthermore, the probe for D5S110

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6. DNA polymorphisms in criminal investigation

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with the probabilities of: (a) AF being the father of the first child; (b) AF being the father of the second child; (c) the father of both children is a different man; (d) both children have different fathers.

Index of concomitant paternity is obtained by the division of the probability of AF being the father by the total of the other probabilities. Such value is usually higher than the product of single paternity indexes.

We suggest to inform in the expertise report not only the probabilities of paternity of AF in relation to each child (calculated as if they were from different trios), but also the probability of his concomitant paternity.

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EXPERTISE REPORT IN DISPUTED PATERNITY CASES WITH TWO OR MORE CHILDREN.

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In expertise reports, when it is not possible to exclude an alleged father (AF), the probability of paternity should be presented, as far as multiple tested systems provide a falsely accused man with a 95% probability of obtaining evidence of non paternity. Although most of disputed paternity cases are composed of trios (AF, mother and child), cases with more than one child are not rare.

Concomitance of non exclusion results of AF in relation to a brotherhood deserves an additional treatment.

In this paper, only the situation involving a quartet (AF, mother and two children) is focused (considering more than two children would produce an extremely high number of possible alternatives).

When the same man is involved in two independent trios, e.g. he is indicated by two different women in relation to their children, it is reasonable to use the multiplication of the paternity indexes, in order to evaluate the probability of paternity in relation to both children. However, when mother indicates the same man as father of her children, the situation is quite different because mother's indication is being tested twice.

We propose to evaluate the probability of concomitant paternity, comparing the probability to observe such quartet

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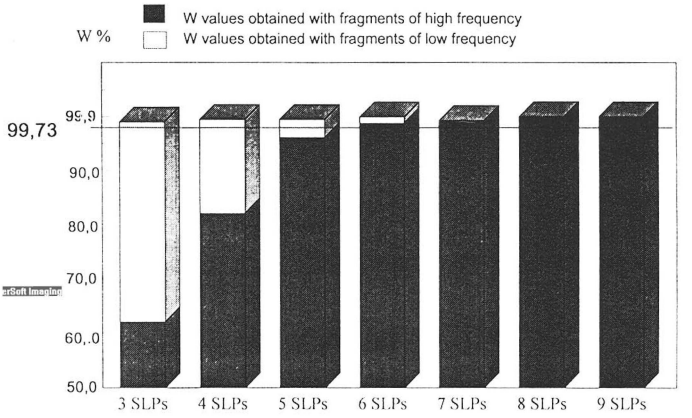

Fig.3: Comparison of W values (Essen Möller)obtained with variable numbers of VNTR's (SLP's) in cases of disputed paternity (n=17)

Tab.1: Comparison of W - values according to data obtained by HLA-. DNA- and HLA + DNA analysis

W - values according to HLA data	HLA	3 SLP's	HLA + 3 SLP's
W(%) 95,0 - 98,5	98,407 98,386 97,736 97,200 96,150 95,225	99,913 99,983 99,680 99,899 99,964 99,733	99,999 99,999 99,994 99,997 99,998 99,987
W(%) 90,0 - 94,5	94,770 94,580 94,284 91,400 91,463 91,883	99,896 99,987 99,692 99,942 99,932 99,901	99,994 99,999 99,980 99,995 99,994 99,991
W(%) < 90,0	88,579 87,740 87,653 87,290 87,100	99,950 98,051 99,842 99,975 99,896	99,994 99,730 99,978 99,996 99,983

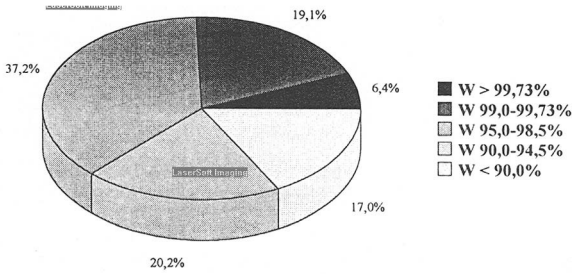

Fig.1: Biostatistical calculation of W values (Essen Möller) using HLA - A and - B frequencies in cases of disputed paternity (n=96)

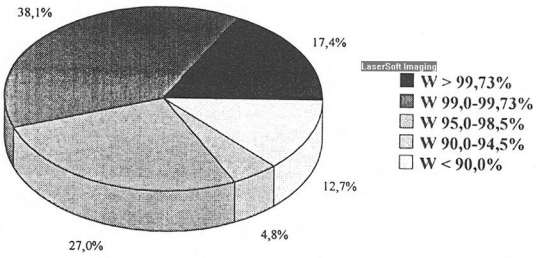

Fig.2: Biostatistical calculation of W values (Essen Möller) using 21 conventional systems in cases of disputed paternity (n=63)

respectively, whereas the application of five SLP's results in W-values of greater than 99,73% in 76,5% of cases. In all of these cases we did not include the situation that all SLP's exhibited either homozygosity of the fragments between the mother and the child ore that both fragments of the child could have been inherited from the mother. If this situation occurs it can lead to W values below 50%. In our analysis this situation did occur only in one case were 3 SLP's with HR's between 0,66 and 0,85 were used. In this instance an extended investigation with 3 additional SLP's (HR 0,94 - 0,98) did correct the W value up to 99,74%. The sole application of the HLA-system in this case resulted in an W value of 95% and the combination of HLA with the worst case DNA analysis did reach an W value of 93.8%. Besides this exception, table 1 summarises the data obtained from various cases where W values were obtained either by HLA or by the combination of HLA with 3 SLP's. The data are grouped according to the W values obtained with the HLA system. Our results demonstrate, that in all of these cases the combination of HLA and 3 SLP's leads to W values of greater than 99,73%. These values were also obtained when HLA typing alone did reach only W values of less than 90,0%. In cases of exclusion (N=10) were probes with high heterozygosity rates between (0.97) and (0.99) were applied (YNH24 , MS1 , MS31 , MS43A, G3) all probes indicated exclusion.

Summary

1. Single application of HLA results in only 25% of cases in W-values of 99,0% and more.
2. The combination of 3 SLP's with HLA reveals in all cases W-values of > 99,73%.
3. The application of SLP's with high heterozygosity rates reduces the number of SLP's that are required to reach sufficient W values.
4. SLP's with a high heterozygosity rate result in high exclusion rates.
5. In all cases were exclusion was found by conventional or HLA-system , the SLP's did also exhibit exclusion patterns.

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Germany) and detection of probes were performed as recommended by the manufacturers (AlphaProbe^{TM} from IMMUCOR/ONCOR, NICE^{TM} from ICI/CELLMARK and GenePrint Light^{TM} from Promega). The results were analysed by an semiautomatic computerised system (DNA-Auswertungssystem Version 2.40 from M.Muche Immucor Medizinische Diagnostik GmbH, Rödermark, Germany) and the biostatistical calculation of paternity was performed with the Essen Möller probability (W) using the allele frequencies (according to J.Henke, Institut für Blutgruppenforschung, Köln) of at least three different probes. The following table summarises the investigated probes and the corresponding heterozygosity rates (HR).

Locus	SLP	HR/RE	Manufacturer
D7S107	pS 194	0,85(PstI)	Immucor
D1S47	pl 336	0,90(PstI)	Immucor
D18S17	pl 159-1	0,74(PstI)	Immucor
D20S15	pl 355-8	0,66(PstI)	Immucor
D21S112	pl 427-4	0,94(PstI)	Immucor
D11S129	pR 365-1	0,66(PstI)	Immucor
D2S44	yNH24	0,97(HinfI)	Promega
D1S7	MS1	0,99(HinfI)	Cellmark
D7S21	MS31	0,98(HinfI)	Cellmark
D12S11	MS43A	0,97(HinfI)	Cellmark
D7S22	G3	0,96(HinfI)	Cellmark

Results and Discussion

We analysed the distribution of W values obtained by typing the HLA - A and -B locus antigens and by using 21 conventional systems (fig.1 and fig.2). In figure 1, the HLA data demonstrates, that in 6,4% of cases W values of 99,73% or more are obtained. In comparison, the analysis of conventional systems shown in figure 2 resulted in 17,4% of cases with W values of 99,73% or above. In 38,1% of cases analysed by conventional systems the W values were between 99,0% and 99,73%, whereas only 19,1% of cases analysed by HLA were in this range. Thus, in comparison to the HLA system, the conventional systems lead in twice of the cases to W values of greater than 99,0%. Since HLA typing is less time consuming and is also cost effective we have chosen to analyse the combination of HLA typing and DNA analysis by SLP's for paternity testing. Previous studies of SLP's have shown that in 23% of cases analysis by 6 SLP's did not result in W values of more than 99,73%. To further evaluate the significance and practicability of SLP's we have extended our panel by SLP's demonstrating a higher heterozygosity rate. The data are illustrated in figure 3. These data have been obtained by selecting fragments with high frequency and fragments with low frequency. Therefore the dark columns represent the W values obtained by worst case analysis whereas the light columns represent the W values by best case analysis. Regarding worst case analysis the application of 6 SLP's was necessary to reach W values of 99,73% in all cases. Application of three or four SLP's can result in W values as low as 62,0% or 82,0%

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APPLICATION OF SINGLE LOCUS PROBES IN CASES OF DISPUTED PATERNITY - THE USEFULNESS OF THE COMBINATION OF HLA AND DNA.

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Introduction

Genetic polymorphism analysis has been a classical technique to identify individual-specific patterns of in heritage in paternity testing. Blood group antigens, plasma protein and erythrocyte enzyme polymorphism's are the conventional systems used for paternity testing. Phenotypic gene frequencies are particularly significant in various populations but most of the systems exhibit only a limited polymorphism. Thus several systems (e.g. about 21) have to be used for accurate calculations of paternity probability. However, in some cases probability values are still not conclusive. Therefore, genetic systems with high polymorphism are of particular interest. In this respect, the human leukocyte antigens (HLA-system) demonstrating a high number of alleles have been used (1). Due to linkage disequilibrium and uneven distribution of haplotypes the W values (Essen Möller) of alleged men are not always sufficient. In recent years variable numbers of tandem repeats (VNTR's) have been analysed by single locus probes (SLP's) (2). We have compared the sole application of HLA and the combination of HLA and SLP's analysis. The results demonstrate that the combination of HLA - A and - B locus typing and 3 SLP's resulted in W values of more than 99,73% .

Materials and Methods

Antigen systems and enzyme polymorphisms (Conventional systems). Blood group antigens (ABO-, MNSs-, Rh-, Kell-, Duffy- and Kidd-system), plasma protein polymorphisms (PLG-, Gm-, Km-, C3-, Tf-, Pi-, Hp- and Gc-system) and erythrocyte enzyme polymorphisms (PGM-, GPT-, ACP-, AK-, ADA-, EsD- and PGD-system) were determined for each case following standard procedures.
HLA-Typing. HLA-A, HLA-B and HLA-C antigens were typed by the microlymphocytotoxicity test (LCT).
VNTR (SLP) polymorphism analysis. Genomic DNA was extracted from 8ml of peripheral blood following the salting-out method (4) and 10µg of DNA were digested with 25 units of the appropriate restriction enzyme (RE) for 16 hours. Subsequently, DNA fragments were separated by gel electrophoresis (44-62 hours at 0.8-1.0 V/cm). Electrophoresis conditions were chosen depending on the range of fragment sizes detected by the various DNA probes. Hybridisation, southern blot transfer to nylon membranes (Quiabrane from Diagen Inc, Hilden,

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TABLE 1. Results of the AMP-FLP analysis. M = mother, C = child, PF = putative father, B = blood sample, N = normal esophageal tissue, Ca = ventricular adenocarcinoma tissue.

SAMPLE	D1S80	D17S30	apoB	col2A1	HumTH01	vWA
M, B	18-18	3-12	36-48	11-23	6-10	5-8
C, B	18-24	4-12	46-48	11-23	6-7	5-5
PF, N	24-31	1-4	31-46	19-23	6-7	5-6
PF, Ca	24-31	1-4	31-46	19-23	6-7	5-6

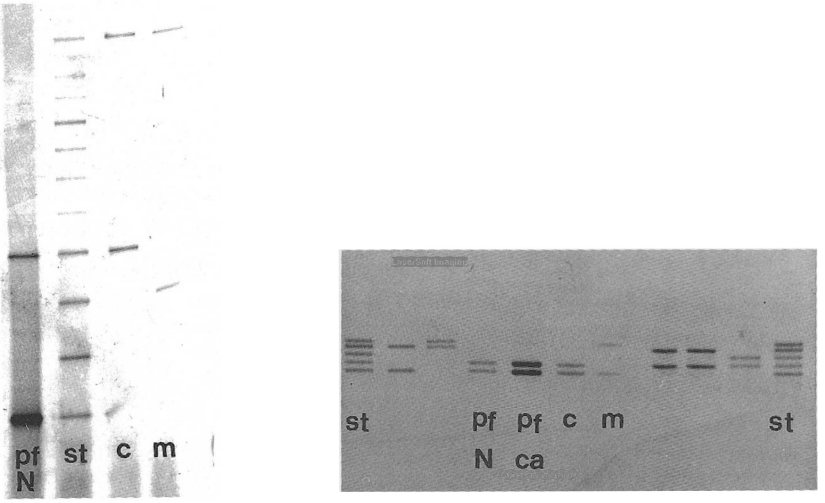

FIGURE 1. A. AMP-FLP analysis of minisatellite D17S30. B. AMP-FLP analysis of microsatellite HumTH01. M = mother, C = child, PF = putative father, B = blood sample, N = normal esophageal tissue, Ca = ventricular adenocarcinoma tissue, L = allelic ladder constructed of known alleles in the Finnish population as previously described (7).

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RESULTS

Four minisatellite loci (D1S80, apoB, D17S30, col2A1) and two microsatellite loci (vWA, HumTH01) were successfully typed for the mother-child-putative father trio (Table 1). The AMP-FLP results from both normal esophageal and ventricular adenocarcinoma tissues from the putative father were consistent in all mini/microsatellite loci (Table 1). Paternity index (6) was 104, providing strong evidence for paternity, and corresponding to the force of evidence of >99 %. An example of AMP-FLP analysis of minisatellite D17S30 and microsatellite HumTH01 is given in Figure 1.

CONCLUSIONS

Our earlier study has shown that genetic typing from soft tissue samples can be successfully performed using AMP-FLP technique (8). Our present results show that formalin-fixed, paraffin embedded tissue samples can also serve as source of DNA for genetic typing in various applications. In paternity cases where the putative father is deceased, analysis of such tissue samples may be the only alternative to prove paternity. The present case is the first paternity case in Finland, where the court has accepted DNA typing from biological sample other than blood as evidence for paternity.

ACKNOWLEDGEMENTS

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PROVING PATERNITY USING FORMALIN FIXED, PARAFFIN EMBEDDED TISSUE SAMPLES FROM A DECEASED FATHER

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INTRODUCTION

DNA testing has had a major impact in forensic medicine, particularly in identification of individuals from crime scene samples and in paternity analysis. Additional convenience for DNA testing can be obtained by using the polymerase chain reaction (PCR) (1) for amplification of DNA. The use of PCR facilitates paternity analysis from old and degraded blood or tissue samples, where conventional serological analysis cannot be performed.

Here we describe a paternity case of a deceased father who had died of ventricular adenocarcinoma 13 months before the analysis.

MATERIALS AND METHODS

DNA extraction

From the putative father DNA was extracted from formalin fixed, paraffin-embedded samples from normal esophageal and from ventricular adenocarcinoma tissue. DNA was extracted by first removing the paraffin with xylene, afterwhich the samples were treated with absolute alcohol, and lysed in the presense of proteinase-K and dithiothreitol (2).

From the mother and the child DNA was extracted from EDTA-blood samples using the Chelex^R method (3).

Analysis of DNA samples

Four minisatellite loci (D1S80, apoB, D17S30, col2A1) and two microsatellite loci (vWA, HumTH01) were analyzed. The analysis was performed using the amplified fragment length polymorphism (AMP-FLP) technique (4). In AMP-FLP analysis the hypervariable mini- and microsatellite loci were first amplified by PCR using the primers described in the litterature. The amplified alleles were then separated according to their size in a high resolution polyacrylamide gel electrophoresis (PAGE), and visualized using simple silver staining (5).

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Alltogether 179 mother - child - putative father trios were investigated. In 42 trios the men had been excluded from paternity by conventional methods. These findings could be confirmed by PCR analysis with one single exception. In this case, the non-excluded man was the brother of the true father.
On the other hand, two father - child exclusions were revealed by PCR but not by the classical marker systems. Based solely on the 24 blood group systems, the probability of paternity had been calculated at 85 and 94% respectively.
The results of all other cases were consistent with the classical genetic systems.

Numbers of analyzed meiosis are: 281 for HLA-DQα, 220 for D1S80 and 106 for Apo B. Concerning the HLA-DQα and Apo B loci, there was no evidence for new mutations; with D1S80 one single new mutation was detected by a mother - child incompatibility. This finding corresponds to a mutation rate of 0.45%. We conclude, that the described loci are genetically relatively stable compared to the more polymorphic VNTR loci detected by Southern blot analysis.

3. Forensic efficiency data

The power of exclusion (PE) of HLA-DQα, Apo B and D1S80 amplification systems was calculated at 0.595, 0.557 and 0.635; the combined value is approximately 0.935. DI - values (discrimination index) estimating the individual specifity of genotypes are 0.07, 0.09 and 0,06, respectively (Table 3). To validate the calculated PE - values, 30 paternity cases with putative father - child exclusions were re - analyzed with the three PCR systems. Exclusions could be verified in all cases at the following rates: 53% (DQα), 63% (Apo B) and 73% (D1S80). Single exclusions were observed with 7 trios, two exclusions with 18 trios and three exclusions with 5 trios. This result underlines the resolving power of these 3 systems which is comparable to the combination of approximately 20 commonly used blood group systems.

Table 3 **Discrimination power of three amplification systems**

amplification system	heterozygosity (calculated, %)	power of excl. PE (%)	discrim. index DI	frequency of most common genotype
HLA-DQα	79.8	59.5	0.07	0.142
Apo B	77.7	55.7	0.09	0.155
D1S80	81.9	63.5	0.06	0.153
combined		93.5	3.8×10^{-4}	3.4×10^{-3}

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limits of +/- 2°C. For the DQα locus allele frequencies ranged from 9.3 - 30.8% which is in close agreement with published data (Table 1). All 21 possible genotypes were observed, heterozygosity rate was 82.1% (79.8% expected). There was no deviation from Hardy-Weinberg equilibrium when expected and observed genotype frequencies were applied in a chi^2 test (Table 2).
With the D1S80 AmpFLP system a total of 23 different alleles could be analyzed, representing 16 - 40 tandemly arranged copies of the 16 bp repeat unit (n = 484). One single allele with approximately 60 - 65 repeats (judged by agarose gel electrophoresis) was observed. The two most common alleles exhibit frequencies of 32.8 and 23.3%, all the other alleles were found at or below 7.6% (Fig 1a). These findings compare favourably to data bases obtained with german (6) and US caucasian samples (32.0 and 26.5% [7]). Chi^2 - tests were carried out as proposed (6) by binning the alleles into 4 groups. Again no significant deviation from the assumed Hardy-Weinberg equilibrium was revealed (Table 2). Observed heterozygosity was 81.8% (81.9% expected).

Fig. 1a

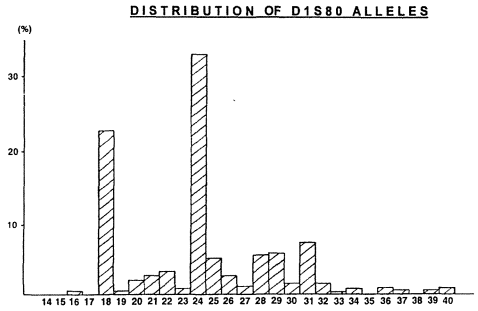

Fig. 1b

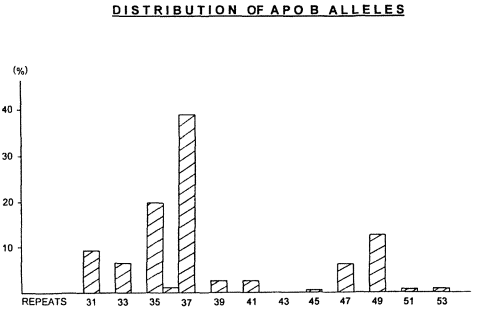

Fig. 1a,b. Allele frequencies at the D1S80 (Fig. 1a) and Apo B loci (Fig. 1b) in a sample of unrelated german individuals

Analysis of the amplified Apo B fragments (n = 240) revealed 12 different alleles representing 31 - 53 repeat units. One single allele with a putative length of 27 repeats was detected in a blood sample of a libanesian man.
Comparison of the allelic data base (Fig. 1b) with results obtained by Schnee-Griese (8) demonstrate a similar distribution, the most common alleles reaching frequencies of 38.5 (37.0), 20.0 (25,4) and 12.5% (8.8%) respectively (values from (8) in parenthesis). As both estimates are based on population samples of similar origin, the observed differences may be due to different sample size. Hardy-Weinberg equilibrium was obviously reached in the Apo B amplification system (Table 2).

Table 2 **Hardy - Weinberg equilibrium at three amplified loci**

locus	$\chi 2$	probability	df
HLA-DQα	6.92	p > 0.99	20
D1S80	3.52	0.95 > p > 0.90	9 *
Apo B	4.22	0.90 > p > 0.80	9 *

* alleles binned into 4 groups

2. Family studies

For this report we carried out extensive family studies, consisting mainly of paternity cases which had been previously investigated with classical blood group markers (24 systems).

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PCR based analysis of HLA-DQα, D1S80 and Apo B loci in paternity testing.

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Introduction

The development of the polymerase chain reaction (PCR) has considerably simplified the analysis of locus specific DNA polymorphisms, generated both by VNTR regions and DNA sequence variation. These kinds of allelism represent informative genetic marker systems which may also be useful in paternity determination. We have focused our interest on the well established HLA-DQα, D1S80 and Apo B amplifications systems (1).
For practical application data bases have been set up with a large german population sample. Observed allele frequencies were tested for Hardy-Weinberg equilibrium and compared to published data. This report further presents forensic efficiency data as derived from extensive family studies and case work.

Material and methods

DNA was extracted from ACD blood samples using a standard salting out procedure (2). Amplification of HLA-DQα and D1S80 alleles was performed with commercially available typing kits (Perkin Elmer Cetus) according to the manufacturer`s protocols with the following modifications: input DNA 25 ng (DQα) and 20 ng (D1S80). Amplified DQα alleles were analyzed by a reversed dot blot assay; D1S80 alleles were run on 5% polyacrylamide gels (1 x TBE, 2 V/cm, 16 h) and subsequently stained with ethidium bromide.
Amplification of Apo B alleles was carried out with primers described by Ludwig (3) under the following conditions: 94°C for 2 min, 62°C for 3 min, 3 cycles; 94°C for 1,5 min, 61°C for 3 min (auto extension 10 sec/cycle) 28 cycles. Analysis of amplified fragments was performed on 1.8% "MetaPhor" agarose gels (1xTBE, 4 V/cm, 8 h). Apo B alleles were identified by coelectrophoresis with an allelic ladder, composed of 9 discrete alleles.

Table 1 **DISTRIBUTION OF HLA - DQα ALLELES**

DQα - ALLELE	Lab Dr. Arnold (%) n=604	Schneider et al (4) (%) n=318	Helmuth et al (5) (%) n=826
1.1	14.0	15.41	13.7
1.2	21.0	22.96	19.7
1.3	9.3	6.92	8.5
2	13.6	10.38	10.9
3	11.2	13.52	20.1
4	30.8	30.82	27.1

(4) south-western Germany, (5) US Caucasians

Results

1. Allele frequency distributions

Frequency estimates for HLA-DQα, D1S80 and Apo B amplification systems were based on a population sample originating from southern and middle Germany.
At the HLA-DQα locus six alleles can be typed by hybridisation of the amplified product to allele specific oligonucleotide (ASO) probes. Identification of the correct genotypes is fast and unequivocal provided that the recommended hybridisation and washing temperature (55°C) is correct within the

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Thanks are given to Cellmark Diagnostics for oligonucleotide synthesis and to P.Debenham for helpful discussions. This work was supported in part by grants from MURST (Fondi 40%).

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RESULTS AND DISCUSSION

An average of 40 repeat units were resolved in each examined individual (Fig. 1). It was found that 1.7% of repeat units scored from 92 separated alleles were "null "or 0-type repeats, compared to 70.20% a-type repeats, and 28.10% t-type repeats. The diploid code 1(aa), 2(tt), 3(at), 4(a0), 5 (t0), and 6(00) was used for parentage testing. Fourteen exclusions and 9 attributions probated by mini/microsatellite analysis were confirmed without ambiguity, with a mean of 6.8 exclusions in the first 35 repeats. Biostatistical values of paternity calculated with traditional DNA markers (range 98 to 99.45%) increased significantly (>99.999%) using the MVR test in the parentage analysis.

Personal experience proves higher efficiency of the single MVR assay compared to traditional mini/microsatellite DNA analysis for testing genetic relationships.

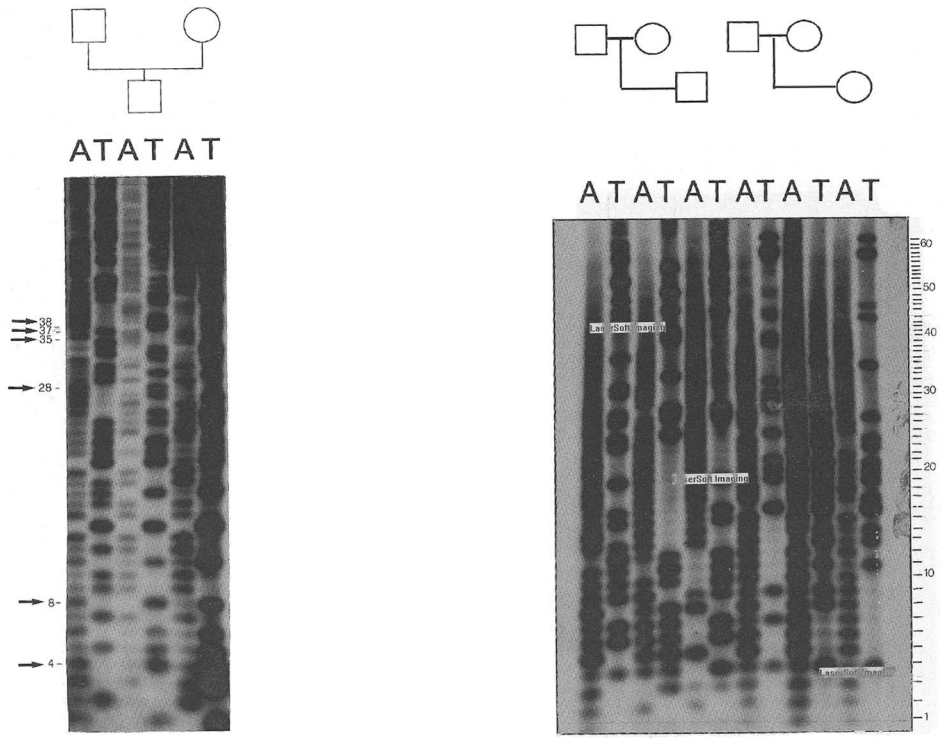

Fig.1. Examples of minisatellite repeat coding by MVR-PCR on total genomic DNA prepared from nuclear families for paternity testing.

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PATERNITY TESTING IN ITALY USING MINISATELLITE VARIANT REPEAT (MVR)

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MVR-PCR provides an innovative and highly discriminating technique to assess individual variation in human DNA (1). Using MVR specific amplimers to amplify the D1S8 (MS32) locus, a ladder of PCR products corresponding to different classes of repeat units, a-type and t-type are generated to produce a diploid digital code derived from the superimposed maps of specific alleles (1). MVR-PCR can be used in pedigrees examination, forensic analysis and population studies (1,3,4).
We have analysed 23 Italian cases of disputed paternity and compared the results founded by MVR technique using those obtained with a set of mini/microsatellite markers (APOB, HLADQa, YNH24, 3' HVR, D5S127, D1S80) (5).

MATERIALS AND METHODS

Peripheral lymphocytes DNA was extracted according to standard protocols. About 200 ng of genomic DNA was amplified using MVR specific amplimers (32-OR, TAG, 32-TAG-A, 32-TAG-T) (1). Reactions were cycled for 1.2 min at 94°C, 1 min at 66°C and 2 min at 70°C for 19 cycles on a DNA Themal Cycler (Perkin-Elmer Cetus). Aliquots of 14μl were subjected to electrophoresis on agarose-gel (1.5% w/v) for approximately 14 hours. DNA was transferred to a nylon-membrane by Southern blotting, and hybridized with a ^{32}P MS32 probe (insert). The MVR code defined by a-type and t-type alleles was established by a computer program designed by Cellmark Diagnostics (U.K.)

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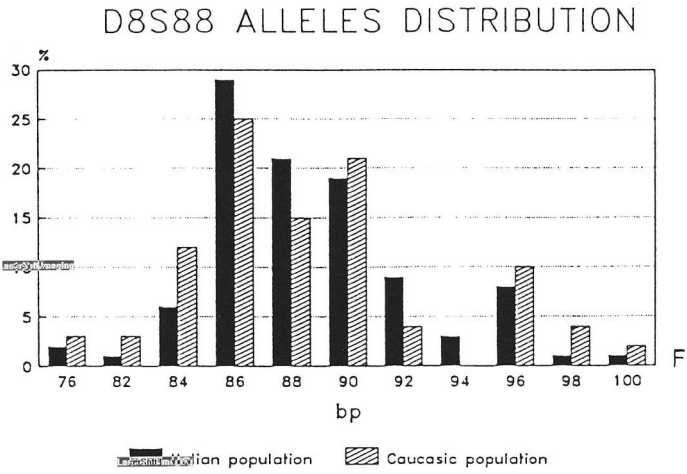

FIGURE 1

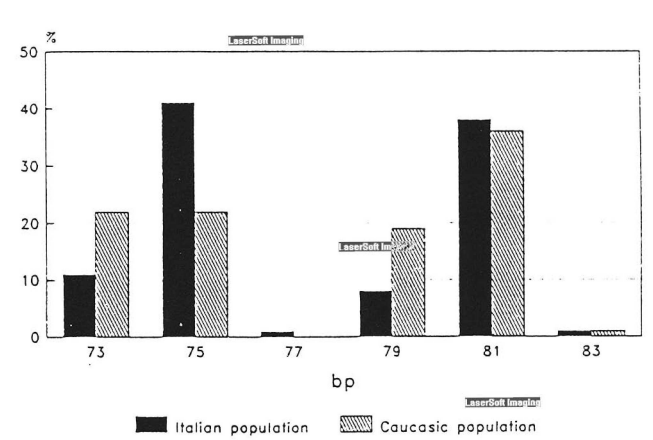

FIGURE 2

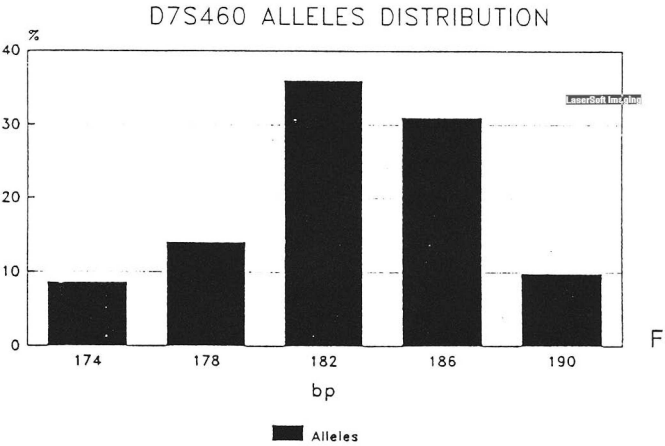

FIGURE 3

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Moreover, we detected an additional 77 bp allele at D8S85 and a 94 bp allele at D8S88. Of the three markers, D8S88 has an high number of alleles with low frequencies and therefore appears as the most informative and potentially useful. On the other hand, being a tetranucleotide, D7S460 was the easiest to resolve and analyse by gel electrophoresis.
The high heterozigosity and potential informativeness of these markers prompted us to evaluate their applicability to paternity testing. In ten cases of mother/father/child trios we observed concordant results with those obtained using three well characterized VNTR, namely D1S80, Apo B and COL21A. These data indicate that D8S85, D8S88 and D7S460 can be valuable, additional markers of biological identity useful for paternity disputes and other forensic medicine applications.

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Tables and figures

LOCUS	PRIMERS (5'-3')	CICLES	AMPLIFICATION CONDITIONS
D8S85	AGCTATCATCACCCTATAAAAT AGTTTAACCATGTCTCTCCCG	27	94°C 1 min 55°C 2 min 72°C 2 min
D8S88	TCCAGCAGAGAAAGGGTTAT GGCAAAGAGAACTCATCAGA	27	94°C 1 min 55°C 2 min 72°C 2 min
D7S460	AATACCCCAAGGGGTGGTAA CATTGATGAACAGTTCAAGCA	30	94°C 1 min 55°C 2 min 72°C 2 min

Table 1: Primer sequences and amplification conditions.

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CHARACTERIZATION OF THREE MICROSATELLITES AND THEIR APPLICATION IN PATERNITY TESTING.

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Introduction

The recent identification of hypervariable sequences interspersed within the human genome has provided new genetic markers useful for the localization of disease genes as well as for biological typing (1).
In previous studies aimed at identifying the parental origin of the extrachromosome found in cases of placental mosaicism, we successfully used several chromosome-specific microsatellites with high polymorphism information content (PIC).
To evaluate their efficacy in paternity testing we selected two CA repeats, D8S85 and D8S88, and one tetranucleotide repeat, D7S460, on the basis of their reported heterozigosity (2, 3). We analyzed their allele distribution and frequency in an italian population and compared it with that available.

Materials and methods

We have collected blood samples from 50 unrelated italian donors. DNA was isolated by standard phenol/chloroform extraction after proteolytic digestion of the cell pellet. 100 to 500 ng of DNA was processed by PCR-mediated amplification using primers specific for D8S85, D8S88 and D7S460. Primer sequences and amplification profiles are illustrated in table 1.
After analyzing amplification products by agarose gel electrophoresis, allelic variants were characterized on a 10-12% non denaturing polyacrilamide gel and subsequently on a modified DGGE based on a 10% polyacrylamide denaturing gel immersed in circulating buffer kept at 60°C.
Allele size was determined by comparison with molecular weight markers, including 10 bp and 100 bp ladders (GIBCO BRL) as well as with an allelic mix previously characterized and provided by J. L. Weber.
Previous reports indicated an heterozigosity of 0.83 for D8S88, 0.75 for D8S85 and 0.95 for D7S460 (2,3). Allele distribution, estimated on a caucasic population, was previously reported only for D8S85 and D8S88 (2).

Results and discussion

Using the modified DGGE, we were able to separate the allelic variants of the highly polymorphic microsatellites and calculate their distribution on 50 italian, unrelated donors (Figures 1-3). Comparing our frequencies to those estimated by Weber et al. (2) on 58 CEPH individuals, we observed significant differencies for D8S88 and D8S85 (Figures 1, 2).

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golden eagle, scarlet macao, cuban amazon, yellow-shouldered amazon, yellow-an-green lorikeet grey-parrot, yellow-crested cackatoo, blue-throated conure, golden-shouldered-parrot, imperial eagle, tucuman amazon, bald eagle, Granada amazon, rose-crested cackatoo, red-tailed amazon, Ecuador amazon) with 1 to 16 siblings for paternity. In more than 50% siblings had to be excluded as breeding success. The DNA fingerprints were mostly analyzed by hand. The patterns of nestlings were compared with the patterns of putative parents. The bandsharing frequency (bsf) was calculated according to the formula bsf%= 2n/(a+b), where n represents the number of common bands and a, b the total number of bands per lane (Jeffreys et al., 1985b). Normally about 20% of bands agree even with non-related individuals. This conformity can be affected either by chance or by sex or is typical for populations or species. In all cases of excluded paternity, we calculated a bandsharing frequency of 10% to 25%, whereas related individuals showed a bandsharing frequency of 55% to 85%.
With RAPD fingerprinting we tested different species of falcons (peregrine falcon, lanner falcon, saker falcon, gyrfalcon and their hybrids) with more than 60 primers. We got the best result with the primer Operon O 11 (5`-GACAGGAGGT-3`). The RAPD analysis of each species of falcon showed specific bands and the hybrid-falcon bands of both species. With many primers RAPD`s showed up to 15 patterns for each sample, but are not polymorphic for individuals and also not for species of falcon. In these cases we could only differ between falcons and eagles or parrots.

Discussion

All species of birds, tested with DNA fingerprinting, polymorphic fragment patterns are detectable, especially with the probes $(GGAT)_4$ and $(GACA)_4$. In all cases of paternity testing we were able to get a clear result. As a consequence dealers which had obtained eggs by robbery or smuggling could be convicted on trial. As a result of our success in paternity testing, a significant reduction in "breeding success" of some dealers occurred.
With RAPD fingerprinting we tested some species of falcons and their hybrids. In some cases the hybrids are phenotypically indistinguishable from their parents. By performing RAPD fingerprinting using the Operon primer O 11 we detected species-specific patterns. As a result we are able to discriminate between the hybrid falcons and true-bred falcons.

Acknowledgments

We thank the Federal Minister of Environment, Natural Protection and Reactor Safety for supporting this work.

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Paternity testing of endangered species of birds by DNA fingerprinting and random amplified polymorphic DNA fingerprinting

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Introduction

Since 1985 DNA fingerprinting is the method of choice to solve forensic problems such as paternity testing (Jeffreys et al., 1986) or identification of immigration cases (Jeffreys et al., 1985 a, b). Its applicability became evident not only in man but also in many biological problems. Jeffreys'probes 33.15 and 33.6 or Epplen's oligonucleotide probes were used in DNA fingerprinting of dogs and cats (Jeffreys & Morton, 1987), non-human primates (Weiss, 1989), birds (Longmire et al., 1991, Miyaki et al., 1993), fishes (Schartl et al., 1993) or even plants (Weising et al., 1991). Especially its application in paternity testing turned to account in many species of animals (Lubjuhn et al., 1991, Mariat & Robert, 1993, Wickings & Dixson, 1992). The new method of random amplified polymorphic DNA fingerprinting (RAPD fingerprinting) allows determination of paternity with very small amounts of DNA such as in the case of insects (Hadrys et al., 1993).
The German Federal Ministry of Environment, Natural Protection and Reactor Safety subsidizes a project to apply DNA fingerprinting in the protection of endangered species, such as birds of prey or parrots (Wolfes et al., 1991, Máthé et al., 1993). The Washington Convention on International Trade in Endangered Species of Fauna and Flora (Cites) only allows selling of rare species by breeding success. These rare species are, however, hardly breeding within captivity. Therefore, dealers are often misled to get eggs or nestlings illegally by robbery to sell them with high profit margins. But now, those dealers could be convicted by DNA fingerprinting (Máthé et al., 1993). In case of getting only very small amounts of blood from nestlings or little birds or tissues from dead birds, the method of random amplified polymorphic DNA fingerprinting should be proved of its applicability.

Material & Methods

We got more than 600 blood and tissue samples of 32 species of birds of prey and parrots, in most cases by confiscation. Peripheral blood (0,5-1,0 ml per sample) was diluted in two volumes of anticoagulant preservative solution (APS) buffer (Arctander, 1988). DNA was extracted by the salting out method of Miller (1988). The DNA was stored at 4°C, diluted in 1xTris-EDTA buffer. About 10 µg DNA of each sample was digested with 50 units *Hinf*I. After electrophoresis in 0,6% agarose gels (1xTBE) DNA was transferred to nylon membranes by Southern blotting or downward gravity blotting (Koetsier et al., 1993). Membranes were hybridized with digoxigenated oligonucleotide probes, mainly $(GGAT)_4$ and $(GACA)_4$. Detection of signals was carried out by an anti-DIG-AP-conjugate and a subsequent color reaction with NBT/BCIP or with the chemoluminescent substrates AMPPD and CSPD.
For RAPD fingerprinting it is useful to dilute DNA in 0,1xTris-EDTA buffer. We took different concentrations of DNA for each sample of 1 ng to 40 ng. As primer (10 bp) we used Operon kits O, E and R. The RAPD analysis was performed according to Williams et al. (1990).

Results

In the last four years we tested more than 100 families of birds (peregrine falcon, lanner falcon, laggar falcon, saker falcon gyrfalcon, prairie falcon, kestrel, common buzzard, long-legged buzzard, sparrow hawk, goshawk, kite, snowy owl, eagle owl, Griffon vulture, Bonelli's eagle,

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Thus we deduced the paternal haplotype in X: A2B18Cw1 which frequency in Spanish population is 0,0002939. GC's phenotype implies this same haplotype (A2B18Cw1) with a segregation probability of 21,377% and so we obtained a PI=0,21/0,0002939=733,62.

CONCLUSIONS

1. The father-sharing probability of X with GC and AC studying DNA markers was 99,7625%.

2. HLA revealed no haplotype shared between GC and AC (this happens in a 25% of cases between siblings) while GC and X shared the highly infrequent HLA haplotype A2B18Cw1. Thus the father sharing probability between GC and X became 99,8638%.

3. From the results for HLA and DNA we came to the conclusion of the existence of father-sharing consanguinity between GC-AC and X with a probability of 99, 99968%, "beyond a reasonable doubt".

Acknowledgments

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We used 8 blood groups, 5 protein polimorphysms, 2 red-cell enzymes, HLA system (A, B, C) and 6 DNA genetic markers (YNH24, TBQ7, MS31, MS43a, D1S80 and ACTBP2).

We used the markers´ frequencies found elsewhere for caucasic population for probability estimation.

RESULTS

1. The results obtained for blood groups, enzymes and proteins did not discard consanguinity but they had no statistical significance. Nevertheless, the results found for HLA and DNA showed a clear sibling relationship between AC-GC and X.

2. Studying the 6 DNA loci mentioned above, we obtained a paternity index (PI) of 419,93 and a consanguinity probability (father-sharing) of 99,7625. There was no incompatibility in any locus. When no coincidence was found, we asigned a value of x=0,25 (PI=x/y). When there was allele sharing, the value for x was 0,25 and for y was the shared allele population frequency.

3. HLA showed the following results:

- GC = A2A29 B18B50 Cw1Cw5
- AC = A29A30 B8B57 Cw6Cw7
- X = A2A29 B18B44 Cw1 C--

In order to find out X´s paternal haplotype we analyzed X´s mother, husband and children (who showed identical HLA phenotype):

- Mother = A2A29 B44B52 C--C--
- Husband = A1A-- B8B38 Cw6C--
- Children = A1A29 B8B44 C--C--

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CASE REPORT OF A DISPUTED PATERNITY WITH NO BIOLOGICAL SAMPLE FROM THE PUTATIVE FATHER

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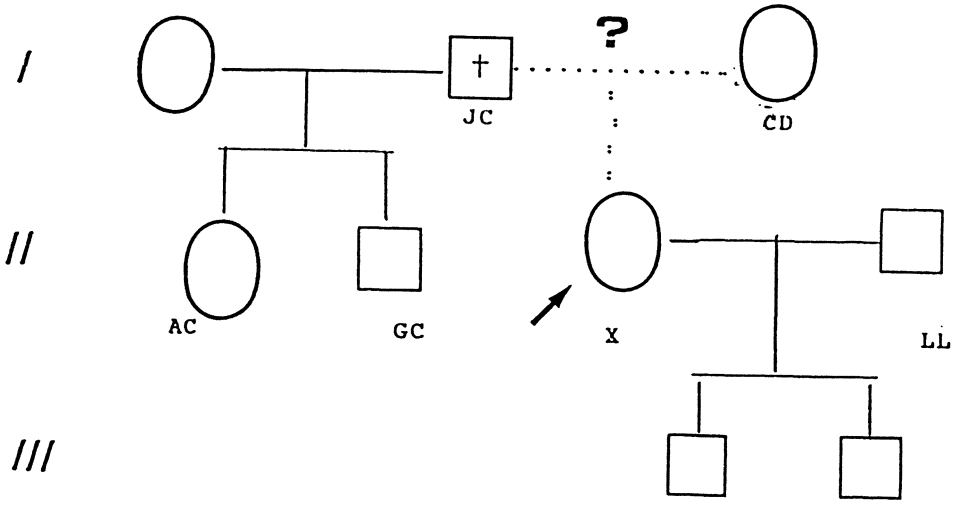
We received in the Paternity Testing Section (University Complutense Madrid) several blood samples in order to solve a parentage problem of some complexity (deceased father with no possibility of obtaining any biological sample from his remains). The problem is shown schematically in Fig 1.



In order to solve the disputed paternity problem we were able to get samples from GC and AC, son and daughter of JC (dead) and presumably X's step-brother and step-sister (sharing father). We found another difficulty, the lack of cooperation from M, biological mother of GC and AC. Therefore we had some trouble in finding the genetic markers received from their father. On the other hand, we could count on blood samples from CD, biological mother of X and also from X's husband and children.

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Locus	P.excl.
1 ACTBP2	0.88
2 HLA B	0.85
3 HLA A	0.78
4 YNZ22	0.69
5 D1S80	0.63
6 HLA DQα	0.60
7 3'ApoB	0.58
8 HLA C	0.35
9 Pi	0.34
10 PGM 1	0.33

Table 2. Probability of exclusion "a priori" of the first ten genetic markers in the population of Catalonia.

Table 3 shows the comparison between the different groups of polymorphisms traditionally used and the PCR group.

Polimorphism group	Chance exclusion
PCR	0.99
HLA ABC	0.98
RBC	0.73
EE	0.61
PP	0.58

Table 3. Probability of exclusion of different polimorphism groups used in paternity testing.

CONCLUSIONS

In the population of Catalonia the locus ACTBP2 shows the highest power of exclusion of all the markers we use in paternity testing.

The exclusion probability of the PCR group is 0.9978, and offers the best statistical values compared with the other genetic marker method groups used in paternity testing in the population of Catalonia.

The accumulated value of chance exclusion obtained by using PCR, HLA ABC, RBC, EE and PP is over 0.9999

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METHODS

After having carried out the research in Catalonian population and getting gene frequencies of all the alleles of each one of the five loci, the following parameters have been calculated:

- "A priori" Probability of Exclusion for every locus (P_{locus}). This value gives the efficiency of the locus studied in paternity exclusion.

- Accumulated Chance Exclusion, gives the efficiency of the five loci in paternity exclusion.

$$P_{accum} = 1 - (1 - P_{D1S80}) \times (1 - P_{YNZ22}) \times (1 - P_{3'ApoB}) \times (1 - P_{ACTBP2}) \times (1 - P_{HLA\ DQ\alpha})$$

- The Essen Möller value is the biostatistical efficiency of the locus.

$$E.M. = 10 + \log Y/X$$

- Accumulated Essen Möller value is the biostatistical efficiency of the loci:

$$E.M._{accum} = \frac{EM_{D1S80} \times EM_{YNZ22} \times EM_{3'ApoB} \times EM_{HLA\ DQ\alpha} \times EM_{ACTBP2}}{10^4}$$

RESULTS AND DISCUSSION

Table 1 shows the probability of exclusion and the Essen Möller value obtained for the five loci independently and as a whole.

Locus	P.excl.	E.M.value
ACTBP2	0.8808	9.0108
YNZ22	0.6977	9.3561
D1S80	0.6367	9.3323
HLA DQα	0.6072	9.5361
3'ApoB	0.5886	9.4717
Five loci	0.9978	7.1064

Table 1. Probability of exclusion and biostatistical efficiency of the five loci studied by PCR in the population of Catalonia.

DISCUSSION

In our population the best performance of all genetic markers is obtained by the ACTBP2. The results obtained by the other four PCR polymorphism studied are only improved by those of the loci HLA A and B. Table 2 shows the relation between these five genetic markers and some of the main classical systems.

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PCR POLYMORPHISMS COMPARED TO OTHERS GENETIC MARKERS IN THE PATERNITY TESTING

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INTRODUCTION

Paternity testing is an important aspect of the Forensic Laboratory in Spain since the reform of the civil code of 1981 about filiation. Peculiarities of the catalan civil law in paternity allowed the Forensic Hemogenetics Investigation Group of the School of Medicine of the Barcelona University to became one of the pioneers in this field in Spain. Ours was the first forensic laboratory in Spain that employed the HLA polymorphism to the paternity testing (1976).

The use of several techniques improves the confiance of the diagnostic so, by employing simultaneously red blood cell groups (RBC), plasma protein systems (PP), red cell enzyme systems (EE) and Human Leucocyte Antigens groups (HLA), a high standard of reliability was achieved in paternity exclusion as well as in inclusion.

The last biotechnological advances allowed to apply methods of DNA polymorphism in the field of the Forensic Medicine. The first DNA polymorphisms applied to Forensic Medicine, were the RFLP's (RESTRICTION FRAGMENT LENGHT POLYMORPHISM). Soon, several problems involved with allelic identification in population studies were resolved by using techniques based on POLYMERASE CHAIN REACTION (PCR). The PCR offered obvious advantages in paternity testing.

In 1990 we included the PCR method in our work in paternity testing. Presently we study five loci: D1S80 (pMCT118), 3'Apo B, D17S5 (YNZ22), HLA DQα and ACTBP2 (SE33). D1S80, D17S5, 3'ApoB and ACTBP2 loci, are VNTR polymorphisms (Variable Number of Tandem Repeats). The HLA DQα locus presents an ASO polymorphism (Allele Specific Oligonucleotide). PCR polymorphisms increase the information in paternity testing because of their variability, thus, reliability is improved.

In this work we have calculated the statistical parameters of the five polymorphisms studied by PCR, and we have compared them to the rest of the classical genetic markers. This allows to know accurately their efficiency in paternity testing.

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Table 2. Outcome of 61 cases of disputed paternity investigated with D1S80 and the five single locus systems D2S44, D5S43, D7S21, D7S22, and D12S11.

Number of men	Number of men/case				
	1	2	2 sibs	3	Total
Non-excluded	45	9	2	0	56
Excluded in D1S80 & other	2	9	1	3	15
Excluded in other systems	2	0	1	0	3
Total	49	18	4	3	74
Number of cases	49	9	2	1	61

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Table 1. Observed distribution of 23 D1S80 alleles among 372 unrelated Danes.

Allele	Observed number	Phenotype frequency	Allele frequency
17	3	0.0081	0.0040
18	148	0.3978	0.2218
19	2	0.0054	0.0027
20	20	0.0538	0.0269
21	19	0.0511	0.0255
22	26	0.0699	0.0363
23	16	0.0430	0.0215
24	214	0.5753	0.3602
25	25	0.0672	0.0336
26	14	0.0376	0.0188
27	6	0.0161	0.0081
28	41	0.1102	0.0551
29	36	0.0968	0.0497
30	13	0.0349	0.0175
31	64	0.1720	0.0887
32	4	0.0108	0.0054
33	4	0.0108	0.0054
34	3	0.0081	0.0040
35	2	0.0054	0.0027
36	4	0.0108	0.0054
37	1	0.0027	0.0013
40	1	0.0027	0.0013
41	3	0.0081	0.0040

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For the D1S80 system RMNE-values (Random Man Not Excluded) were calculated in two ways: i) for the 62 mother-child pairs, RMNE = 0.380, ii) by means of random reallocation of the 74 men, RMNE = 0.297. The average value observed was RMNE = 0.339, or a rate of exclusion of 66,1%. Using the genefrequencies given in table 1, a theoretical rate of exclusion of 64.0% was calculated. For comparison the observed exclusion efficiency of the five VNTR systems used routinely in 70 cases were: D2S44 87.1%, D5S43 51.4%, D7S21 91.4%, D7S22 92.9%, and D12S11 78.6%. (Hansen & Morling 1993).
No mother-child exclusions were observed in the D1S80 system, and all paternal exclusions were confirmed by at least two other DNA systems. No evidence of the presence of a "blank" allele was found.

CONCLUSION

The system D1S80 (MCT118) has proven a reliable system for use in cases of disputed paternity. The amount of material required is small, the technique is not time-consuming, and together with the high exclusion efficiency this makes the system well fit for use in the first step of paternity investigations.

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PATERNITY TESTING WITH DNA SYSTEMS: APPLICATION OF D1S80 PHENOTYPING TO DANISH PATERNITY CASES ANALYSED WITH FIVE VNTR SINGLE LOCUS SYSTEMS.

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INTRODUCTION

This study presents the results of examination with D1S80 (MTC118) in 61 Danish cases of disputed paternity involving 74 men. The calculations were based on 372 unrelated Danes. The exclusion efficiency of the system D1S80 has been compared with the efficiency of the VNTR single locus systems D2S44 (YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a).

MATERIAL AND METHODS

The details of the technique used for D1S80 examinations has been described elsewhere (Thymann et al. 1993). Briefly the AMP-FLP technique used comprised DNA extraction from 3 ul blood by the chelex method followed by PCR amplification according to Budowle et al. (1990). Typing of the PCR products were performed by vertical electrophoresis in polyacrylamide gels and silverstaining. Paternity testing using the five VNTR-single locus systems has been published previously (Morling & Hansen 1993, Hansen & Morling 1993)

RESULTS AND DISCUSSION

Among 372 random, unrelated Danes 23 different D1S80 alleles were observed. The frequencies are given in table 1. Out of the 276 possible phenotypes 72 were observed. In 297 two D1S80 alleles, and in 75 persons only one allele were observed, giving a heterozygosity of 79.8%.
The investigations of 61 cases of disputed paternity with the system D1S80 and the five single locus systems used routinely in paternity investigations are summarized in table 2. Of the 74 men 18 were excluded. The comparatively high proportion of possible fathers (56/74) can be explained by the fact that the DNA-investigations were used as a second step for men not already excluded by the conventional genetic marker systems. All the 18 men excluded by D1S80 were also excluded by at least two of the other DNA systems used. Three men were excluded by the other DNA systems but not by the D1S80 system.
The positive evidence **for** paternity was examined by calculating the paternity index (PI) for 60 men not excluded by the D1S80 system. The PI-values were calculated as described by Gürtler (1956). A paternity index, PI <10, was found in 47 cases (78%), and in 10 cases 11 <PI< 50. In only 3 cases PI > 100.

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Ziegle, J.S., Su, Y., Corcoran, K.P., Nie, L., Mayrand, E., Hoff, L.B., McBride, L.J., Kronick, M.N., Diehl, S.R. (1992): Application of automated sizing technology for genotyping microsatellite loci. Genomics 14: 1026-1031.

Table 2. The results from conventional blood typing, DNA fingerprinting and microsatellite genotyping in 25 parentage control cases. In cases **01 - 18**, the cow was subject to two AI (artificial inseminations) within 24 days with semen from two bulls; in cases **19 - 22**, parentage of a father or mother had to be confirmed; in case **25**, 4 bulls were indicated as possible fathers; in two cases (**23** and **24**) one parent (the mother) was not available for typing. ["-" indicates exclusion, "+" indicates, that the questionable animal(s) cannot be excluded from being the parent. The number in parentheses indicates the number of microsatellite loci leading to an exclusion].

Case no.	Conventional blood typing	DNA-fingerprinting	Microsatellite genotyping
01	bull1 + bull2 +	bull1 + bull2 +	bull1 -(1) bull2 +
02	bull1 + bull2 +	bull1 + bull2 -	bull1 + bull2 -(1)
03	bull1 + bull2 +	bull1 - bull2 +	bull1 -(2) bull2 +
04	bull1 + bull2 +	bull1 - bull2 +	bull1 -(2) bull2 +
05	bull1 + bull2 +	bull1 - bull2 +	bull1 -(2) bull2 +
06	bull1 + bull2 +	bull1 - bull2 +(?)	bull1 -(3) bull2 +
07	bull1 + bull2 +	bull1 - bull2 +	bull1 -(3) bull2 +
08	bull1 + bull2 +	bull1 - bull2 +	bull1 -(3) bull2 +
09	bull1 + bull2 +	bull1 - bull2 +	bull1 -(3) bull2 +
10	bull1 + bull2 +	bull1 - bull2 +	bull1 -(3) bull2 +
11	bull1 + bull2 +	bull1 - bull2 +(?)	bull1 -(3) bull2 +
12 a)	bull1 + bull2 +	bull1 + bull2 +	bull1 -(3) bull2 +
13	bull1 + bull2 +	bull1 - bull2 +	bull1 -(3) bull2 +
14	bull1 + bull2 +	bull1 - bull2 +	bull1 -(4) bull2 +
15	bull1 + bull2 +	bull1 + bull2 +	bull1 -(4) bull2 +
16	bull1 + bull2 +	bull1 - bull2 +	bull1 -(5) bull2 +
17	bull1 + bull2 +	bull1 - bull2 +	bull1 -(5) bull2 +
18	bull1 + bull2 +	bull1 - bull2 +	bull1 -(6) bull2 +
19	bull -(?) (chimerism ?)	bull -	bull -(1)
20	bull +(?) (chimerism ?)	not typed	bull +
21	bull - (?)	not typed	bull -(5)
22	mother - (disputed)	not conclusive	mother -(2)
23	bull1 - bull2 +(?)	bull1 -(?) bull2 +(?)	bull1 -(2) bull2 +
24	bull - (disputed)	not typed	bull -(2)
25	bull1 +(?) bull2 - bull3 - bull4 -	bull1 + bull2 - bull3 - bull4 -	bull1 + bull2 -(4) bull3 -(3) bull4 -(3)

a) Bull2 is the son of bull1. The same case also showed probable allele-non-amplification (Koorey et al., 1993) in the mother and in the calf. Non-maternity appeared to be a very unlikely explanation considering the constellation at 7 other microsatellite loci (2 more were investigated). Furthermore, the fingerprints of the calf and the cow were very similar.

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Table 1. Characteristics of the microsatellite loci and PCR reactions

PCR system [AT[a)]]	Locus	Chromosome	Microsatellite designation	No. of alleles	Size range (base-pairs)	Labelling dye	Reference[b)]
1 [58°C]	BOLADRBP1	23	MHCII	7	208-226	FAM	1
	DU17S2	U17	ETH121	7	173-211	TAMRA	2
	D21S4	21	ETH131	8	141-163	JOE	2
2 [65°C]	D5S3	5	ETH10	6	210-224	JOE	3
	DU2S1	U2	ETH225	6	141-159	TAMRA	2
	D19S2	19	ETH3	6	114-126	FAM	3

a) Annealing temperature. b) 1, Creighton et al. (1992) 2, Steffen et al. (1993); 3, Solinas et al. (1993).

Results and Discussion

Details on the estimation of the allele sizes based on the electrophoretograms and the calculated fragment sizes, the estimation of allele frequencies in the Swiss cattle population, and a study of the variation of the fragment sizes in different gel runs will be presented elsewhere (Glowatzki-Mullis et al., manuscript in preparation). However, in general run to run variations of the fragment size were minimal, which is very important when the results from newly analysed samples have to be compared to stored data. Table 2 summarizes the results from conventional blood typing, DNA fingerprinting and microsatellite genotyping in 25 parentage control cases. All cases were solved using the genotypes of the 6 microsatellite loci. Therefore, the set of microsatellites used in this study is an extremely powerful system for parentage control in cattle. Multiplexing of the PCR and the automated fragment analysis make this system very efficient and attractive for routine parentage control in cattle. In terms of time to perform the test (and this without the use of radioactivity), power of the results, costs and possibilities of computerization, it seems that in the area of DNA profiling microsatellite genotyping is the most promising approach. A PCR-based system also has other advantages over the conventional blood typing and minisatellite fingerprinting. Only minute amounts of DNA are required for PCR-based analyses. As opposed to the DNA fingerprints, the microsatellites represent a closed system, allowing consideration of both alleles and therefore providing complete genotype information for each locus. DNA-based typing is not affected by the age of the animals, in contrast to certain conventional systems. DNA can be extracted from blood, semen and other tissues and can be stored for practically unlimited periods of time, thus allowing the setup of DNA banks. Another disadvantage of conventional typing over microsatellite genotyping is the diversity of methods that need to be mastered. Furthermore, blood group testing depends on the production and maintenance of a panel of test sera. Based on the above findings and considerations, the blood typing service at the Institute for Animal Breeding in Berne, which handles about 3000 bovine parentage control cases a year, intends to gradually replace conventional blood typing with microsatellite genotyping.

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PARENTAGE CONTROL IN CATTLE BY GENOTYPING MICROSATELLITES

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Summary. *Microsatellites are highly polymorphic, readily detectable markers that offer great promise for paternity testing and individual characterization in humans and domestic animals. We present here our experiences with a system consisting of 6 microsatellite loci, as a new approach to parentage control in cattle. The microsatellite polymorphisms were visualized by automated fluorescence detection on the Applied Biosystems 373 DNA Sequencer with 672 Genescan Analysis software, and allowed us to solve all tested bovine paternity cases not solveable by conventional blood typing, and only partially solveable by multilocus DNA fingerprinting (pV47). We propose microsatellite genotyping as the sole method for parentage control in cattle.*

Introduction

Blood typing for parentage control has been offered as a service to breeders and herdbooks in many laboratories throughout the world since about 1950. Parentage control is carried out to verify the relationship of animals for pedigree registration, to determine the father in cases of multiple insemination or when mixed semen was used, and in animals originating from embryo transfer. Correct parentage among breeding stock is a prerequisite for an efficient breeding program.

Most questions of parentage in cattle can be solved adequately by current conventional testing: in our lab (Berne) conventional blood typing consists of the determination of blood group antigens, serum proteins and red cell enzymes; cases which are not conclusive in the routine analysis are completed, if possible, by the determination of bovine leucocyte antigens (BoLA). Nevertheless, in a few cases, the application of the mentioned polymorphisms does not allow a clear conclusion and should be extended by utilising other genetic marker systems.

Recently, a new type of genetic polymorphism in the area of DNA profiling (Easteal et al. 1991), based upon microsatellites, (i.e., repetitive 1 - 5 base pair motifs) has been described in several species, including cattle. These polymorphisms are due to the variable number of repeat units and can be visualized using the polymerase chain reaction (PCR) and subsequent polyacrylamide electrophoresis. Microsatellite genotyping can be automated by fluorescence-based fragment sizing, e.g., on the Applied Biosystems 373 DNA Sequencer equipped with 672 Genescan Analysis software (Ziegle et al. 1992). We now present a system of 6 polymorphic microsatellites of the $(CA)_n$ type that allowed us to solve 25 parentage control cases which could not be solved by conventional typing, and for which even DNA fingerprinting was not in every case conclusive.

Material and Methods

Blood and semen samples were obtained from routine paternity cases not solveable by conventional blood typing and only partially solveable by DNA-fingerprinting. DNA extraction was carried out as described by Dolf et al. (1992). Six polymorphic (number of alleles ≥ 6) microsatellite loci located on 6 different chromosomes were chosen (Table 1). PCR amplification was performed using a Perkin Elmer DNA Thermal Cycler in two multiplex reactions, each containing the primer pairs for three loci. The primers were synthesized and labelled at their 5'-ends (one of each pair) by a commercial oligonucleotide synthesizing service with the fluorochromes as indicated in Table 1. 50-100 ng of template DNA were amplified in 25 µl standard PCR reactions containing 2.5 units Taq polymerase (Boehringer Mannheim) and 0.5 - 0.8 µl of each fluorescent-dye-labelled forward and each unlabelled reverse primer (20 µM). Samples were subjected to 28 cycles of denaturation at 94° C for 30 sec, annealing for 30 sec at the temperature indicated in Table 1, and extension at 72° C for 1 min. The last polymerization step was extended to 7 min. The co-amplified PCR products were diluted 1:4 . To 1 µl of the mixture (combined 1:1 from the two diluted multiplex reactions) 4 µl of formamide and 0.5 µl of the Genescan 2500 Rox internal lane standard (Applied Biosystems) were added. Each mixture was heated at 95° C for 4 min, chilled on ice and applied to one lane of 6% polyacrylamide gel containing 8.3 M urea installed in a 373 DNA Sequencer of Applied Biosystems. The fluorescent signals were collected and analysed on a Macintosh computer equipped with the Genescan 672 software (Applied Biosystems). The fragment size was automatically calculated based on the internal lane standard, as described by Ziegle et al. (1992).

Blood group determination, BoLA-typing, and protein electrophoresis were carried out using standard procedures, and DNA fingerprinting (probe pV47) was as described by Dolf et al. (1992).

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Case 3

Prostatic chips from two patients were accidentally mixed in a surgical pathology laboratory. Histological examination revealed malignancy in some of the chips. Our laboratory was asked whether it was possible to match the DNA types of the benign and malignant tissues to the bloods of the two patients. The PCR-SSOP based HLA-DQA DNA typings (Fig 4) of the wax-embedded tissues and bloods of the two patients resolved the problem(Table 1).

CONCLUSION

Our experience illustrates that a variety of techniques and strategies are essential to resolve clinical as well as forensic problems.

Table 1 HISTO-PATHOLOGY CASE
HLA-DQA DNA TYPING

Patient	Blood	Tissue	
		Benign	Malignant
P1	1.2, 2	1.2, 2	-
P2	4, 4	-	4, 4

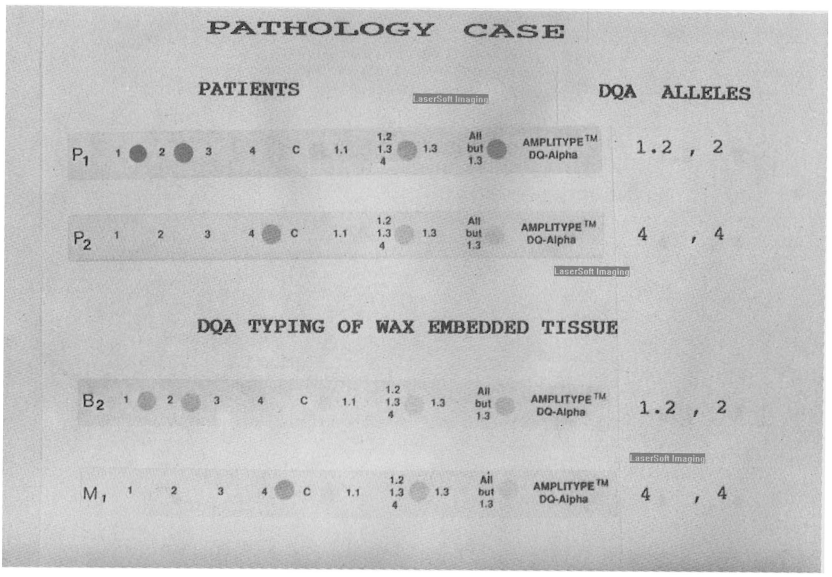

Fig. 4

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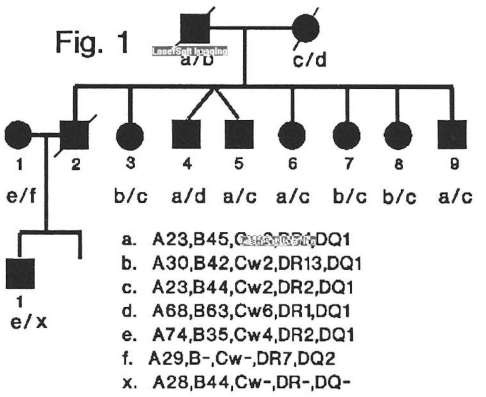
EXAMPLES

Case 1

A woman claimed that a millionaire, who had been murdered and buried a year earlier, had fathered her son. This was disputed by the other heirs and the insurance company. Exhumation seemed unnecessary as the deceased had seven siblings. The red cell and plasma protein results were uninformative. The red cell enzyme PGD*AC phenotype, present in the child was absent in the siblings of the deceased as well as the mother (all phenotype PGD*A). This was not conclusive and hinted at a possible exclusion. The HLA haplotype "x" which the child must have inherited from his biological father was not present in the siblings of the deceased putative father (Fig 1). The HLA "d" haplotype of the twin brothers of the deceased was an added proof that they all had the same parents. The results (not shown) of at least 2 VNTR probes also enabled us to exclude the child from the family.

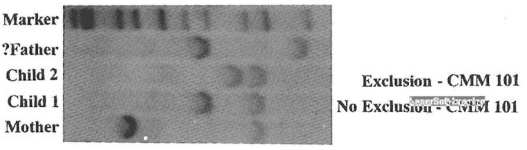
Fig. 1



Case 2

In a paternity dispute involving twins the putative father was excluded as the father of the one twin, II-2, (Fig 2), in the HLA system as well as with the restriction enzyme *Hae*III and the VNTR probes YNH24, CMM101 (Fig 3) and TBQ7.

Fig. 3



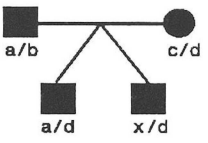


Fig. 2

a. A23,B57
b. A30,B-
c. A23,B49
d. A24,B18
x. A1,B52

DNA TYPING - STRATEGIES IN PATERNITY AND FORENSIC CASEWORK

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Since 1972 we have tested 7272 cases of doubtful paternity. Most cases were adequately resolved by testing the conventional red cell antigen, plasma protein, red cell enzyme and HLA polymorphisms. During the last year we have added DNA typing using single locus VNTRs in all cases not resolved by conventional methods or where the probability of paternity was too low.

DNA testing in forensic casework was recently introduced in our laboratory. This was particularly as a result of an increasing demand due to newspaper publicity of developments in DNA technology. The only other laboratory doing DNA tests in forensic casework in South Africa is the State forensic laboratory.

MATERIALS AND METHODS

Paternity cases:

The salting-out method (Miller *et al*, 1988) was used to obtain genomic DNA from peripheral blood. The DNA was digested using restriction enzymes *Pvu*II and *Hae*III, Southern blotted and hybridised to six VNTR probes YNH24 (D10528), CMM101 (D14513), TBQ7 (D10528),(Promega Corporation), Mucin, HaRas and 3'alpha globin. Fragment sizes were determined using a digitised video-image system developed in our laboratory. A fixed-bin approach was used in which the increments were 2%.

The results in 300 paternity disputes were used to evaluate probe and enzyme combinations in the local populations.

Forensic cases:

We have done a variety of forensic case studies over the last 12 months. These include rape, murder and body identification. DNA samples have been isolated successfully from vaginal swabs, underwear/clothing, organs/muscle/brain, drug abuser's syringes, amniotic fluid, bone marrow and wax-embedded tissue.

The problems were resolved using a combination of techniques, which include the reverse dot-blot technique (Cetus HLA DQA Forensic DNA Typing Kit), PCR-SSP, PCR-RFLP, PCR-SSOP as well as single and multilocus VNTR analysis.

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RESULTS

28 of the 41 cases gave no exclusions on conventional or DNA testing.

In 2 cases (see case 8 and 11) clear exclusions were found using only conventional systems; in 1 case (see case 4) DNA testing was useful for the exclusion of paternity.

In table 1 13 of 41 cases of disputed paternity are reported with exclusion.

Table 1 - Exclusion in 13 cases of disputed paternity

case 1 - exclusion: Duffy, PGM_1, DQalfa

case 2 - exclusion: Rh, Alfa-1-AT, Gc, ORM, DQalfa

case 3 - exclusion: Kidd, DQalfa, MCT118

case 4 - exclusion: DQalfa

case 5 - exclusion: AB0, Hp, MNSs, Kidd, Tf, Alfa-1-AT, C3, DQalfa, YNH24, MCT118

case 6 - exclusion: AB0, MNSs, Hp, Tf, DQalfa, YNH24, MCT118

case 7 - exclusion: MNSs, Kidd, PGM_1, Tf, Alfa-1-AT, DQalfa, YNZ22, ApoB

case 8 - exclusion: ADA, Alfa-1-AT, PGM_1

case 9 - exclusion: Rh, AcP, ORM, Tf, DQalfa

case 10 - exclusion: Rh, Alfa-1-AT, DQalfa, MCT118

case 11 - exclusion: MNSs, Kidd, ADA, PGM_1, Alfa-1-AT

case 12 - exclusion: Rh, Duffy, ApoB

case 13 - exclusion: AcP, YNZ22

The analysis by DNA polymorphisms appears to be quite valuable in the study of paternity cases.

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APPLICATION OF CONVENTIONAL AND DNA POLYMORPHISM TO PATERNITY CASES

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INTRODUCTION

Multilocus and single locus minisatellite probes have been applied to the paternity expertise for sometime.

DNA profiling has been routinely used in cases of disputed paternity at the Institute of Legal Medicine of Padua since 1991.

A series of 41 cases of paternity testing have been investigated using a single locus probe (pYNH24), up to 3 AMP-FLPs (ApoB, YNZ22, MCT118), HLA-DQalfa, and also 21 systems comprising red cell antigen, red cell enzyme and serum protein polymorphisms.

In the present study we have assesed and compared the use of both DNA testing and conventional markers in each of the above mentioned paternity cases.

MATERIALS AND METHODS

The material comprised 41 cases of disputed paternity.

Routine paternity testing consisted of the conventional analysis of the red cell antigen systems: ABO, MNSs, Rhesus, Kell-Cellano, Duffy, Kidd, Lutheran, the red cell enzymes AcP, PGM_1, GLO, EsD, ADA, AK, 6-PGD, the serum proteins Hp, Tf, Pi, Bf, ORM and PLG.

DNA was extracted from EDTA-blood using a non-organic solvent procedure. Genomic DNA was digested with HinfI, submitted to electrophoresis and Southern blotting, and hybridized with ^{32}P-labelled DNA probe, YNH24. The radioactive fragments were visualized by autoradiography.

PCR amplification of MCT118, ApoB and YNZ22 was performed using previously published conditions and primers (1-3). PCR products were separated by electrophoresis in 1x TBE Buffer using 6% polyacrylamide (29:1 Acrylamide/Bis) for ApoB, 8% for MCT118 and YNZ22. Electrophoresis was performed at 150 V in a C.B.S. apparatus for about 3-4 hours (depending on the genetic system). The marker used was Marker VI Boehringer. The amplification products were visualized directly after staining the gel in ethidium bromide.

HLA-DQ alfa were amplified by PCR using the Amplitype^{TM}DQalfa Kit (CETUS) according to the recommended protocol.

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allele isolated from buccal swabs of the child and the corresponding maternal allele isolated from whole blood is 0.9993. Furthermore, the absolute percent difference between the child's allele and the corresponding mother's allele is .84% with a standard deviation of .56%. The average percent difference between the child's allele (buccal) and the mother's allele (blood) is -0.37% of the maternal allele size (Standard deviation is 0.83). Finally, there was no correlation between the percent difference of correponding alleles of the mother and child and the size of the allele. These results are similar to what is observed when the mother's and child's alleles are both derived from blood samples.

III. Case Observation

A. Age of Samples:

DNA from buccal swabs that had been stored for over 6 months at room temperature was successfully analyzed for RFLP results corresponding to the locus D2S44. DNA from buccal swabs that had been stored frozen for over one year was successfully analyzed using PCR results for DQ alpha and the loci corresponding to D1S80, SE33, CYP-19, YNZ22,APO-B, and Col2A1.

B. Production History:

Genetic Design Inc. performed its first buccal swab during 1991 on an geriatric individual that could not be bled for medical reasons. Since then we have processed over 1000 cases using buccal swabs. Currently we are processing over 100 buccal swab cases per month (mother, child,and alleged father) using RFLP analysis of the loci D2S44, D10S28, D17S26, and D4S139 with an average turnaround time of 17 days and a reswab rate of less than 1%. The observed heterozygosity frequencies for the four loci tested using buccal swabs were similar or identical to the frequencies observed for cases tested with blood samples. Of the 1000 cases tested, 32% were exclusions and 68% were inclusions.

IV. Summary and Conclusion

A. Buccal cells are suitable for RFLP and PCR.

B. Genetic results from buccal swab are the same as blood.

C. Eight buccal swabs yield an average of 8.6 micrograms.

D. More than 1000 cases analyzed (less than 1% reswab).

E. SAFE, painless and effective.

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to the Buccal Swab Mailer Kit. Each envelope is sealed and initialed by the individual and the phlebotomist to maintain the chain of custody. The buccal swab kit is mailed to Genetic Design Inc. by 2 day express mail.

C. Isolation of DNA from the cotton swab:

The cotton material is cut off of each swab and collected into a 15 ml conical tube. Cell lysis buffer (.01 M Tris, .01 M EDTA, .1M NaCl, 2% SDS, and Proteinase K) is added to the cotton and incubated for 1 hour at 56° C. The cell lysate is recovered from the cotton by centrifugal filtration. The DNA is purified using phenol/chloroform extraction followed by ethanol precipitation. DNA concentration is determined by UV absorbance at 260/280 for RFLP testing and by the Life Technologies Human Quantitation blot for PCR analysis. Following purification and quantitation of DNA, the samples are further processed for either RFLP or PCR analysis.

D. Variation of DNA concentration:

Buccal Swabs were collected from nine individuals over a fourteen day period. Eight buccal swabs were collected per individual on days 1,5,10, and 14. The DNA was isolated and processed for RFLP analysis for the loci D10S28 and D2S44. Individual RFLP alleles are analyzed for size and amount of DNA by comparing the density of the alleles to quantitation standards. The amount of DNA varied from 2.3 micrograms to 14 micrograms of DNA per eight buccal swabs. An average of 8.6 micrograms of DNA is obtained from 8 buccal swabs.

E. Comparison of RFLP sizes between DNA isolated from buccal swabs and whole blood:

1. Comparisons of DNA RFLP results between whole blood and buccal swabs were analyzed. The standard deviation of measurement in allele size when comparing blood size measurements from the same individual versus buccal size measurements is 0.60% of the allele size, which is similar to the standard deviation from two different blood specimens from the same individual (0.66%).
2. The size of the obligate maternal allele from the child's buccal cells are compared with the size of the allele obtained from the mother's whole blood. One hundred comparisons were made between the obligate maternal allele of the child (buccal) and that allele detected in the mother (blood) and analyzed for the loci D10S28 and D2S44. The correlation of allele measurements between the obligate maternal

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DEVELOPMENT AND VALIDATION OF BUCCAL SWAB COLLECTION METHOD FOR DNA TESTING FOR PATERNITY TESTING

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I. Introduction

One significant advantage of DNA typing as compared to classical serological testing is that tissue other than blood can be used to obtain DNA. The use of buccal swabs has several advantages over the collection of blood samples. Buccal swab collection is a noninvasive method of tissue collection which is less painful and traumatic than venipuncture. It is a safe alternative for collection of tissues from newborns, children, and adults who cannot have blood drawn for medical or religious reasons. Furthermore, it reduces the risk involved in shipping liquid blood by eliminating the risk of tube breakage. Finally, buccal swabs are resistant to extreme temperatures which can affect the results from liquid blood.

The purpose of this presentation is to present the development and validation of our buccal swab procedures. This study will present the method of extracting DNA from the buccal cells collected from the swab. Additionally, it will discuss the variation in the amount of DNA collected from buccal swabs as well as compare the allele size measurements of DNA obtained from buccal swabs and blood. A summary of buccal swab cases will be presented with regards to observed heterozygosity, exclusion rates, turnaround time, and retest frequencies.

II. Development and Validation of Buccal Swab Test

A. Choice of swab collection device:

GDI investigated four different types of brushes: cervical brush, dacron swab, cotton swab, and a toothbrush. The cotton swab (Baxter Scientific Products S/P Sterile Cotton Tip Applicator catalogue #A5002-5) was chosen as the specimen collection device due to consistent high yield of DNA and its safe and nonabrasive texture.

B. Specimen Collection Kit:

8 buccal swabs are collected per individual. Two swabbings of each quadrant of the cheek are collected . The eight swabs are placed in a paper envelope that is attached

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Table 3. DNA profiling test results (observed fragment sizes, kb)

	Probe					
	Mucin	YNH24	MR24/1	TBQ7	MS43a	3'α HVR
DP (Husband)	5.01	3.66	4.36	4.18	9.23	2.93
	4.62		3.39	1.41	4.31	2.24
JP (Mother)	4.47	4.25	**4.83**	**4.49**	7.52	**4.02**
	1.92	**2.83**	4.41		**4.66**	1.37
C (Child)	4.65	3.72	**4.83**	**4.51**	9.24	**4.03**
	1.94	**2.84**	3.40	1.42	**4.67**	2.96
BP(Brother)	4.98	3.70	4.31	5.57	9.29	ND
	4.61	3.60	4.22	3.56	7.70	

Discussion

This case demonstrates the value of DNA profiling in establishing beyond doubt the paternity of a child in a situation where other factors strongly suggest a different outcome. It was important that a high relative chance of paternity was obtained to counter this *à priori* evidence. For the same reasons it was also important to exclude the man's brother from possible paternity, and again the high discriminating power of the DNA systems employed enabled this to be the case.

This is an important demonstration of fertilisation by a man deemed infertile by the criteria usually accepted for defining a successful vasectomy.

Failed vasectomies are normally characterised by the time post-operation at which the failure is observed and by the nature and numbers of any spermatozoa seen. This case belongs to a very small subset of failures where initial sperm counts were zero and only later (in this case following the pregnancy of the man's wife) were any spermatozoa identified. Even at this later stage the spermatozoa seen were very few and were all non-motile and would normally satisfy criteria for infertility. Alderman (2) states that when such sperm characteristics were observed he would "cautiously advise" that the operation had been a success. This is supported by Edwards and Farlowe (3) who showed no associated pregnancies in their study of 200 such technical failures. Schmidt (4), however, disagrees and would "find it difficult to believe that these patients [showing the presence of any spermatozoa of any kind] are totally safe".

This single case does nothing to detract from the view that men with very few non-motile spermatozoa following vasectomy are likely to be infertile but clearly demonstrates that this conclusion is not immutable.

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Methods

Sperm counts were carried out by Dr T. Kelly of the Dept of Clinical Microbiology, Mayday Hospital, Surrey. Semen samples were stored for a maximum of two hours at room temperature. The whole film was scanned at 400x magnification by two members of staff.

Paternity testing was performed at LHMC. Conventional grouping was carried out using the following systems: ABO, Rh, MNSs, Fy, K, Lu AK, ADA, EAP, Glo, ESD, PGM, Hp, Gc, Pi, Tf, PLG and α2HSG. DNA profiling tests were performed using the methods described by Syndercombe Court *et al* (6). Six single locus probes (Mucin, MR24/1 (Amersham), YNH24, TBQ7, 3'α HVR (Promega) and MS43a) were utilised.

Results

Sperm counts

The results of the semen examinations are summarised in table 1. They show that no motile spermatozoa were observed in seven separate examinations over a period of three years, both before and after JP's pregnancy.

Table 1. Summary of semen examinations

Time post-op	Observations
12 weeks/ 18 weeks	Initial semen examinations. Spermatozoa not seen
28 months	JP confirmed pregnant
2½ years	5 further semen examinations. No spermatozoa seen in 2 samples. Few non-motile spermatozoa seen in 3 samples
3 years	Child Born

Paternity testing results

Conventional tests:

The results of the conventional red cell antigen, serum protein and red cell enzyme tests do not exclude DP from paternity of the child (results not shown). They indicate a relative chance of paternity of about 94%.

The conventional tests also fail to exclude the man's brother, BP, from possible paternity of the child.

DNA Profiling tests:

The results of the DNA profiling tests are shown in table 3.

These results do not exclude DP from paternity of the child using fragment size matching criteria established in this laboratory (10). They indicate a relative chance of paternity of about 99.99%. When combined with the figure from the conventional typing this figure is increased to over 99.999%. Such figures are usually considered to offer virtual proof of paternity.

The results for the man's brother, BP exclude him from paternity on the MR24/1 and TBQ7 systems. In both these tests the child has a fragment which must have been inherited from its true father which BP does not possess.

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AN UNUSUAL CASE OF FAILED VASECTOMY CONFIRMED BY DNA PROFILING

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Introduction

The increasing use of DNA polymorphisms in recent years has greatly improved the ability of parentage testing to confirm paternity in those cases where the named man is not excluded from fathering the child. The high discriminating power of such systems is of great benefit in cases where other evidence suggests that the man is not the father. Such a case is presented here where the putative father had undergone an apparently successful vasectomy.

Vasectomy is a safe and effective means of sterilisation. The success of the operation is monitored by the examination of semen samples at suitable intervals post-operation. Failed vasectomies are identified by the presence of spermatozoa in the semen and they can be classified according to the time post-operation that sperm are identified and the condition of those sperm.

The majority of failures are identified in the first sperm counts carried out post-operation. Late failures can be defined as those in which spermatozoa, in any condition, are identified after the operation has been declared a success. This normally means after two consecutive semen samples have been completely free of spermatozoa.(1,2)

These late failures can be further divided into those in which high levels of active spermatozoa are observed and those in which non-significant numbers of non-motile spermatozoa are present. The mechanism by which these latter "technical" failures occurs is unclear but they have previously been reported as being of no clinical significance (3) and no confirmed associated pregnancies have been identified in the studies cited.

Case History

A 30 year old man (DP) and his 25 year old wife (JP) had decided that their three child family was complete and in December 1987 DP underwent a vasectomy under local anaesthetic. Semen samples at 12 and 18 weeks post-operation were clear of spermatozoa and he was told that the vasectomy had been successful.

Two and a half years later, in 1990, JP became pregnant. Repeated examinations of DP's semen showed the presence of small numbers of non-motile spermatozoa; a pattern normally thought to be indicative of infertility. No motile spermatozoa were observed in five specimens. The assumption of many of their associates was that JP had been unfaithful, which she always denied. DP was convinced of his wife's fidelity. A summary of these events is shown in table 1.

After an uneventful pregnancy, a healthy male child was born on April 10 1991. It was decided to carry out tests including DNA profiling to confirm the paternity of the child.

These tests provided no evidence that DP was not the father of the child. A relative chance of paternity of over 99.99% was calculated. Such a figure is normally considered to offer virtual proof of paternity, provided that a close relative of the man is not a possible father.
In order to exclude this possibility it was decided to test DP's brother, to which DP, JP and the brother BP all willingly consented. The brother was duly excluded from paternity on two of the five DNA tests used.

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(AMP-FLD). Frequency distribution in Danes. Intra and Inter laboratory reproducibility of the technique. *Forensic Science International.*

Table 1: Allele frequencies in 111 unrelated English from north-east England compared to other Caucasians

Allele (number of repeats)	Danes (a) n = 210	US-Caucasians (b) n = 99	Finns (c) n = 140	East Slovenian (d) n = 100	North-east England (e) n = 111
17	0.005	-	-	-	-
18	0.224	0.293	0.307	0.235	0.283
19	0.002	0.010	0.011	0.030	-
20	0.028	0.020	0.032	0.025	0.023
21	0.014	0.025	0.018	0.015	-
22	0.041	0.045	0.014	0.045	0.045
23	0.019	0.020	0.014	0.025	0.018
24	0.371	0.328	0.311	0.375	0.324
25	0.036	0.035	0.075	0.045	0.045
26	0.017	0.015	0.011	0.025	0.081
27	0.012	0.056	0.007	0.040	0.013
28	0.050	0.066	0.068	0.045	0.049
29	0.057	0.056	0.032	0.040	0.058
30	0.010	0.010	0.043	0.010	0.005
31	0.088	-	0.079	0.010	0.090
32	0.005	0.010	-	0.015	0.005
33	-	0.005	-	0.010	0.005
34	0.005	0.005	-	0.010	-
35	0.005	-	-	-	0.005
36	0.007	-	-	-	0.005
37	0.002	-	0.007	-	0.005
..	....	....	....	....	0.005
..	....	....	....	....	-
41	0.002	-	-	-	-
41	-	-	-	-	-
					0.005

(a) Thymann et al., 1993; (b) Budowle et al., 1981; (c) Sajantila et al., 1992; (d) Nasikov et al., 1992; (e) present study

Table 2: Phenotype of five paternity cases each excluded with three single locus VNTR probes (g3, MS1, MS43)

Relation	Case 1	Case 2	Case 3	Case 4	Case 5
Putative father	24, 31	24, 29	24	23, 36	18, 25
Child	22, 24	24, 29	24, 25	18, 22	18, 24
Mother	22, 24	18, 29	24	18, 25	18, 24
Conclusion	NE	NE	E	E	NE

NE not excluded; E excluded

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(0.05%). A single allele with more than 41 repeat units was also found in heterozygote combination with allele 23.

The allele frequencies of north-east England were very similar to the Caucasian populations of Finns, East Solvenians, Danes and US Caucasians (Table 1). However, there are significant differences compared to the Italian sample (Tagliabracci et al., 1992). In the Italians, the allele with 20 repeats was described as most frequent and both alleles 18 and 24 showed significantly low frequencies. Whether these differences are due to population structure or to the difference in technique needs further confirmation.

Heterozygosity and Power of Exclusion: The heterozygosity for the D1S80 locus in the population of north-east England is 75% and this value is similar to that in the Danes (77%) and other white Caucasian populations listed in Table 1. The theoretical chance of exclusion of non-father in the population of north-east England is 62%, a value once again very similar to that found for the Danish population (Thymann et al., 1993) and the match probability of the two randomly selected individuals is 6%.

Paternity Testing: The phenotypes of the five paternity cases where the exclusions were confirmed by three single locus probes are given in Table 2. Two cases (40%) were excluded by the D1S80 system.

It is evident from the results that the D1S80 locus is highly polymorphic and extremely useful in population and medical genetic studies. The discrimination power of D1S80 locus is 94%, suggesting its potential efficiency in forensic testing, however the proportion of cases independently excluded indicate that D1S80 can be used as an additional marker to confirm results.

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ANALYSIS OF THE LOCUS D1S80: FREQUENCY DISTRIBUTION IN NORTH-EAST ENGLAND AND APPLICATION TO PATERNITY TESTING

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Analysis of the VNTR locus D1S80 was first performed by Budowle et al. (1991) and the genetic and forensic applications were studied by Sajantile et al. (1992). Recently the reproducibility of the amplified fragment length polymorphism (AMP-FLP) technique to study the D1S80 locus has been tested by Thymann et al. (1993). However our knowledge about the variation of allele frequencies for the D1S80 locus is still very limited. The study describes the distribution of D1S80 alleles in the population of north-east England. A retrospective analysis of five paternity cases (trios), each excluded with three single locus VNTR systems detected by RFLP techniques, was also undertaken to assess the efficiency of the D1S80 locus in paternity testing.

Materials and Methods

High molecular weight DNA was extracted using phenol/chloroform procedure from 10ml EDTA blood samples, collected from 111 random individuals born and living in north-east England. Five paternity cases (trios) investigated previously, and each excluded with g3, MS1 and MS43 probes, were also investigated. DNA samples were air lifted to the Institute of Forensic Genetics, Copenhagen, where the characterisation of D1S80 phenotype was carried out.

Amplification of the D1S80 locus was performed using the following primers:

5'-AAA CTGG CCTC CAAA CACT GCCC GCCG-3'

5'-GTCT TGTT GGAG ATGC ACGT GCCC CTTGC-3'

The details of the PCR conditions, characterisation of the amplified product by polyacrylamide gel, visualisation by silver staining, and the allelic ladders used for assignment of the phenotype, are discussed in detail by Budowle et al. (1991) and Thymann et al. (1993).

Results and Discussion

Population Variation: The allele frequencies in the population of north-east England and other white Caucasian populations already studied are given in Table 1. In the population of north-east England a total of 18 alleles was observed for the D1S80 locus. The most frequent allele was with 24 repeats and its frequency was 32%, followed by the 28% frequency of the allele with 18 repeats. All other frequencies were less than 9% and alleles with 32 or more repeats all had very low frequencies

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Table 1 Phenotypes of the individuals involved in this case

	mother	child 1	child 2	pat.grandmother
AB0	A1	0	A1	0
RH	ddccee	DCcee	ddccee	DCcee
MNS	MNs	MNs	MNs	NSs
P1	P2	P2	P2	P2
KEL	K+	K-	K+	K-
FY	a+b+	a+b-	a+b+	a+b+
JK	a-b+	a-b+	a+b+	a+b+
LU	a-b+	a-b+	a-b+	a-b+
HP	1	2-1	1	2
C3	2-1	2	1	2-1
GC	2-1S	1F-1S	2-1S	1S
PI	3-2	3-2	3-1	1
GM	1,-2,4,5	-1,-2,4,5	-1,-2,4,5	1,-2,4,5
ACP1	B	AB	B	AB
ADA	1	1	1	1
6PGD	1	1	1	1
GPT	1	2-1	2-1	2-1
ESD	1	1	1	1
PGM1	1	1	2-1	2-1
HLA	A3,10; B13,38	A3,28; B13,51	A3; B7,13	A2,9; B15,17
TBQ7/HinfI	3,3-2,4	3,3-3,3	3,3-2,1	4,8-2,1
YNH24/HinfI	3,3-3,3	3,5-3,3	3,3-2,8	2,8-2,5
PH30/HinfI	6-3,2	9-6	3,2-2,7	5,2-2,7
MS31/HinfI	11-7,5	11-8,4	11-11	11-11
MS43/HinfI	11-5,4	10,5-5,4	11-5,4	13,5-13,5
G3/HinfI	10-4,5	4,5-4,5	25-10	25-6

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From this table, it can be seen that the deceased putative father cannot be the biological father of both children because of the results in the systems MS43/HinfI and HLA-AB:
- in MS43/HinfI, child 1 inherited a paternal band with 10,5 kb, child 2 a paternal band with 5,4 kb or 11 kb, while the putative father must possess the gene coding for a 13,5 kb band, and
- in HLA-AB, child 1 inherited the paternal haplotype A28,B51, child 2 the paternal haplotype A3,B7 (or AX,B7), while the father must carry on one chromosome the genes defining A2,B15 or A2,B17 or A9,B15 or A9,B17.
From these data, it is clear that the putative father cannot be the biologic father of both children.

The computation of the plausibility of grandmotherhood according to Essen-Möller of the paternal grandmother (W) to the two children of this case gave the following results: W for child 1 = 0.0003% and W for child 2 = 72%. As the latter value is a typical value for such cases, there are no indications that the paternal grandmother is not the biological grandmother of child 2; the odds for the hypothesis that she is the grandmother of child 2 and not of child 1 (excluding the possibility that she is not related to both children) are 240000:1.

This case shows clearly that the use of genetic polymorphisms with a high polymorphism, i.e. the HLA system and DNA polymorphisms, can solve complex paternity problems, especially cases in which the putative father is not available for direct testing and in which his genotype cannot be completely reconstructed.

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CLARIFICATION OF A PATERNITY CASE WITH THE MOTHER, TWO CHILDREN AND THE MOTHER OF THE PUTATIVE FATHER

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In a paternity case, the biologic fatherhood of a deceased man with respect to two children was questionable; for solving this question, only the following individuals could be tested: two children, the mother of the children and the mother of the deceased putative father - the father of the putative father was also already dead.
From a theoretical point of view, it is not possible to exclude a putative father from the paternity to a single child if only his mother (and not his father) can be included in the investigations as the paternal allele of the child could have been present in the father of the putative father. In case that two children are involved, it is possible that one allele (or haplotype) of the putative father can be deduced from the phenotype of his mother and that the children inherited two other alleles (or haplotypes) from their true father; such informations, however, can only be obtained by investigating genetic systems with at least 3 alleles (or haplotypes). If so, it would be important to compute the probability of grandmotherhood of the mother of the putative father in order to see whether or not there are some hints for a kinship with child 1, child 2, both or neither of both children. A paternity case demonstrating these problems is presented in the following.

The results of the phenotyping for several markers (in order to compute the biostatistics, systems with 2 alleles have also been included) are presented in Table 1.

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blood samples, according to standard protocols. The PCR reactions were carried out according to the conditions previously described (6). Native 10% 25-cm long polyacrylamide gel electrophoresis and ethidium bromide staining (2) of the PCR products were employed to analyze the Y-27H39 locus.

Results. Allele frequencies for the Y-27H39 locus were first determined. Among the 202 unrelated Italian males studied, 5 different alleles were detected: A, B, C, D, E, with frequencies of 0.07, 0.51, 0.31, 0.10, 0.01, respectively. The size interval and alleles detected were similar to what previously reported for the Brazilian (6) and German (7) populations. The frequency distribution in our sample differs: allele A is significantly less frequent and allele C is more frequent in our population sample.

80 father-son pairs with established paternity from among the 202 individuals were analyzed with the Y-27H39 polymorphism. The inheritance of the same allele was confirmed in all cases.

To determine the informativity of the Y specific locus in paternity testing we performed a retrospective analysis with Y-27H39 polymorphism in 52 Italian father-son pairs, selected among 94 disputed paternity ceses, which we previously studied with different autosomal VNTR and microsatellite regions (2). In all the 25 attribution cases there was allelic concordance, as expected. For the exclusion cases, 19/25 were confirmed: a 76% informativity rate.

Conclusion. The number of Y-27H39 alleles, their frequency distribution in the Italian population and the informativity derived from our paternity data, indicated that this is a good marker for individual and sex typing. The PCR technique and the non isotopic detection advantages allow this system to be used for several purposes, expecially for forensic case work with minute biological samples.

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Application of a Y-linked tetranucleotide repeat polymorphism to paternity cases.

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Abstract. The polymorphic microsatellite Y-27H39 was analyzed in a large Italian population sample by the polymerase chain reaction amplification technique and a simple method of native polyacrylamide gel electrophoresis followed by ethidium bromide fluorescence. Five alleles were distinguished in 202 males, with frequencies ranging from 1% to 51%. In 52 father-son pairs, selected among 94 disputed paternity cases, previously tested with different autosomal VNTR and microsatellite regions, the Y-27H39 polymorphism confirmed all the 27 attributions and 19 of the 25 exclusions, indicating 76% informativity in Y chromosome exclusion testing.

Introduction. Many highly informative human microsatellite polymorphisms with a motif length of 1-5 bp have been described to date, and most of them are detected by PCR analysis (1). We have used several microsatellites (2) and other markers (3,4) in the analysis of Italian population samples. In order to expand the informativity to sex determination we have now introduced a Y specific chromosome marker. The microsatellite Y-27H39 is based on the repetition of a GATA motif and was recently discovered on the short arm of the human Y chromosome (5).

Materials and Methods. All subjects studied were coming from several Italian regions. In all father-son pairs, the paternity was previously established by autosomal VNTR and microsatellite PCR determinations (2). DNA was prepared from peripheral

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Table 1: Allele frequencies with standard errors, chromosome location, heterozygosity, power of exclusion and the percentage of cases excluded by the STR loci

Loci	HUMHPRTB(AGAT)n (n = 182)		HUMRENA4(ACAG)n (n = 193)		HUMFABP(AAT)n (n = 205)		D10S89 (n = 148)	
	allele	frequency & standard error	allele	frequency & standard error	allele	frequency & standard error	allele	frequency & standard error
	9	0.003±0.003	8	0.808±0.020	10	0.524±0.025	140	0.003±0.003
	10	0.023±0.008	10	0.078±0.014	11	0.151±0.018	142	0.017±0.007
	11	0.160±0.018	11	0.104±0.016	12	0.029±0.008	144	0.358±0.028
	12	0.338±0.024	12	0.010±0.005	13	0.222±0.021	146	0.044±0.012
	13	0.282±0.023			14	0.049±0.011	148	0.095±0.017
	14	0.137±0.017			15	0.024±0.008	150	0.331±0.027
	15	0.053±0.011					152	0.125±0.019
	16*	0.005±0.004					154	0.014±0.007
							156	0.014±0.007
cho	Xq26		1q32		4q31		10p	
H	.6888**		.3419		.6439		.7568	
PE	-		0.1747		0.4138		0.6099	
%	-		30		60		43	

* new allele;
** for females;
Cho = Chromosomal location;
H = heterozygosity;
PE = power of exclusion;
% = percentage of exclusion

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Results and Discussion

The STR loci investigated, their chromosomal locations, total number of alleles tested, allele frequencies, with their SE, heterozygosity, probability of exclusion of each system and the proportion of exclusions given by each autosomal locus are given in Table 1. The *PRTB* locus is on the X chromosome and a total of eight alleles were found with allele sizes between 263 and 299 bp. Allele 12 gave the highest frequency (34%) which is similar to the white Caucasian sample by Edwards et al. (1992) in the U.S. The frequency of allele 13 is second highest which also agrees with the Edwards study. For this allele the Asians investigated in the U.S. gave the highest value (51%). This locus therefore shows significant heterogeneity in different racial groups although the white Caucasians on either side of the Atlantic seem to be homogeneous. For loci RENA4 and FABP, alleles 8 and 19 respectively gave the highest frequencies (81% and 52%). Once again the patterns of allele frequencies for the two loci are similar to the white Caucasian population of the U.S. No data is available for the direct comparison of locus D10S89. In the present study of 148 individuals, 9 alleles were found and the allele sizes ranged from 140 to 156 bp. The highest values were found for alleles with 144 and 150 bp. Both showed similar frequencies, together constituting 68% of the total genetic variation at this locus.

The power of exclusion (PE) for the three autosomal loci was calculated (Garber and Morris, 1983). RENA4 with four alleles gave the lowest PE value (17%) while the dimeric loci D10S89 with nine alleles gave the highest value of power of exclusion (61%). The FABP locus showed an intermediate PE value. The cumulative PE value for the three autosomal loci is 81%

Paternity cases excluded by each of the three VNTR probes (g3, MS1 and MS43) were examined for exclusion with each of the three autosomal STRs investigated here. Sixty percent were excluded with the trimeric STR, while 43% (3 out of 7) and 30% of cases were independently excluded by the dimeric and tetrameric STRs. Three cases (43%) were excluded with two STR systems while the other four (57%) showed exclusion with only one system.

In conclusion, the magnitude of polymorphism for STR markers differs considerably, as does their power of exclusion. These systems are certainly useful for genetic differentiation studies, but the three autosomal STRs studied can not replace three long VNTRs for individual identification. These three STR systems could however be used as additional markers for confirmation of results.

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POPULATION GENETIC STUDIES OF SHORT TANDEM REPEAT LOCI (STRS): EFFICIENCY IN PATERNITY TESTING

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Tandemly repeated DNA sequences are extensively polymorphic, due to variability in reiteration number (Jeffreys et al., 1985). There are two types of variable number of tandem repeats (VNTRs), long and short. Long VNTRs, also known as minisatellites, are *generally* detected by the technique of Southern hybridisation, but due to technical limitations the allele sizes of the long VNTR loci cannot be determined with precision, which complicates their application. In contrast, short trandem repeats (STRs), also known as microsatellites (Litt and Luty, 1989), are widely scattered throughout the human genome and provide a valuable source of polymorphic markers (Weber and May, 1989). These STRs can be precisely amplified by the polymerase chain reaction (PCR) and the variability of allele size can be determined faithfully in repeated experiments. Several STRs can simultaneously be studied by multiplex PCR (Chamberlain et al., 1988), making the STR analysis both rapid and less expensive. For both technical and economic reasons STR analysis provides an important and valuable tool for individual identification in paternity and forensic testing. This study presented here provides population frequency data for two tetrameric loci, one trimeric and one dimeric locus. The efficiency of these systems in paternity testing was also analysed by a retrospective analysis of ten paternity cases (trios) in which the exclusion had already been established with each of three long VNTR probes.

Materials and Methods

DNA was extracted by phenol/chloroform extractions, from 10ml EDTA blood samples, collected from 205 random individuals born and living in north-east England. For the timeric HUM*FABP*(AAT)n and tetrameric HUMH*PRTB*(AGAT)n and HUM*RENA4*(ACAG)n loci the primers and the conditions of PCR used were as described in Edwards et al. (1992). For the dimeric (CA)n locus D10S89, the primers were supplied by Professor S.S. Bhattacharya (London) and the amplification conditions were: denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, and extension at 72°C for 1 minute for 30 cycles. Phenotypic characterisation was carried out in DNA sequencing gels containing 6% acrylamide with 8M Urea.

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CONCLUSION

Our study demonstrates that paternity testing with the ML-oligonucleotide probe $(CAC)_5/(GTG)_5$ is reliable and allows decision making that is robust against methodological variations. Rare, undecisive cases may nevertheless occur and should be solved by alternative means, e.g. single-locus testing. We also demonstrated that the major arguments put forward against the likelihood approach in quantitative paternity testing are invalid for this ML system.

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Nevertheless, paternity cases could be clearly divided into two subgroups on the basis of (i) the proportion of offspring bands that could not be assigned to either parent, and (ii) the extent of band sharing between child and alleged father. This partitioning, which most likely corresponds to true and false paternity, confirms previous findings obtained for Jeffrey's ML-probes (1) and can be achieved without biometrical analysis.

The assumption of statistical independence of gel positions in the formal analysis of ML-patterns has been shown to be not always justified. However, allelism and linkage between loci are of only minor importance for the following reasons. Loci contributing to DNA fingerprints generated with probe $(CAC)_5/(GTG)_5$ are evenly scattered throughout the genome (4), and the majority of these comprise a large number of alleles. However, only a minor fraction of alleles, representing also the rarer ones, is larger than 4kb (5) and therefore actually scored. Thus, only a small number of the evaluated DNA fragments can be allelic.

Finally, theoretical considerations and simulations (3) indicate that partial homozygosity, allelism and linkage do not alter paternity probabilities to an extent relevant for decision making, i.e. in the range of 0.1% and 99.9%. It should be noted in this context, that confining the numerical analysis to large fragments also renders invalid one of the major arguments put forward against the likelihood approach, namely that ML DNA fingerprints are merely overlays of single-locus patterns (6)

Under the above assumptions, position-wise phenotypes are approximately independent from each other, and the number of bands per individual should consequently follow a Bernoulli distribution. However, the empirical distribution of the normalized number of bands per individual differs slightly from it's standard normal expectation although no systematic deviation is apparent. Further, all position-wise phenotype patterns, as introduced above, have been observed at frequencies not dissimilar to their theoretical expectations. This result not only supports the likelihood approach but specifically justifies the highly debated inclusion of positions lacking a band in mother, child and alleged father (pattern M-C-F-) in the calculation of paternity probabilities. Such positions could have been occupied by a band in the pattern of the alleged father, and since not transmitted, would have argued against paternity.

The distribution of $\log_{10}$-likelihood ratios (LR) allows a clear-cut distinction between two groups of cases. A total of 43 cases yielded LR-values smaller than zero, with a maximum value of -3.9288 (which corresponds to a paternity probability of 0.02% according to Essen-Möller). The remaining cases are characterized by LR values larger than 1.9 (Paternity probability of 98.87%). This partitioning coincides perfectly with that described above. Variations of LR between laboratories can be explained by different laboratory protocols, beeing reflected especially in different numbers of gel positions scored.

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PATERNITY TESTING BY OLIGONUCLEOTIDE DNA FINGERPRINTING: A MULTI-CENTER STUDY PROVING RELIABILITY AND VALIDITY

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INTRODUCTION

While Jeffreys and co-workers (1) demonstrated in an extensive study of 1702 paternity cases that paternity testing with multi-locus (ML) probes 33.6 and 33.15 is effective and reliable, this technique nevertheless remained an issue of controversial debate.
The aims of our multi-center study (2), comprising 256 paternity cases submitted to 7 different German laboratories was: (i) to assess whether or not the positive results obtained for Jeffrey's probes also hold true for the multi-locus oligonucleotide probe $(CAC)_5/(GTG)_5$, (ii) to evaluate the robustness of paternity testing against differing laboratory protocols and (iii) to determine the extent of correspondence between observed data and parameters expected under analytical models, e.g. whether the assumption of statistical independence is valid for electrophoretical bands on the same gel.

MATERIAL AND METHODS

DNA was digested with restriction enzyme HinfI and hybridized with oligonucleotide $(CAC)_5/(GTG)_5$ (Fresenius, Oberursel). Transformation of entire ML banding patterns into series of position-wise phenotype patterns (e.g. band present in the patterns of mother and child, but not for the alleged father: M+C+F-, or M-C+F+, M+C+F+, etc.) followed the procedure as described in (2). Only fragments larger than 4 kb were evaluated. Paternity probabilities were calculated from the likelihood ratios of paternity vs. non-paternity which, in turn, are based on the observed numbers of the different, position-wise phenotype patterns (3).

RESULTS

All parameters characteristic of ML DNA fingerprints were found to differ significantly between the contributing centres, e.g. the number of analyzed gel positions, the number of bands scored per individual, the probability of occurrence of a band at a particular position of a gel, and the band-sharing probability between the mother and both the child and the alleged father.

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5. DNA polymorphisms in paternity testing

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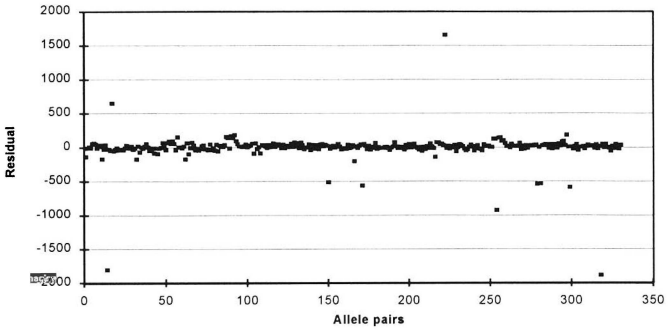

Figure 1. Plot of residuals from the average difference in the analyzed population (0 in Y axis is equivalent to 1063 bp).

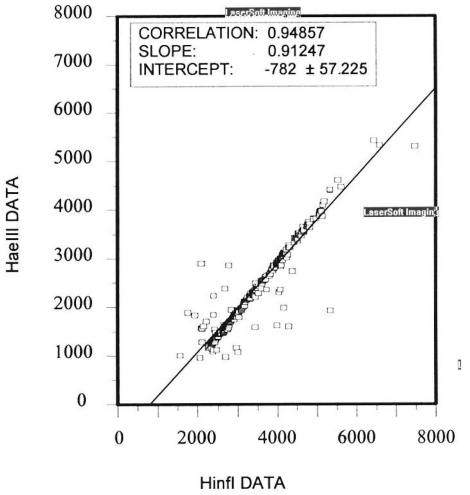

FIGURE 2. Regression of *HaeIII* data over *HinfI* data (whole population).

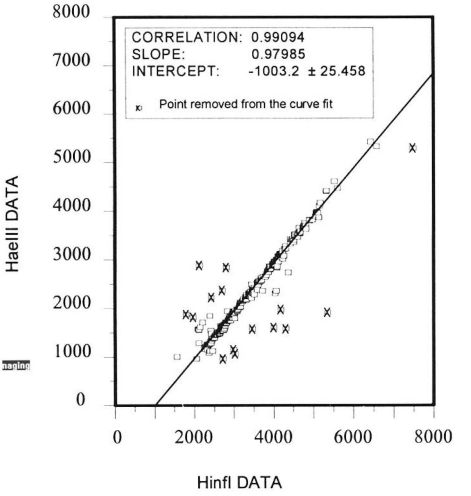

FIGURE 3. Regression of *HaeIII* data over *HinfI* data (modified population).

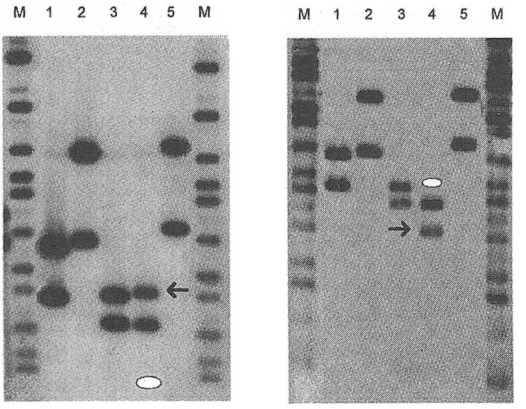

FIGURE 4. Lane 4 shows an individual carrying a "rare" allele (arrows). Blank ellipses indicate the expected positions for the "normal" alleles.

RESULTS AND DISCUSSION

1.- Comparing the two data sets studied, the HinfI fragments are longer than the corresponding HaeIII ones. The average value of the difference is 1063 bp (SD = ±7.74), (Figure 1).

2.- The regression analysis suggests a linear relationship between the two data sets (Figure 2). This fact is in agreement with the existence of a VNTR polymorphism. However, some data pairs show a marked divergence with respect to the average value, and lie out of both confidence and prediction limits for the straight line fitted. When these data (3.03% of the total) are removed from the plot, the regression analysis gives significant differences respect to the formerly made, and the parameters defining the relationship are closer to the linearity (Figure 3), as could be expected in a pure VNTR polymorphism.

3.-The divergent data are listed in Table 1. In all cases, only one of the two alleles shows a significant difference from the average value (Figure 4). There are two reasons that justify the existence of these "rare" alleles:

- Loss of small HaeIII fragments (Chakraborty and Jin, 1992).
- Creation/elimination of restriction sites for the used enzymes (Waye and Fourney, 1990).

4.- The loss of small HaeIII fragments is possible in three individuals, which are homozygous for HaeIII and have HinfI alleles with length close to 2 kb (# 1, 2 and 6). The corresponding HaeIII allele must have around 1 kb in length, and the limit of the detection, using the standardized electrophoretic protocols, is in this range.

5.- Mutation of a restriction site could explain the divergence in almost all cases. Significantly, half of the divergent alleles have similar lengths (2-2.2 kb for HinfI) and show similar differences (≈500 bp). This suggests that a linkage between the mutated restriction site and a VNTR allele must exist.

6.- The analysis of the HinfI database showed that the only alleles in the range 2127-2086 bp were "rare" alleles. The alleles found in the HaeIII database in the range 1450-1750 bp, were a mixture of normal and mutant alleles. These data support the existence of a new HinfI site. However, this fact will be only proved by sequencing the 5′ and 3′ flanking regions of the D2S44 VNTR locus.

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TWO DIFFERENT MECHANISMS ARE INVOLVED IN THE POLYMORPHISM SHOWN BY THE LOCUS D2S44

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INTRODUCTION

Tandemly repeated sequences (VNTRs) represent a rich source of highly polymorphic markers useful in genetic linkage analysis, genome mapping and personal identification. These VNTRs are hotspots of homologous recombination and are present throughout the human genome (Wahls et al., 1990). Mutations in the flanking region can generate or delete new restriction sites, hence contributing to increase the polymorphism.

The polymorphic locus D2S44, recognized by the YNH24 probe, was first described by Nakamura et al (1987). D2S44 locus polymorphism has been demonstrated using different restriction enzymes, e.g. MspI (Nakamura et al., 1987), BamHI (Henke et al., 1990), HaeIII (Budowle et al., 1990), HinfI (Gill et al., 1991), etc. In our study we have analyzed the databases of the D2S44 alleles in the spanish population, using two different restriction enzymes, HaeIII and HinfI, over a sample of 177 individuals. The distribution profiles obtained have been compared, in order to observe the correlation between the fragment lengths in both samples. From 354 analyzed alleles, 10 have shown a different size with regard to those expected. In 5 of these 10 individuals, the new alleles are likely generated by the creation of a new HinfI site.

MATERIAL AND METHODS

SAMPLE ANALYSIS: DNA from 177 Spanish unrelated individuals were extracted from whole blood. Two alliquots of 5-10 μg of DNA each were digested with HaeIII or HinfI, and the resultant fragments electrophoresed and blotted following the EDNAP protocol recommendations. The YNH24 probe was labelled with ^{32}P by random priming or with alkaline phosphatase.

DATA ANALYSIS: Band profiles were analyzed using a densitometer (Elscript 400, Hirschmann), as well as a computerized scanner system (EquiDNA, Pharmagen/Filosoft). The values obtained were pooled into a database that was used for the statistical analysis. A simple regression analysis was performed, taking arbitrarily the HaeIII values as the dependent variable. Those values that were out of the confidence intervals for the straight line fitted were rejected. Finally, we performed a comparison between the two equations obtained.

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changes at the contiguous codons encoding the amino acid residues 650 and 651 were found not to result from artifact during sequencing reaction.

Polymorphic single strands of exon 11, 12 and 14 were successfully detected by SSCP analyses under the conditions as follows; electrophoresis at 150V overnight in 20% polyacrylamide gels without glycerol for exons 11 and 12, and with 5% glycerol for exon 14.

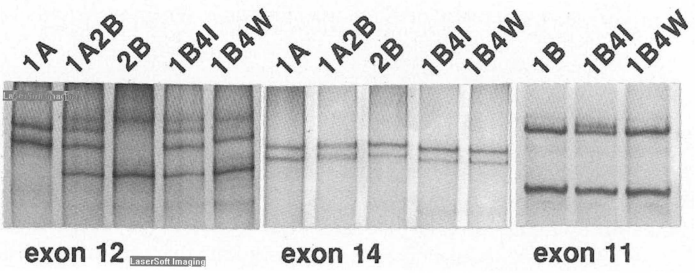

Fig.3. SSCP analysis of three exons of the F13A gene.

Figure 3 shows SSCP patterns of the three exons. SSCP analysis is a powerful technique to reveal a base substitution harbored in a short DNA stretch of a few hundreds bases but is not a completely potent one because monomorphic separation of single-stranded DNAs by SSCP analysis does not always mean that there are no base substitutions along the sequence tested. To separate polymorphic single strands in this study, three parameters, acrylamide/*N-N'*-methylenebisacrylamide ratio, gel concentration and temperature during electrophoresis, were tested. Then the two selected conditions described above were applied to SSCP analysis of all the coding exons of F13A alleles. There were no SSCPs in the 12 exons other than exon 12 and 14 for the four common alleles, the findings which are compatible with sequence data showing allelic base changes only in the 2 exons. Fortunately, SSCP analysis under the same conditions could separate polymorphic bands in exon 11 for *F13A*4I* and in exon 12 for *F13A*4W,* the sequences of which were presented elsewhere in this volume by the same authors.

DNA typing of conventional markers by PCR-based methods is one of potent tools for genetic characterization of forensic materials owing to the feasibility of their detection, ample accumulation of population data of many ethnic groups and stability of allelic sequences of a gene against de novo mutations although DNA typing of conventional markers is not so powerfully discriminative as tandem repeat DNA markers such as VNTR, STR or DNA fingerprint. Thus, DNA typing of the F13A gene provides an additional chance for forensic identification of individuals.

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harboring type "A" exon 12, type "1" exon 14 and type "2" exon 14, respectively (fig.1). Figure 2 presents *Pst* I and *Eco*R I RFLPs that define allelic versions of exon 12 and 14, respectively. Ten F13A genotypes deduced from the four alleles were defined by the sum of the two RFLPs but F13A 1A2B and 1B2A could not be discriminated from each other.

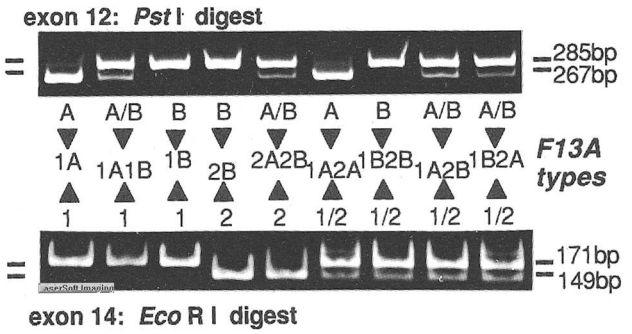

Fig.2. RFLPs of exons 12 and 14. Note that F13A 1A2B and 1B2A show the same RFLPs for both of the two exons.

F13A types of randomly collected blood samples from unrelated 134 individuals were defined by both AGIF for plasma and PCR-RFLPs for genomic DNA. Phenotype distribution and allele frequencies determined from the samples were listed in table 1. F13A type of each individual determined by the two methods was completely coincide with each other except for the two types, 1A2B and 1B2A, both of which were defined as heterozygote for each exon by PCR-RFLPs.

Table 1. Phenotype distribution and allele frequencies calculated from the results by plasma typing and by DNA typing.

phenotypes	no.obs.	no.exp.	allele frequencies
1B	49	47.76	*F13A*1A* = .2948
1A1B	43	47.17	*F13A*1B* = .5970
1B2B	18	16.11	*F13A*2A* = .0075
1A	14	11.65	*F13A*2B* = .1007
1A2B	8	7.96	
1B2A	1-------		
2A2B	1		
2B	0	3.35	
1A2A	0		
2A	0-------		

$\chi^2 = 1.641$, $.30 < p < .50$ (d.f. = 2)

Hinc II RFLP defined by cleaving the "1" version of exon 14 showed together with *Eco*R I RFLP that the contiguous two codons specifying the two antithetic versions, GTTGAG and ATTCAG, are homogeneous in their sequences because all of the F13A*1A and *1B alleles were cleaved by *Hinc* II and all of the F13A*2A and *2B were cut by *Eco*RI. Thus, the two nucleotide

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DNA TYPING OF COAGULATION FACTOR XIII "a" SUBUNIT BY PCR-RFLP AND SSCP

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INTRODUCTION

Data on nucleotide sequences of conventional polymorphic markers leads to development of PCR-based typing methods such as PCR-RFLP, PCR-ASO, or SSCP. RFLP usually requires loss or gain of a recognition sequence of a restriction enzyme in which a polymorphic site is included, but artificially introduced mismatch of a single nucleotide in a PCR primer can produce a recognition site of a restriction enzyme (Kumar and Dunn 1989).

Polymorphism of the "a" subunit of the coagulation factor XIII is defined by four alleles, *F13A*1A, *1B, *2A,* and **2B* (Suzuki et al. 1988). Sequence analysis demonstrated that a single nucleotide change in exon 12 specified the "A"/"B" difference and two nucleotide changes at contiguous codons in exon 14 determined the "1"/"2" difference as described in this volume by the same authors. Thus four combinations of the two antithetic versions of the two exons were found to correspond to the four respective alleles. In this study, we present PCR-RFLP and SSCP analyses for specifying F13A alleles.

MATERIALS AND METHODS

Three primers with a single mismatch were designed so that recognition sequences were introduced through amplification of sequences of two polymorphic exons by PCR (fig.1).

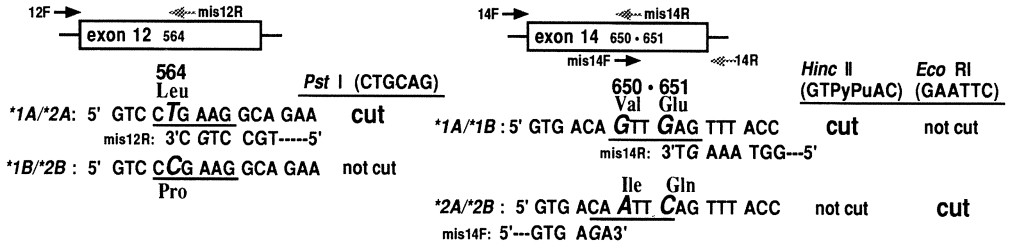

Fig.1. Schematic presentation of regions amplified by PCR with three sets of primers. Italicized nucleotides in the allelic sequences and in the three mismatched primer sequences indicate polymorphic sites and mismatched nucleotides, respectively.

After digestion of PCR products with appropriate restriction enzymes, RFLPs were detected by polyacrylamide gel electrophoresis and ethidium bromide stain. SSCP analysis was performed in polyacrylamide gels (12x10x0.1 cm) that were continuously cooled at 15℃. DNA bands were visualized by silver stain.

RESULTS AND DISCUSSION

Pst I, *Hinc* II and *Eco*R I recognition sites were artificially introduced in the PCR products

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Tab.1 PCR and PAGE conditions for 6 microsatellite loci

Locus	APOB	COL2A1	D1S80	YNZ22	SE33	TC11
syn.	2p24-p23	12q13.1	pMCT118	D17S5, D17S30, 17p13.3	ACTBP2, Ac-psi-2	HUMTH01, 11p15.5
Lit.	Ludwig et al 1989	Wu et al 1990	Nakamura et al 1988 Budowle et al 1991	Horn et al 1989	Polymeropoulos et al 1992	Edwards et al 1992
Primer sequences	5´-ATG GAA ACG GAG AAA TTA TG-3´ 5´-CCT TCT CAC TTG GCA AAT AC-3´	5´-CCA GGT TAA GGT TGA CAG CT-3´ 5´-GTC ATG AAC TAG CTC TGG TG-3´	5´-GAA ACT GGC CTC CAA ACA CTG CCC GCC G-3´ 5´-GTC TTG TTG GAG ATG CAC GTG CCC CTT GC-3´	5´-AAA CTG CAG AGA GAA AGG TCG AAG AGT GAA GTG-3´ 5´-AAA GGA TCC CCC ACA TCC GCT CCC CAA GTT-3´	5´-AAT CTG GGC GAC AAG AGT GA-3´ 5´-ACA TCT CCC CTA CCG CTA TA-3´	5´-GTG GGC TGA AAA GCT CCC GAT TAT-3´ 5´-ATT CAA AGG GTA TCT GGG CTC TGG-3´
PCR-protocol	50 ng template DNA, 0.4 µM each primer, 200 µM each NTP, 5µl 10xPCR buffer[3], 2U DNA Polymerase[4], total volume 50 µl, overlaid with 50 µl oil					
Amplification conditions	94°C - 1 min 58°C - 1 min 72°C - 4 min 29 cycles	94°C - 1 min 60°C - 1 min 70°C - 1.5 min 25 cycles	94°C - 1 min 65°C - 1 min 72°C - 8 min 27 cycles	94°C - 1 min 63°C - 1 min 72°C - 5 min 29 cycles	93°C - 1 min 61°C - 1 min 72°C - 1.5 min 28 cycles	94°C - 1 min 64°C - 1 min 70°C - 2 min 30 cycles
polyacrylamide gels composition size(mm) rehydration buffer electrode buffer	 T5% C3% 230x110x0.45 Tris-P TBE	 T5% C3% 230x110x0.45 Tris-P TBE	 T6% C3% 230x110x0.45 Tris-P TBE	CleanGel[2] 110x250x0.43 Tris-Ac Tris-Tric	 T5% C3% 230x110x0.45 Tris-Ac Tris-Tric	 T6% C3% 230x110x0.45 Tris-Ac Tris-Tric
Electrophoresis[1] ramp final	 1200V,7mA, 30W 10 min 1200V,15mA, 30W	 1200V, 7mA, 30W, 10 min 1200V,15mA, 30W	 1200V, 7mA, 30W, 10 min 1200V, 15mA, 30W	 none 600V, 50mA, 30W	 1200V, 12mA, 30W, 10min 1200V, 25mA, 30W	 1200V, 12mA, 30W, 10min 1200V, 25mA, 30W
number of observed alleles	23	11	26	15	30	8

[1] Electrophoresis was stopped when the bromphenolblue-front reached the anode
[2] Pharmacia Biotechnology, consists of a T5% stacking gel and a T10% resolving gel
[3] Finnzymes Oy
[4] DynaZyme^{tm}

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Results and discussion

Sufficient resolution of the polymorphic alleles was achieved in all cases after modifications of the electrophoresis conditions, the rehydration and the sample buffers (Tab 1). Allelic cocktails that allow allele assignment were obtained for all systems. The number of alleles identified in a local population sample is listed in Tab 1. However, for D1S80 and SE33, allele assignment was difficult when comparing the results of the population studies (Schwartz et al 1993) to those of other workers. For SE33, a considerable high number of new high molecular weight alleles was observed which is presumably due to the enhanced electrophoretic separation conditions. The typing of D1S80 revealed microheterogenity of the V22-Allele and several new interalleles. From the results of our studies, we may conclude that PAGE with subsequent silver staining offers a reliable and convenient possibility to type for VNTR and STR-loci in forensic practice in the future. Since the combined Mean Exclusion Chance (Krueger at al 1968) of the 6 systems is 0.99975, it is possible to solve most forensic cases using only this technique with 6 systems. As a next step, sequencing of the individual alleles is necessesary to define a final nomenclature and cocktails composed of alleles with known sequences should be produced and shared by all workers in this field to further establish this procedure for forensic paternity and identity testing.

Acknowledgements

Thanks to B. Brinkmann and P. Wiegand for providing allelic cocktails for D1S80 and YNZ22 and for fruitful discussions.

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SIMPLE AND RAPID TYPING FOR VNTR POLYMORPHISMS USING HIGH RESOLUTION ELECTROPHORESIS OF PCR PRODUCTS ON REHYDRATABLE POLYACRYLAMIDE GELS

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Introduction

The invention of the Polymerase Chain Reaction (PCR, Saiki et al 1988) has opened a wide new field of applications in molecular biology. Many new PCR-based techniques to type for polymorphic markers are now available and thus forensic haemogenetics is certainly moving to a new age. The highly polymorphic Variable Number of Tandem Repeat (VNTR) -loci offer a system of markers with high informativeness and have already been employed for paternity and identity testing for a few years. So far, most work has been done by analysis of enzyme-digested amplification products (RFLP) which is a cumbersome method with limited sensitivity. Therefore, typing of VNTR´s and STR´s (Short Tandem Repeats, a subset of VNTR´s with very short repeat sequences: 2-4bp) by Amplification Fragment Length Polymorphism (AMPFLP) is about to become the method of choice, since DNA-sequencing, of course the most precise way to type for a genetic polymorphism, will presumably not be available to most workers in the forensic field in the near future. We have undertaken studies to optimize conditions for high resolution electrophoresis on rehydratable polyacrylamide gels for the VNTR marker systems ApoB, COL2A1, D1S80, YNZ22 and for the STR loci SE33 and TC11 in order to obtain a simple protocol that leads to reproducible and reliable results and to further employ this protocol for local population and family studies and for the forensic casework in the future.

Material and methods

Samples

Genomic DNA was extracted from peripheral blood of healthy unrelated individuals by the salting out method described by Miller at al (1988). The DNA concentration was adjusted to 1 ng/µl.

PCR

The PCR protocols for the individual loci as well as the amplification conditions are listed in Tab 1.

Allelic Cocktails

were produced by coamplification of genomic DNA of 4-5 individuals.

Gel preparation

For typing of YNZ22 a commercially avaiable rehydratable polyacrylamide gel (CleanGel 48S, Pharmacia Biotechnology, FRG) was used. For typing of the other loci rehydratable gels were produced as described by Westermeier (1990).

Buffers

Rehydration and electrode buffers as originally described for the use of CleanGel (Pharmacia, FRG) were used:

Tris-Phosphate (Tris-P): 100mM Tris, 2mM Na_2EDTA, adjusted to pH 8.0 with phosphoric acid

Tris-Acetate (Tris-Ac): 112 mM Tris, adjusted to pH 6.4 with acetic acid

TBE: 0.27M Tris, 0.27M H_3BO_3, 7.5mM Na_2EDTA

Tris-Tricine (Tris-Tric): 0.2M Tris, 0.2M Tricine, 19mM SDS

Polyacrylamide gel electrophoresis

Horizontal electrophoresis was carried out at a temperature of 10°C on a Multiphor II electrophoresis equipment (Pharmacia Biosystems, FRG). The electrophoresis conditions are listed in Tab 1.

Silver staining

A simple and sensitive silver staining protocol (Bassam et al 1991) was used to visualize the amplification products.

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Table 2: Characteristics of the phenol/chloroform and the Chelex DNA extraction method.

Chelex 100	phenol/chloroform
rapid and simple extraction	long DNA isolation procedure
non hazardous	use of organic solvents
no loss of DNA	higher possibility to loose DNA during isolation
whole cell extract, may contain inhibitors, further time consuming and expensive purification may be necessary	more or less pure DNA, less inhibitors
DNA is single stranded, therefore not suitable for RFLP	DNA ist double stranded, RFLP can still be performed

Our results show that DNA extraction with Chelex from stamps, envelopes, saliva samples and cigarette butts was comparable to the conventional phenol/chloroform extraction method. It does not matter that the DNA is single stranded because ist is rare to get enough DNA for a RFLP-analysis from these stains. Therefore we think that the Chelex extraction method provides a good and fast alternative to the conventional DNA isolation method.

DNA extracted with Chelex from post coital samples and blood also amplified well, but required an additional purification and washing step through Centricon 100 to get a reliable amplification. Alternatively the gel had to be blotted. This reduces the differences in speed between the two extraction methods. Furthermore DNA from blood sometimes failed to amplify even after purification and post-coital samples require an extra treatment to separate the female from the male fraction which ist not practical if they are extracted together with saliva stains. Therefore, at the moment it seems to be the most practical way in our lab to continue to isolate all - even small - semen/vaginal secretion and also blood stains with phenol/chloroform. In any cases we use this conventional method for RFLP-analysis. An additional advantage of this procedure is the possibility to perform a RFLP analysis if more DNA than presumed was isolated.

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Table 1: Comparison between the conventional phenol/chloroform and the Chelex DNA extraction method. The table shows the percentage of stains which could be amplified and analyzed in ethidiumbromide stained agarose gels (left) or after blotting and hybridizing with a digoxygenin labeled Apo B probe (right).

	ethidiumbromide-stained gel		blot	
DNA extracted with	phenol	Chelex	phenol	Chelex
stamps	45 %	59 %	97 %	97 %
cigarette butts	63 %	57 %	97 %	100 %
saliva samples	97 %	97 %	97 %	93 %
sperm	79 %	97 %	100 %	100 %
vaginal secretion	97 %	57 %*	100 %	100 %*
blood	75 %	65 %*	100 %	85 %*

*: without further purification

Saliva stains:
While incubating the stain in PBS and washing the cells once seemed to be sufficient to remove potential inhibitory substances from stamps and saliva samples on filter paper, DNA from cigarette butts did not amplify without further purification such as ethanol precipitation or spin dialysis with centricon 100. With this treatment more than half of the amplification products could be typed in ethidiumbromide stained agarose gels, all resulted in a signal after blotting. From nearly all saliva samples enough DNA could be extracted to be typed in ethidiumbromide stained gels. These results show that the Chelex method works well for saliva stains and that there is no difference between Chelex and phenol/chloroform extracted DNA.

Post-coital samples, blood:
DNA from sperm isolated with the phenol/chloroform method amplified well and reliably. However, when DNA was extracted with Chelex without further purification the amplification was weak and bands were only seen in the blot at high dilutions. Ethanol precipitation improved the amplification rate, but only centrifugation through Centricon 100 lead to results comparable to or even better than those obtained with the phenol/chloroform method.

The same was true for DNA from vaginal epithelian and blood cells: without further purification the amplification was less effective and interpretable results were often only seen in the blot. In some cases DNA isolated from blood failed to amplify even after spin dialysis.

DISCUSSION

The most striking advantage of the Chelex method ist the simplicity and speed (DNA extraction and amplification within one day) especially for a lab where many DNA preparations have to be managed by a small number of assistants. However, there are also some disadvantages. Table 2 summarizes the main features of both methods:

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DNA EXTRACTION FOR PCR: PHENOL/CHLOROFORM VS. CHELEX - A COMPARATIVE STUDY

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INTRODUCTION

Chelex 100 is a chelating resin with high affinity for polyvalent metal ions which can cause degradation of DNA at high temperatures (1). Boiling of DNA in the presence of Chelex prevents the degradation of DNA.

The Chelex method has been described as a simple, rapid, non hazardous and efficient method to extract DNA (1, 2, 3). To test whether it would be feasible to replace the phenol/chloroform extraction procedure routinely used in our lab by the Chelex method, we extracted DNA with both methods from various forensically relevant stains. The DNA was then amplified (Apo B 3'locus) and the results compared regarding to reliability of amplification, speed, sensitivity to inhibitors and range of DNA concentration which will amplify.

MATERIAL AND METHODS

With small modifications the Chelex extraction method was performed as described by Walsh et al. (2). The stains (stamps, cigarette butts, 6 cm^2 of saliva samples on filter paper, 5 mm^2 of sperm stains, one third of swabs with vaginal epithelian cells and different amounts of blood) were cut into small pieces and incubated for one hour in 1 ml of PBS. The fabric or paper was then removed by centrifuging the PBS containing the cells through a small hole into a second Eppendorf tube. The cells were pelleted at 9000 g for 5 min and the supernatant discarded. 100 μl of 5 % Chelex and 5 μl of 10 mg/ml Proteinase K was added to the cell pellet which was then vortexed at high speed and incubated for at least one hour at 56°C. After vortexing again the cells were incubated in a boiling water bath for 8 min, vortexed again and then three different DNA concentrations (30, 5 and 1 μl or - for post-coital samples - 5, 1 and 0.2 μl) were amplified at the Apo B-3'locus as described (4). If necessary the DNA was purified by centrifugation through Centricon 100 tubes or ethanol precipitation was performed.
In the case of post-coital samples sequential DNA extraction was performed as described (2).

Main criterion for the success of the method was the percentage of stains which were successfully amplified and not the amount of DNA isolated because this does not always correlate with the amplification rate if inhibitors are present.

RESULTS

DNA from more than 200 stains was extracted with phenol/chloroform as well as with Chelex. Generally DNA from all kinds of stains investigated could be amplified with both methods. Table 1 summarizes the results:

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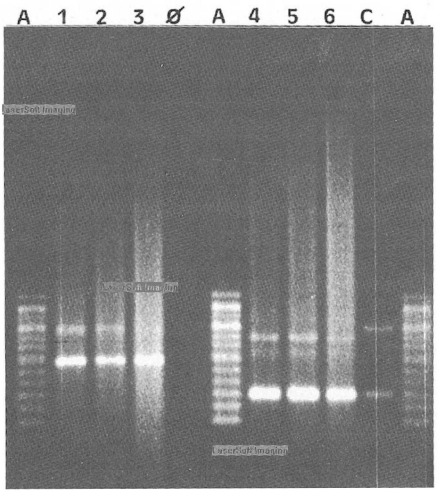

Fig. 4: Amplification of D1S80 locus
1-6: DNA from bloodstain on three carpeting makes (2 persons)
A: Allelic Ladder
C: control-DNA

Discussion

Although the extraction procedure described above produced a better yield of DNA, the number of complete restrictions remained about equal. As was expected, the largest amount of DNA was extractable from new carpeting, the least amount from carpeting stored under humid conditions.
The influence of the stained material was considerably greater than expected. Clearly less DNA can be extracted from absorbent materials (e.g. wool, carpeting sample 1) than from close-pile, less absorbent carpeting (carpeting samples 4 and 5).
In the case of partially degraded DNA, good results were obtainable in some cases using the two single-locus probes, but using the AmpFLP system produced considerably better results.
The present study has again confirmed the known fact, that BSA greatly improves DNA-restriction.

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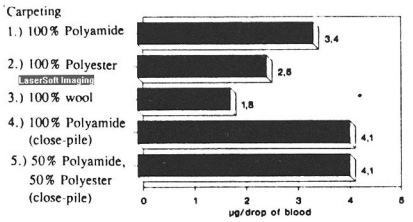

Fig. 1a: Comparison of amounts of DNA extracted from bloodstains on different carpeting makes

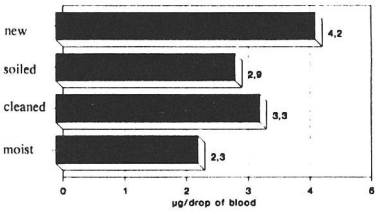

Fig. 1b: Comparison of amounts of DNA extracted from bloodstains on differently treated carpetings

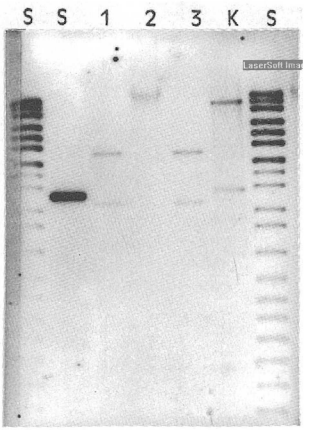

Fig. 2: Hybridisation of DNA extracted from bloodstains on differently treated carpeting
1: DNA from bloodstain on new carpeting
2: DNA from bloodstain on soiled carpeting
3: DNA from bloodstain on cleaned carpeting
S=molecular weight standards
K: K562-DNA restricted with *Hinf* I

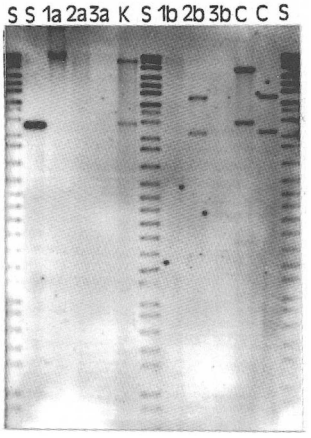

Fig. 3: Hybridisation of DNA from one donor extracted from bloodstains on three different carpeting makes
1a, 2a, 3a: restricted without BSA (restriction inhibited)
1b, 2b, 3b: restricted with 0.25% BSA (complete restriction in lane 2b)
S: molecular weight standards
K: K562-DNA restricted with *Hinf* I
C: control-DNA from two donors (bloodstain on cotton)

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DNA-Profiling on carpeting - Methods of purification, restriction and detection

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Introduction

In practice, bloodstains on carpeting are not very often submitted for investigation (approx. 5 % of all stained materials). However, carpeting is frequently very soiled or has been treated chemically (cleaned). In contrast to bloodstaines on smooth surfaces or cotton, DNA-extraction and restriction from bloodstaines on soiled and highly absorbent materials is often difficult (Holtz and Olek, 1987; Kantner et al, 1988; Prinz and Berghaus, 1990; Scheithauer and Weisser, 1991a). Problems of identification of DNA from bloodstained materials previously treated with purification media have been demonstrated by Scheithauer (1991b).

The aim of the present study was to test and, if possible, improve the extraction and purification procedures applied to bloodstaines on carpeting. A further objective was to determine the degree, on which extraction depends upon the substrate material and its composition.

Material and methods

A total of 160 extractions was performed on bloodstains applied to five different makes of carpeting (each make available factory-new, soiled, cleaned or stored under humid conditions). The size of the stain was that produced by a drop of blood. The bloodstains were first incubated in extraction buffer (10.0 mM Tris, 10.0 mM EDTA, 100.0 mM NaCl, pH 8.0) for 3 hours at room temperature then treated with Proteinase K (final concentration 100 μg/ml) for 1 h at 50 C and 4 h at 37 C. Genomic DNA was isolated by Phenol/Chloroform extractions or by deproteinisation with 6M NaCl solution. DNA precipitation was carried out with ethanol under standard conditions. Further purification occured by membrane dialysis against TN-buffer (10.0 mM Tris, 10.0 mM NaCl, pH 8.5). The amount of DNA extracted was estimated by comparing with a concentration standard in agarose gel. Finally, the DNA was restricted with *Hinf* I (10 U/μg DNA) in a volume of 40 μl containing 0.25 % BSA (Pflug et al, 1991). In some of the extracts, RFLP analyses using two single-locus systems (MS43a and YNH24) and investigations with the AmpFLP system D1S80 were performed.

Results

The results of the examinations showed that the improved procedure yielded an average of 10 % more DNA than the standard protocol. The amount of DNA extracted from the bloodstains on the carpeting was highly dependent on the material of the carpeting (Fig. 1a). The difference between carpeting 3 (wool) and carpeting 5 (short and close polyamide fibre pile), for example, was highly significant. In general, the highest DNA yield was achieved from new, untreated carpeting, and the lowest yield from carpeting stored under humid conditions. The results obtained are shown in Fig. 1b. Fig. 2 and Fig. 3 show the results of hybridisation examinations using the probe MS43a. Fig. 4 shows the results of an experiment using the AmpFLP system D1S80.

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FIGURE 1. a) AMP-FLP analysis of the minisatellite D1S80. L= D1S80 Allelic Ladder (Cetus Corporation, CA, USA).

b) AMP-FLP analysis of the microsatellite vWA. L=allelic ladder constructed of known alleles in the finnish population prepared as described by Sajantila et al.(11) A=boiling method, B=rapid lysis, C=organic extraction, 1-3 =number of samples.

Figure 1a)

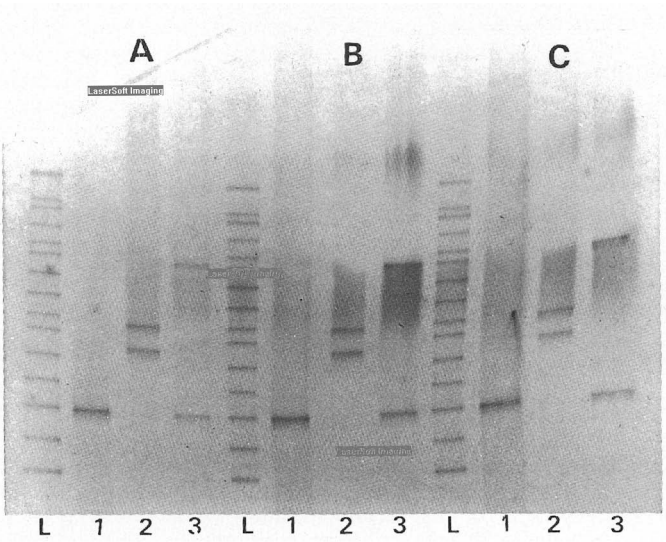
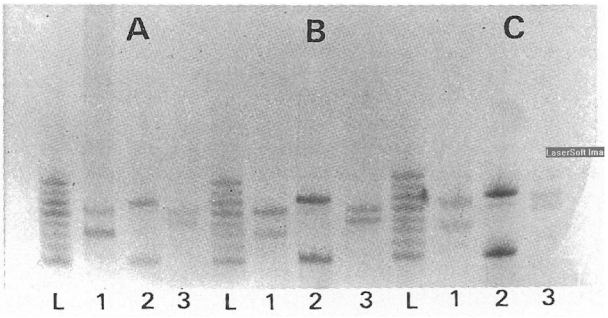


Figure 1b)



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RESULTS

Human DNA was obtained from all samples using the three extraction methods (Table 1). The treatment of powdered bone with EDTA before DNA extraction was essential for reproducible results. AMP-FLP analysis of several mini-and microsatellite loci was successful from all samples and concistent results were obtained using the three different DNA extraction methods (data not shown). Summary of DNA yield and results of the AMP-FLP analysis is given in Table 1. Examples of AMP-FLP analysis from the samples using D1S80 and vWA loci are given in Figure 1.

CONCLUSIONS

Our preliminary results show that DNA typing from compact bone is possible. The use of EDTA before extraction is essential. Our present results show that several extraction methods can be used to obtain reproducible results. Quantitation of human DNA content is useful for assessing the amount of initial DNA template for PCR.

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Inc^{R}, USA) according to the manufacturer's instructions. After all three methods the extracts were centrifuged, washed twice with water and concentrated using Centricon^{TM} 30 microconcentrators.

Quantitation of DNA

Human DNA content was determined by hybridization with a human specific DNA probe (Photo Probe ^{TM} Human DNA Quantitation system, Gibco BRL, NY, USA) according to the manufacturer's instructions.

Analysis of DNA samples

Two minisatellite loci (D1S80, D17S30) (5,6) and two microsatellite loci (vWA, HumTHO1) (7,8) were analysed using the amplified fragment length polymorfism (AMP-FLP) technique (9). The amplification was performed with Gene Amp^{TM} PCR System 9600. The amplified fragments were separated in polyacrylamide gel electrophoresis (PAGE) according to their size. Simple and sensitive silver staining (10) was used to visualize the PCR-products.

TABLE 1. Yield of human DNA from compact bone tissue using three extraction methots and genotypes of the samples using D1S80 and vWA loci.

	Boiling			Rapid lysis			Organic extraction		
	DNA ng/g	typing D1S80	vWA	DNA ng/g	typing D1S80	vWA	DNA ng/g	typing D1S80	vWA
Sample 1	>6000	1 - 1	4 - 6	10	1 - 1	4 - 6	>200	1 - 1	4 - 6
Sample 2	1500	6 - 10	2 - 7	20	6 - 10	2 - 7	>200	6 - 10	2 - 7
Sample 3	3000	1 - 14	5 - 6	20	1 - 14	5 - 6	>200	1 - 14	5 - 6

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COMPARISON OF DNA-EXTRACTION METHODS FROM COMPACT BONE TISSUE

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INTRODUCTION

Identification of highly decomposed remains of a corpse can be accomplished by DNA-typing (1). In some cases the only source of DNA is sceletal bones. A simple and rapid DNA-extraction method for bone tissue would have use in routine casework as well as in working with a large number of mass disaster victims. In this work we have compared three methods to extract DNA from compact bone tissue.

MATERIALS AND METHODS

Femural bone samples were obtained from routine medicolegal autopsies. Soft tissue and bone marrow were removed using a scalp and a sandpaper before further processing. Thin bone slides were frozen with liquid nitrogen and crushed to fine powder. Two 0.5 g samples of the powder were taken and the other one was decalcified with 0.5 M EDTA overnight before extraction.

DNA extraction

Three DNA extraction methods were used. First, we applied a simple boiling method modified from Maijer et al., 1992 (2). In this method the samples were boiled 20 minutes in buffer containing detergents. Second, we used a rapid lysis method modified from Higuchi et al., 1989 (3). In order to lyse the cells, the samples were incubated at 56^{O}C for 1.5 hours in the presence of proteinase K. Third, we performed an organic extraction of the samples (4) with phenol-chloroform-iso amylalcohol using Phase lock gels (5Prime-3Prime

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FIGURE 1: CE Typing Data - The CE traces for MMH, DLF, and RKR were overlaid onto the HUMTHO1 allele specific ladder run on the same day, on the same capillary. The asterisk in each overlay represent the alleles which line up with the ladder. Each set of overlays is labeled with one of the three individuals and the resulting genotype.

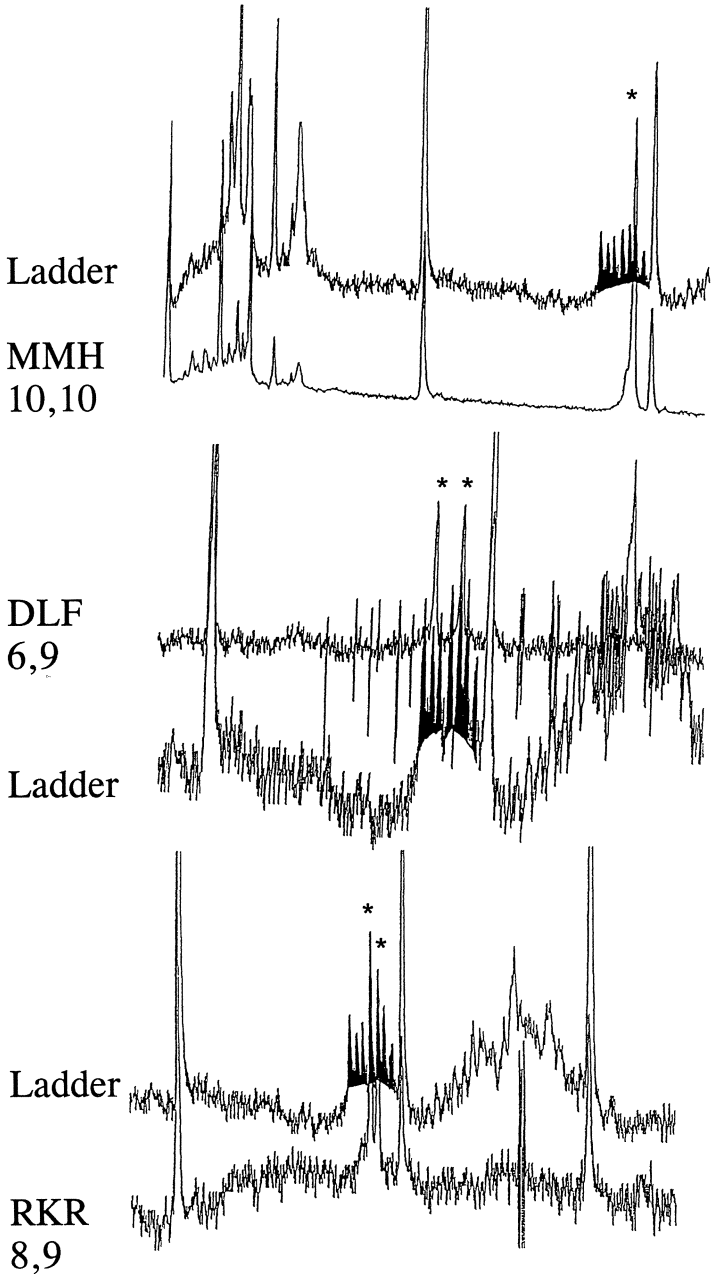

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TABLE 2a: Applied Biosystems 373A Gene Sequencer Precision Data

Allele Number (Repeat Units)(Expected Size Value)	Size Variance (Largest Difference)	Mean & Standard Deviation (309 Data Points, 6 Gels on 6 Different Days)
6 (159 bp Expected Size)	0.48 bp	157.97 bp & 0.089 bp
9 (171 bp Expected Size)	0.42 bp	170.41 bp & 0.074 bp

TABLE 2b: DNA Typing of Three Individuals (MMH, RKR, DLF) Using the Applied Biosystems 373A Gene Sequencer

DNA Identifier	Allele Size Value	Allele Number Designations Using the Binning Listed Below*
MMH	173.56 bp (173.56)	10,10
RKR	166.37 bp 170.56 bp	8,9
DLF	158.16 bp 170.42 bp	6,9

*Bin Boundaries: Allele 5 - 153.00-155.15; Allele 6 - 157.00-159.15; Allele 7 - 161.00-163.15; Allele 8 - 165.00-167.15; Allele 9 - 169.00-171.15; Allele 10 - 173.00-175.15; Allele 11 - 177.00-179.15.

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alleles using the molecular weight marker is relatively poor. Nevertheless, the reproducibility of the sizing information from run-to-run is excellent. While the run-to-run data is reproducible, the day-to-day reproducibility is variable. Consequently, allele specific ladders are required as a daily standard in order to accurately type DNA samples. The results from Figure 1 illustrate that when using the allele specific ladder in conjunction with the PCR-based molecular weight marker, individual genotypes can be accurately and reproducibly determined. CE profiles of the allelic ladder and three individuals were overlaid, and based on retention times and size determinations, allele designations were made. The genotypes determined for the three individuals were consistent with the AB Gene Sequencer results.

In contrast to CE, the day-to-day, gel-to-gel reproducibility of the AB Gene Sequencer is excellent as reported in Table 2a. In addition, reproducibility is consistent across most of the STR loci tested (STR loci which exhibit micro-heterogeneity are more difficult to analyze). PCR conditions were similar to those described above, with the exception of the relative position of the primer pair and the thermal cycler parameters (7). Allele size determination is more precise when using the Gene Sequencer, although the observed values are still slightly different from the expected values. In general, the observed values differ from the expected by 1-2 base pairs in denaturing gels, and by 3.5-6 base pairs within native (non-denaturing) gels (8). This is consistent with the data presented here from native capillary gels. Nevertheless, the genotypes determined for the three individuals using a binning technique were identical to the genotypes determined by CE analysis (Table 2b). The Gene Sequencer and CE results were verified by conventional vertical polyacrylamide gel electrophoresis and silver staining. The PCR product was run next to a lane containing the identical allelic ladder used in the CE study.

The development of CE for forensic casework is continuing. Software specifically targeted for sizing STR alleles using retention times of allelic and molecular weight markers may enhance the day-to-day precision. Numerous generations of AB 672 Gene Sequencer software have dramatically improved sizing reproducibility. In addition, data management software (similar to the AB GenoTyper), which allows for unbiased typing and minimizes transcription errors, must be developed for CE. Through fluorescence detection and multi-column instrumentation, CE should be a powerful method for rapid, precise typing of DNA in the near future.

TABLE 1: Dionix CES1A CE Precision Data

Allele Number (Repeat Units)(Expected Size Value)	Day 1 (Mean & Standard Deviation)(7 Data Points)	Day 2 (Mean & Standard Deviation)(3 Data Points)	Day 3 (Mean & Standard Deviation)(3 DataPoints)
5 (179 bp Expected Size)	179.23 bp & 0.24 bp	178.36 bp & 0.03 bp	177.25 bp & 0.02 bp
6 (183 bp Expected Size)	183.34 bp & 0.15 bp	182.02 bp & 0.13 bp	179.84 bp & 0.15 bp
7 (187 bp Expected Size)	186.09 bp & 0.16 bp	184.75 bp & 0.21 bp	182.60 bp & 0.05 bp
8 (191 bp Expected Size)	189.01 bp & 0.18 bp	187.90 bp & 0.13 bp	186.36 bp & 0.04 bp
9 (195 bp Expected Size)	192.75 bp & 0.10 bp	191.61 bp & 0.05 bp	189.12 bp & 0.03 bp
10 (199 bp Expected Size)	195.82 bp & 0.27 bp	194.42 bp & 0.05 bp	191.84 bp & 0.02 bp
11 (203 bp Expected Size)	199.03 bp & 0.35 bp	197.44 bp & 0.04 bp	195.24 bp & 0.02 bp

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Typing Human DNA Using Capillary Electrophoresis: Comparison of Slab Gel and Capillary Formats

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Capillary electrophoresis (CE) is a high resolution microanalytical technique which can be used to separate biological molecules such as DNA (1-3). Compared to conventional slab gel electrophoresis, CE provides rapid run times and similar run-to-run precision. Slab gel electrophoresis of PCR products generally requires 2-5 hours for separation and detection. Thin-walled fused silica capillaries allow for greater heat dissipation and consequently higher voltages (as high as 30,000 Volts). An increase in voltage results in run times of approximately 30-50 minutes for PCR amplified short tandem repeat (STR) alleles or even larger restriction fragments (4). Instruments available on the market to date, however, only provide single column capabilities. Therefore, with a single column instrument 29-48 injections can be made in a twenty-four hour period. With auto-sampling capabilities, the amount of hands-on time is minimized. Nevertheless, the current level of through-put cannot compete with manual or automated slab gel analysis. Development of multi-capillary instruments (24-96 capillary formats), will allow for high through-put analysis in the near future. Consequently, on a multi-capillary instrument greater than 3000 injections could be made in a single day.

The results presented in this short paper attempt to illustrate the precision and reproducibility of the Dionix CES1A CE. The CE results were then compared to data generated on the Applied Biosystems (AB) 373A Gene Sequencer. Comparisons were made using the short tandem repeat locus HUMTHO1 (5,6). This locus has received a favorable evaluation by the forensic community and was chosen as a model for the development of CE for forensic casework. PCR conditions for CE experiments were as follows; 50 μl PCR containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP, dTTP, 2.5 units of Taq DNA Polymerase (Cetus), 5-10 ng of target DNA, 0.01% gelatine, and 1 X HUMTHO1 Primer Pair (Promega; a HUMTHO1 allele specific ladder was also provided by Promega). Thermal cycler parameters (Perkin Elmer 9600) were as follows; 95°C hold for 2 minutes, 10 cycles of 94°C for 30 seconds, 64°C for 30 seconds, and 70°C for 45 seconds, and 20 cycles of 90°C for 30 seconds, 64°C for 30 seconds, and 70°C for 45 seconds. Separation of PCR product was performed on a non-denaturing μPage3^{TM} (3%C,3%T) polyacrylamide gel column with a 40 cm effective length, using μPage3^{TM} buffer (J&W Scientific). Ethidium bromide was added (10 μM) to the buffer prior to separation. PCR product or allelic ladder was subjected to dialysis to remove excess salts using VS 0.025 μm membranes (Millipore) according to Williams (1992). Nanoliter (nl) volumes of PCR product were loaded onto the column using electrokinetic injection. Separations were performed at 210 V/cm in the presence of a molecular weight marker (Boehringer Mannheim, DNA Marker XI, 50-1000 base pairs).

Initial questions were addressed concerning the accuracy and reproducibility of CE when sizing AmpFLP alleles. The data presented in Table 1 suggests that when using non-denaturing polyacrylamide gel capillaries (3%T,3%C), the accuracy of sizing HUMTHO1

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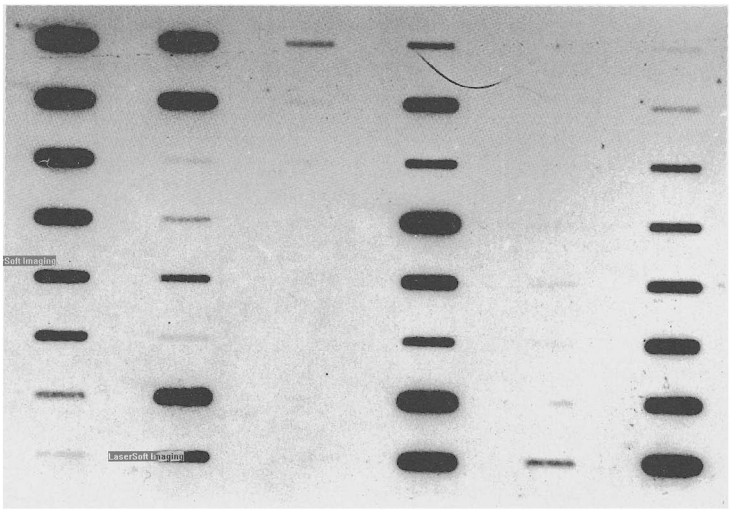

Figure 3:
Hybridization result after rehybrization with the GPL human Y-specific probe.

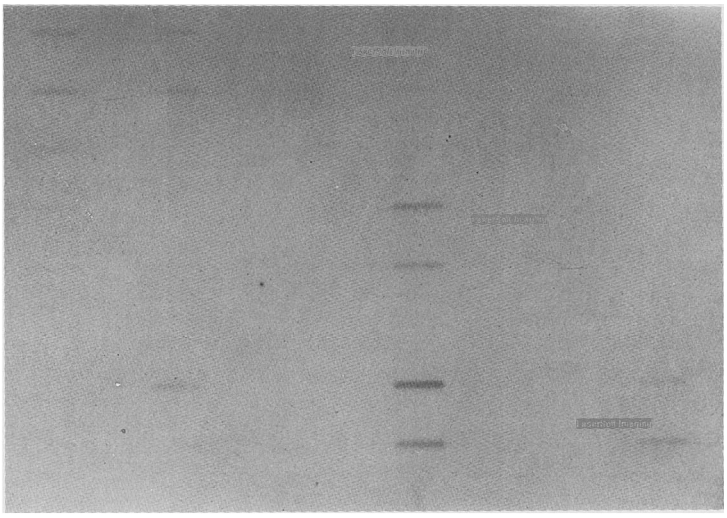

Figure 4:
Hybridization result after rehybridization with the self-construted, alkaline phosphatase labelled Y40 oligonucleotide probe.

	1	2	3	4	5	6
A	♂ 100 ng	Vag.Swab	♂ 1.0 ng	Vag.Swab	E.coli 20 ng	♀ 2 ng
B	♂ 80 ng	Vag.Swab	♂ 0.4 ng	Vag.Swab	E.coli 20 ng	♀ 4 ng
C	♂ 60 ng	Vag.Swab	♂ 0.2 ng	Vag.Swab	Bovine 40 ng	♀ 10 ng
D	♂ 40 ng	Vag.Swab	♂ 0.1 ng	Vag.Swab	Dog 40 ng	♀ 20 ng
E	♂ 20 ng	Vag.Swab	♀ 0.1 ng	Vag.Swab	Pig 40 ng	♀ 40 ng
F	♂ 10 ng	Vag.Swab	♀ 0.2 ng	Vag.Swab	Rat 40 ng	♀ 60 ng
G	♂ 4 ng	Vag.Swab	♀ 0.4 ng	Vag.Swab	Mouse 40 ng	♀ 80 ng
H	♂ 2 ng	Vag.Swab	♀ 1.0 ng	Vag.Swab	Chicken 40 ng	♀ 100 ng

Figure 1:
Slot Blot Arrangement of the different DNAs: Standard female and male DNA, bacterial DNA and animal DNA, DNA extracted from vaginal swabs (V.S.) after differential lysis.

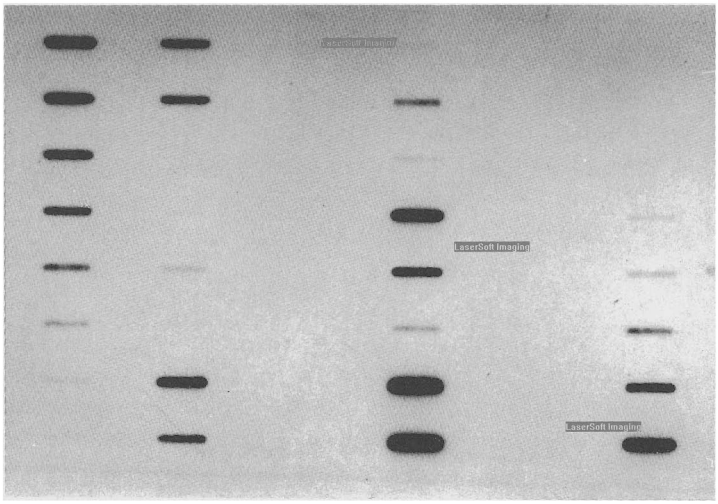

Figure 2:
Hybridization result after hybridization with the human specific probe (D17Z1)

Conclusion

After determination of quantity and human specifity of DNA by slot-blot hybridization with a human specific probe the information can be used for a further investigation. The results indicate that the same DNA can be sex-determined by a further hybridization with a human Y-specific probe. In case of vaginal swabs an additional information can be obtained about the quality of the differential lysis.

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The second rehybridization was carried out with a self-constructed Y40 probe. This probe is 40 bp long and contains eight times the pentanucleotide sequence described in Nakahori et al. (1988) [3] as human and Y-specific. This probe was labelled with the Lightsmith luminescence engineering system for oligonucleotides (Promega Cat.No.F1280). A hybridization protocol analogous to the PhotoProbe system from Gibco BRL gave the best results. The final concentration of the probe per one ml hybridization solution was 1200 fmol.
At last the membrane was hybridized with the human Y-specific probe from Promega (Cat.No.DK5141). The probe was labelled with digoxigenin analogous the Feinberg and Vogelstein method [4]. The concentration of the probe was 5 ng/ml hybridization solution. The hybridization was carried out analogous to the protocol of Boehringer Mannheim at 72°C. The membrane was exposed for 3 hours at 40°C.
To validate the results the extracted DNA from vaginal swabs was amplified by PCR. Different loci such as D1S80, TC11, SE33 and YNZ22 were amplified.

Results and Discussion

Figure 1 shows the slot blot arrangement and figure 2-5 four different hybridization results. In figure 2 the hybridization result of the human specific probe D17Z1 is visible. The results indicate the human specifity of the probe. No signal is visible for bacterial and vertebrate DNA. Human DNA up to 1 ng can be detected. A difference in intensity for the signal of the female and male fraction after differential lysis of the same vaginal swab is visible. If the signal of the female fraction of the vaginal swab is stronger more female epithel cells are detected in the cytological investigation. If more sperm cells are detected in the cytological investigation, a corresponding stronger signal of quantified DNA is visible in the male fraction.
The first rehybridization of the same membrane with the alkaline phosphatase labelled GPL Y-specific probe indicates that this probe does hybridize to animal DNA as well. The strongest non-human signal is visible for chicken DNA. The signal intensity is increased for both, female and male standard DNA, so that amounts up to 0.1 ng DNA can be detected. With this probe no sex-determination is possible because both female and male DNA give a strong hybridization signal.
A second rehybridization with the self-constructed Y40 probe another alkaline phosphatase labelled oligonucleotide probe, indicates human-specifity. The signal for human DNA is less intense than the signal after hybridization with the human specific probe. In case of male standard DNA amounts up to 1 ng can be detected. In case of female standard DNA the limit is 2ng. No sex-identification is possible with this probe, because there is no signal difference for female and male DNA.
The digoxigenin-labelled probe indicates no human specifity because even for bacterial and animal DNA a hybridization signal is detected. The signal intensity is the same for different amounts of bacterial DNA and for the animal DNAs. This probe gives the best results with respect to Y-specifity. The hybridization signals of the human male standard show a higher signal intensity in comparison to the hybridization with the human specific probe. In case of male DNA amounts up to 0.2ng can be detected. The signals of the human female DNA standard are less intense for DNA amounts from 60-100ng. No increase of signal intensity is visible for DNA amounts less than 40ng. For the DNA extracted from vaginal swabs an increase in signal intensity can be observed for the male and the female fraction. This indicates that the female fraction still contains male DNA, meaning no good separation of female and male fraction through differential lysis. This is confirmed by the following PCR reaction (data not shown).

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Studies on Sex Determination with Different Y-Specific Probes After Quantifying Human Genomic DNA with the Slot Blot Hybridization Method

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Introduction

Sex determination can be performed routinely by Southern blot analysis, by the PC Reaction or by dot/slot blot analysis. In forensic science the method for sex identification has to be fast and the amount of DNA available is sometimes limited. In case of vaginal swabs additionaly a good separation of female and male cells after differential lysis is required. At first the extracted DNA from forensic specimen has to be quantified. One possibility is the slot blot hybridization method with a human specific probe. It represents a method to detect small amounts of degraded DNA and to test its human specifity [1]. In this study a rehybridization of the membrane with different human Y-specific probes is investigated to identify the sex, to test human Y-specifity and to proof the quality of the differential lysis. Two alkaline phosphatase labelled human Y-specific probes are tested for their usefulness and one longer digoxigenin labelled human Y-specific probe. This method requires no additional DNA material for sex determination and quality control of DNA extracted from vaginal swabs.

Material and Methods

DNA from vaginal swabs was extracted according to the FBI protocol [2] with an organic extraction procedure. As female standard K562 DNA from Promega was used. Male standard DNA was human placenta DNA from SIGMA (Cat.No.3160). Bacterial DNA from E.coli was from Gibco BRL (Cat.No.220-4220). The vertebrate animal DNA from chicken, mouse, rat, dog, pig and bovine was from Promega (Cat.No. G3121, G3091, G3131, G3051, G3071, G3081). The bacterial and animal DNA were used to examine human specifity of the different probes. One µl from each fraction of the DNA extracted from the vaginal swab was used for slot blot quantification. All DNAs were diluted in distilled water up to 25 µl final volume. The different concentrations and the arrangement of the slot blot is visible in figure 1. All DNAs were denatured for 5 min. at r.t. with 500 µl 0.5M NaCl; 0.5M NaOH per sample. As a nylon membrane Biodyne A was used (Gibco BRL Cat.No.4866 SA). The membrane was prewetted for 5 min. in 2x SSC. After placing the membrane in the slot blot apparatus (Gibco BRL Convertible Slot Blot Vacuum Apparatus Cat.No.580-1055) the samples were loaded in the different slots. Each slot was rinsed with 500 µl of the denaturation solution. The membrane was removed from the apparatus and placed into a neutralization solution for 5 min.. Then the membrane was air dried and the DNA was fixed by UV crosslinking at a wavelength of 302 nm for 90 sec. and baked at 80°C for 30 minutes.

The first hybridization was carried out with the human specific probe D17Z1, an alkaline phosphatase labelled oligonuceotide probe (Gibco BRL Cat.No.4220 SA), analogous the PhotoProbe human DNA quantitation system protocol from Gibco BRL at 50°C. The exposition was carried out at 40°C overnight. Then the membrane was stripped with 0.1% SDS at 80°C for 30 minutes.

At first the membrane was rehybrized with the Gene Print Light human Y-specific probe from Promega in a concentration of 5 µl/10 ml hybridization solution of the PhotoProbe system. The hybridization protocol and exposition was the same as for the human specific probe D17Z1.

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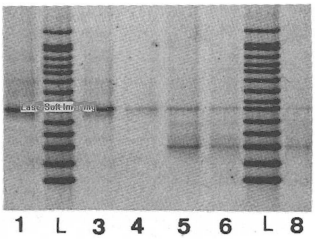
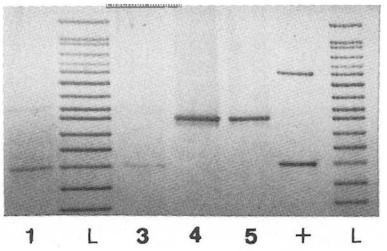


Figure 2.-D1S80 typing of a serial dilution of DNA. Allelic ladder (L). DNA from individual 1(T24,T24): lane 1 (50 ng), lane 3 (25 ng), lane 4 (5 ng). DNA from individual 2 (T18,T24) : lane 5 (50 ng), lane 6 (25 ng), lane 8 (5 ng).



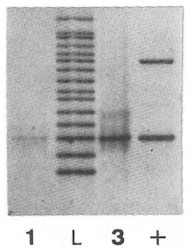


Figure 3.-D1S80 typing of different forensic samples. Allelic ladder (L). Positive control (+). (A) Case 1(T18,T25): lane 1 (DNA from whole blood extracted by the phenol-chloroform method), lane 3 (DNA from a hair root extracted by rapid digestion). Case 2 (T24,T24): lane 5 (DNA from whole blood extracted by the phenol-chloroform method), lane 4 (DNA from a semen stain extracted with 30 µl of 30% Chelex resin). (B) Case 3 (T18,T18): lane 1 (DNA from a blood stain extracted with 30 µl of 30% Chelex resin), lane 3 (DNA from whole blood extracted by the phenol-chloroform method).

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RESULTS and DISCUSSION

Up to 22 different alleles were found in the Basque sample, ranging in size from 17 to 40 repetitions. The alleles of 34 and 39 repetitions were not observed, while the alleles of 35 and 38 repetitions, not previously reported in the studied European populations, have been detected. The gene frequency distribution of the sample here studied and those from the European populations of Galicia and Portugal (Lareau et al, in press), Germany (Skowash et al., 1992), Finland (Sajantila et al., 1992) and that from the U.S. Caucasian population (Budowle et al. 1991) are shown in Fig.1. All the populations display similar distributions of their D1S80 gene frequencies.

The Hardy-Weinberg equilibrium test was performed by binning alleles together (Brenner and Morris, 1990) and using a three-allele model (17-23, 24 and 25-40; $X^2_{5d.f.}$= 1.584).

The observed Heterozygosity in the Basque population sample for the D1S80 locus was 0.74, the expected Heterozygosity being 0.794±0.016 (Nei, 1978). The Index of discrimination, calculated as by Wong et al. (1987) in the Basque population was 0.076. This Index is slightly higher than in other populations, and therefore, this system is equally useful for forensic purposes in our population. The Chance of Exclusion, calculated as by Smouse and Chakraborty (1986) is 0.621, a figure a little higher than that observed in other populations.

Genotyping of the amplified 5 ng samples of DNA both from homozygous and heterozygous individuals was possible using the procedure here described (Fig. 2), while the resolution was not enough in the case of the 2.5 ng samples. These results show that the lowest limit of DNA that can be amplified using this technique is 5 ng.

The application of the micro thermal cycler technique to criminal cases requires the modification of the DNA extraction protocol for stains with Chelex resine. The concentration of DNA extracted by Chelex resin was increased using a 30% resin stock solution, and adjusting the final volume of extraction to 30 µl. The same modification can be applied to semen stains, which enables stronger signals to be obtained after silver staining. Figure 3 shows the results obtained when forensic samples are amplified using this new procedure.

To summarize, based on a micro thermal cycler apparatus, we have developed a new protocol for amplification of the D1S80 locus.The advantages of this system consist of a reduction in time and costs of the amplification process, which greatly facilitates the analysis of the D1S80 locus at the population level as well as in paternity testing and forensic identification.

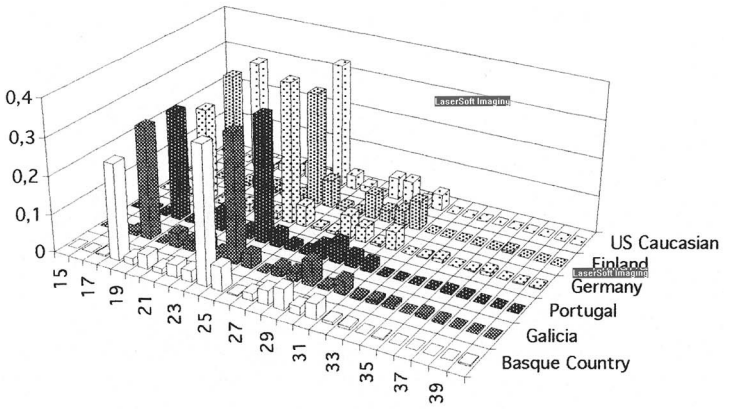

Figure 1.-Gene frequency distributions of several populations

POLYMORPHISM OF THE D1S80 LOCUS IN BASQUE COUNTRY POPULATION AND ITS FORENSIC APPLICATIONS

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INTRODUCTION

The D1S80 locus is a VNTR region, whose moderate repetition (units of 16 bp repeated in tandem 15-40 times) allows its analysis after amplification by the polymerase chain reaction (Kasai et al. 1990) and its genotyping after fractionation of the resulting fragments in high resolution PAGE followed by silver staining (Budowle et al. 1991). The possibility of analyzing this locus by PCR makes it a useful tool for the analysis of forensic samples which provide degraded or very low quantities of DNA. Moreover, the D1S80 locus displays a high number of different alleles in all the populations studied, and due to its high power of discrimination, this locus is becoming a widely used system in genetic identification testing. So, in order to develope a simple system of amplification of the D1S80 locus, we have developed a new protocol using a micro thermal cycler, its advantages consisting of a reduction in time and costs. On the other hand, a study of the allele distribution of the D1S80 locus in a sample of 150 individuals residing in the Basque Country has been carried out using our procedure. We can also see that this technique is applicable to DNA samples obtained by different extraction procedures (phenol-chloroform, Chelex resin, rapid digestion) as well as from different sources (blood, blood or semen stains, hair roots).

METHODOLOGY

Extraction of DNA from different samples

Whole blood samples were obtained by venipuncture from 150 healthy and unrelated individuals resident in the Basque Country. DNA was extracted using the phenol-chloroform method. The DNA from blood or semen stains was extracted with Chelex resin (Jung et al. 1991). With the aim of increasing the concentration of the DNA extracted, the following modifications were made: a 30% stock solution of Chelex resin was prepared and 30 µl of this were added to the sample. The DNA from hair roots was extracted by rapid digestion.

Amplification

4 µl of the DNA (100ng) extracted using the phenol-chloroform method or 4 µl of each sample extracted by the above mentioned methods were amplified with 6µl of the reaction mix containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM of each dNTP, 1µM of each primer (Budowle et al. 1991) and 0.2 U of Taq I polymerase (Ampli Taq ™ Perkin-Elmer Cetus) without adding mineral oil.

Alternatively, 6 µl of D1S80 PCR reaction mix from Cetus Corp. were used adding 0.1 U of Taq polymerase. Amplification was carried out using a micro thermal cycler (Linus Microcycler) with the following cycles: one cycle of 94°C 2 min - 65°C 5 s - 72°C 35 s, five cycles of 94°C 30 s - 65°C 5 s - 72°C 35 s , thirty cycles of 94°C 5 s - 65°C 5 s - 72°C 35 s -30 cycles and one final cycle of 72°C 1min.

PAGE and silver staining

PAGE was performed using 0.4 mm thick, 12 cm long polyacrilamide-piperazine gels (4% T, 3% C), 0.375 M Tris-HCl pH 8.8 buffer, with 3% agarose electrodes in 0.25 M Tris-glicine buffer pH 8.8. The gels were run at 15 mA, keeping the current constant. Detection of the genotypes was perfomed by silver staining (Budowle et al. 1991).

Statistical calculations

Hardy-Weinberg equilibrium was tested using the three-allele model of Skowash et al. (1992). The expected number of alleles was estimated as by Chakraborty et al. (1991) and the expected heterozygosity as by Nei (1978).

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cance level is small (less than 0.05), the hypothesis that all group means are equal is rejected.

TAB. 3

Canonical Discriminant Functions

Fcn	Eigenvalue	Pct of Variance	Cum Pct	Canonical Corr	After Fcn	Wilks' Lambda	chisquare	DF	Sig
					: 0	.0199	336.768	30	.0000
1*	11.6281	81.52	81.52	.9596	: 1	.2516	118.678	18	.0000
2*	2.5005	17.53	99.05	.8452	: 2	.8807	10.929	8	.2058
3*	.1355	.95	100.00	.3455	:				

* marks the 3 canonical discriminant functions remaining in the analysis

If the observed significance level is less than 0.05, the discriminant function is "powerful".

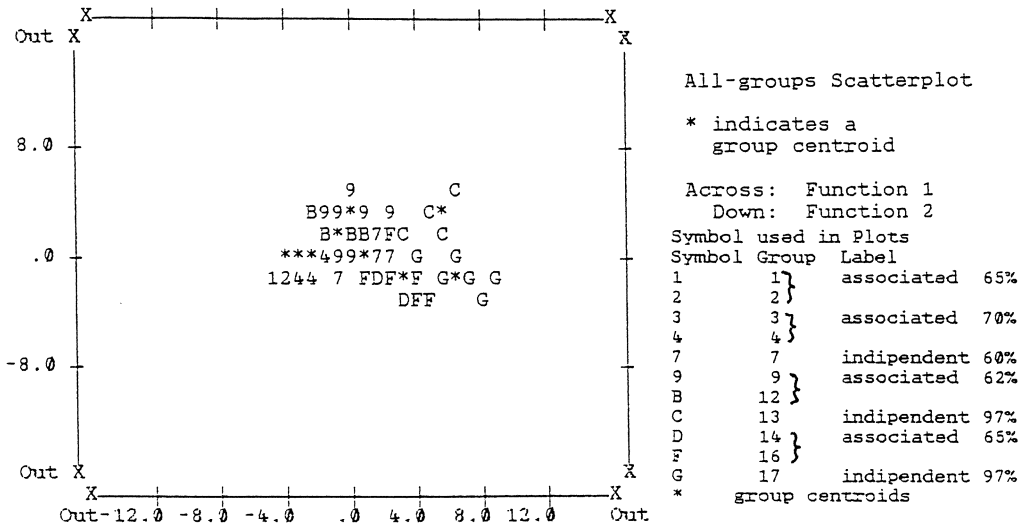

Fig.1 : Plot of the values of the two discriminant scores for each cases. Cases are identyfied by their group member. When several cases fall into the same plotting location, only the symbol of the last cases is printed.

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dy are very encouraging and significative.
Therefore we programmed a further investigation both on genealogy and on population study in order to allow to introduce this methodology in forensic medicine.

TAB. 1

CL.	AN	AT	RATIO	;	AN	AT	RATIO	;	AN	AT	RATIO
1	215	576	.3732		232	615	.3772		258	669	.3856
	453	1187	.3816		303	881	.3439		460	1138	.4042
	382	1001	.3816		277	805	.3440				
2	386	879	.4391		336	815	.4122		322	729	.4417
	269	785	.3426		306	840	.3642		344	921	.3735
	361	926	.3898		320	780	.4102		282	688	.4098
3	411	986	.4168		506	1186	.4266		430	1041	.4130
	448	950	.4715		458	1030	.4446		394	905	.4353
	424	1012	.4189		409	960	.4260				
4	353	949	.3719		421	1009	.4172		399	873	.4570
	422	986	.4279		400	999	.4004		347	919	.3775
	335	879	.3811		457	1012	.4515		517	1155	.4476
	442	1166	.3790		492	968	.5082		472	1015	.4650
	372	961	.3870		429	844	.5082				
7	773	2141	.3610		1020	2319	.4398		948	2279	.4160
	908	2090	.4344		1037	2619	.3960		997	2285	.4363
	777	2061	.3770		1064	1926	.5524		663	1373	.4828
	819	1579	.5186		620	1308	.4740		695	1560	.4455
	1050	2727	.3850								
9	738	1609	.4587		841	2476	.3397		774	2536	.3052
	771	2340	.3295		673	1583	.4251		775	2391	.3241
	664	1820	.3648		1062	2872	.5673		710	2291	.3099
	610	2105	.2898		573	1675	.3420				
12	818	2246	.3642		667	1932	.3452		513	1831	.2802
	593	1710	.3468		842	2118	.3975		758	1970	.3848
13	1153	2783	.4143		1466	3868	.3790		1422	3373	.4216
	1338	3364	.3978		1407	3443	.4087				
14	1054	1985	.5310		1126	1879	.5993		1006	1782	.5645
	1196	2070	.5777		1063	1962	.5418		922	1726	.5342
16	1045	1837	.5689		1061	2579	.4114		1191	2236	.5326
	1175	2173	.5407		1187	2038	.5824		859	1603	.5359
	1181	2180	.1417								
17	1517	2891	.5247		1595	2659	.5998		1150	2438	.4717
	1319	2339	.5810		1439	2915	.4926		1708	3232	.5285
	1466	2872	.5104								

Values of the three variables AN, AT, RATIO for each case (cluster).

TAB. 2
Wilks' Lambda (U-statistic) and univariate F-ratio with 10 and 83 degrees of freedom

Variable	Wilks' Lambda	F	Significance
AN	.08001	95.44	.0000
AT	.11953	61.14	.0000
RATIO	.52126	7.623	.0000

Significance test for the quality of group means for each variable (between-groups variability). If the observed signifi-

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METHOD FOR THE EVALUATION OF C-BANDS HETEROMORPHISM IN HUMAN CHROMOSOMES BY IMAGE PROCESSING (I.P.).

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The I.P. method allows the C-bands computerized evaluation heteromorphisms of Y chromosome by reducing the operator variability and the measurements of the relationship between the heterochromatic region (AN) and the total chromosome area (AT).
The I.P. method (1) is articulated in the following steps: 1) image gain of each photo by camera and digitalization of analogic image; 2) interactive extraction of working area (Y chromosome); 3) preelaboration to reduce the background and to get a better contrast of digital image; 4) automatic segmentation of the specific area; 5) evaluation of AN, AT, RATIO parameters.
Eleven male subjects (94 Y chromosomes)(Tab.1) were analyzed by I.P.: all data from I.P. will be processed by a multivariate statistical method (M.S.M.).
At first we performed the outliers analysis (2) to reduce the measurement error of observator due to sensor noise, film gains, etc.
The masking effect (i.e. the inability of a testing procedure to identify even a single outlier in the presence of several suspected values) is solved by the Median test (2).
Finally we analized the data by M.S.M. (3) to identify the variables (AN, AT, RATIO) that are important for distinguishing among the groups (i.e. subjects) and to individuate among several mutually exclusive groups.
Using the discriminant score, it is possible to obtain a rule for classifying cases into one of the groups. The technique used is based on Bayes' rule.
The demonstration of maximum between group variability of C-bands heteromorphism and the minimum within group variability is obtained by the ratio eigenvalue = between group s.s./within group s.s. (Tab.2).
Infact, the coefficients of the discriminant functions are chosen so that the ratio of the between group sum of squares to the within group sum of square is as large as possible (Tab.3).
By this mathematical process we reach an exact classification for all 94 measurements (Fig.1).
Comparing the different clusters with the subjects the results was confirmed: infact, patients 1 and 2 are broters; 3 and 4 are father and son; 9 and 12 are father and son; 14 and 16 brothers, while remaining don't show any classification.
In conclusion, the results obtained from this preliminary stu-

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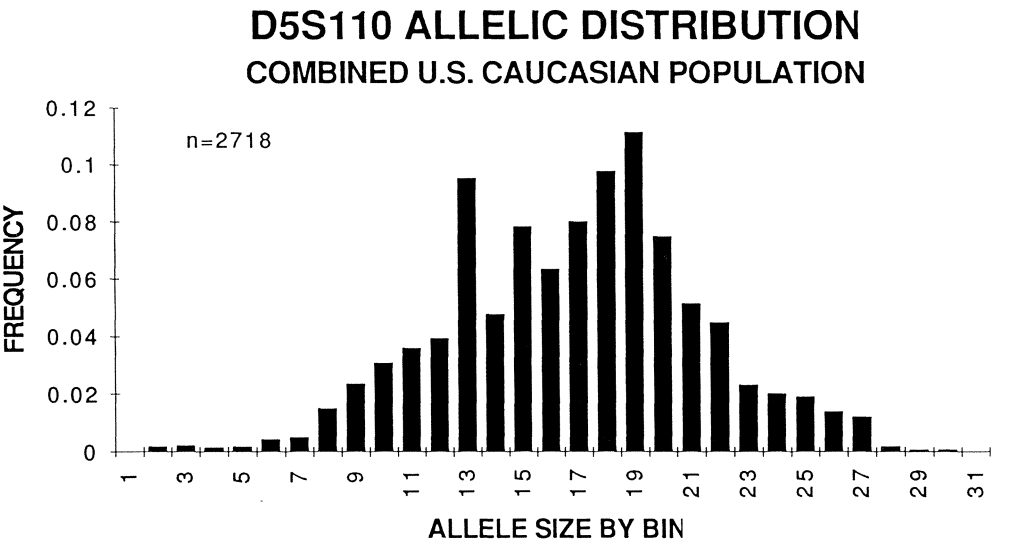

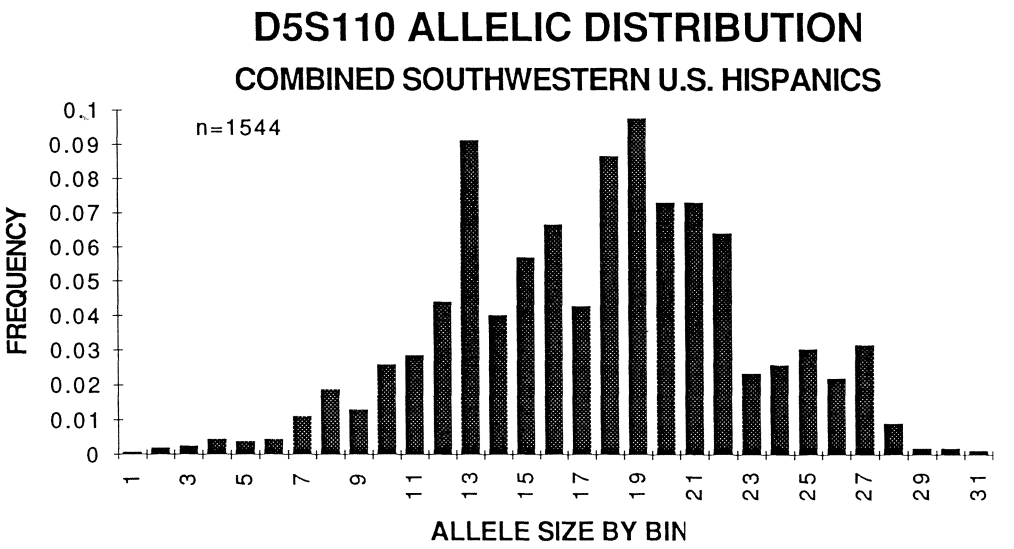

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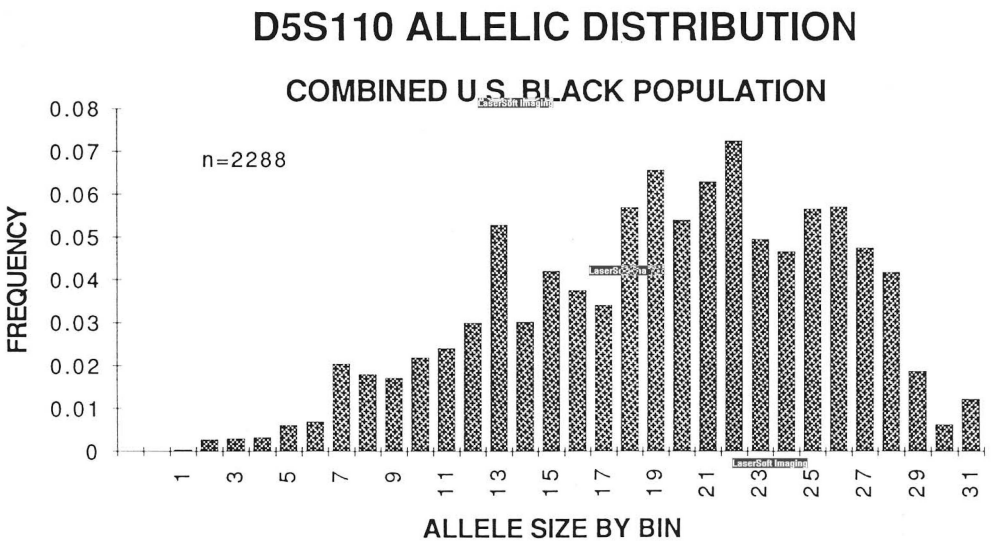
combined with a very high exclusionary power would indicate that an analysis of the D5S110 locus would be extremely useful for the determination of paternity.

The utility of the LH1 probe in forensic casework has been examined at the Tarrant County Medical Examiners Office (Texas, USA). In several cases where a marginal amount of DNA was recovered from an evidentiary sample, a DNA profile was detectable with the LH1 probe. Other commonly used single locus probes such as YNH24, MS1, and PH30 occasionally failed to produce a profile either due to an insufficient or partially degraded sample. The sensitivity of the LH1 probe combined with the broad distribution of alleles between 640 and 10,000 base pairs makes it ideally suited for RFLP forensic casework. Forensic laboratories should strongly consider D5S110 as one of the primary loci analyzed in forensic samples.

CONCLUSION

The analysis of the D5S110 locus is extremely useful for human identification. The availability of the LH1 probe in both a radioactive and chemiluminescent format makes it one of the most powerful probes for DNA typing.



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FURTHER CHARACTERIZATION OF THE VNTR PROBE LH1 (D5S110) AND APPLICATIONS FOR DNA TYPING

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The single-locus probe LH1 (D5S110) originally described by Armour, J.P., et al, Genomics, Vol. 8, p.501, 1990 was evaluated for its effectiveness in DNA typing assays for human identification. A cloned, purified, 6-Kb DNA fragment containing sequences complementary to the D5S110 locus was labeled with ^{32}P-dCTP by the random primer method. LH1 appears to be one of the most sensitive single-locus probes observed, capable of detecting a DNA profile in 10 ng of Hae III restricted genomic DNA in a 24-hour autoradiographic exposure. An enzyme-linked oligonucleotide probe was constructed for use in a chemiluminescent hybridization and detection format. Nine repeats within the D5S110 cloned insert were sequenced, and a 30-base oligonucleotide was synthesized and labeled with alkaline phosphatase. The sensitivity and specificity of the alkaline phosphatase labeled oligonucleotide under the ACES 2.0 hybridization and detection conditions is comparable to that of the ^{32}P labeled LH1 probe. The enzyme-linked oligonucleotide probe was capable of detecting the correct banding pattern in 25 ng of Hae III restricted genomic DNA in a 3-hour exposure following a 3-hour ramp.

The LH1 probe was used in population studies at the University of North Texas Health Science Center at Fort Worth (UNTHSCFW, Texas, USA, and Genetic Design Incorporated (GDI, North Carolina, USA). A total of 3280 individuals were probed between the two labs (1144 African Americans, 1359 Caucasians, 777 Hispanics). The Hae III allelic distributions for the African American, Caucasian, and Hispanic ethnic groups were very similar between UNTHSCFW and GDI, with a linear correlation of 0.95, 0.97, and 0.91 respectively. The majority of alleles (African American 95%, Caucasian 99%, and Hispanic 98%) were broadly distributed between 640 and 10,000 base pairs. The observed heterozygosity at the D5S110 locus was also very similar in both population studies. The heterozygosity was greater than 95% for each of the three ethnic groups.

In 1800 cases examined between the two parentage testing laboratories, 19 maternal exclusions were observed for a maternal recombination rate of 0.0106. The combined maternal and paternal recombination rate observed at UNTHSCFW was 0.008. In cases were the alleged father had been excluded as the biological father, the probe LH1 failed to exclude the man approximately 4% of the time. The observed Exclusionary Power was greater than 95% for each of the three ethnic groups examined. Although the overall recombination rate is higher than some of the other single locus probes currently used for parentage testing in the United States, the broad distribution of alleles

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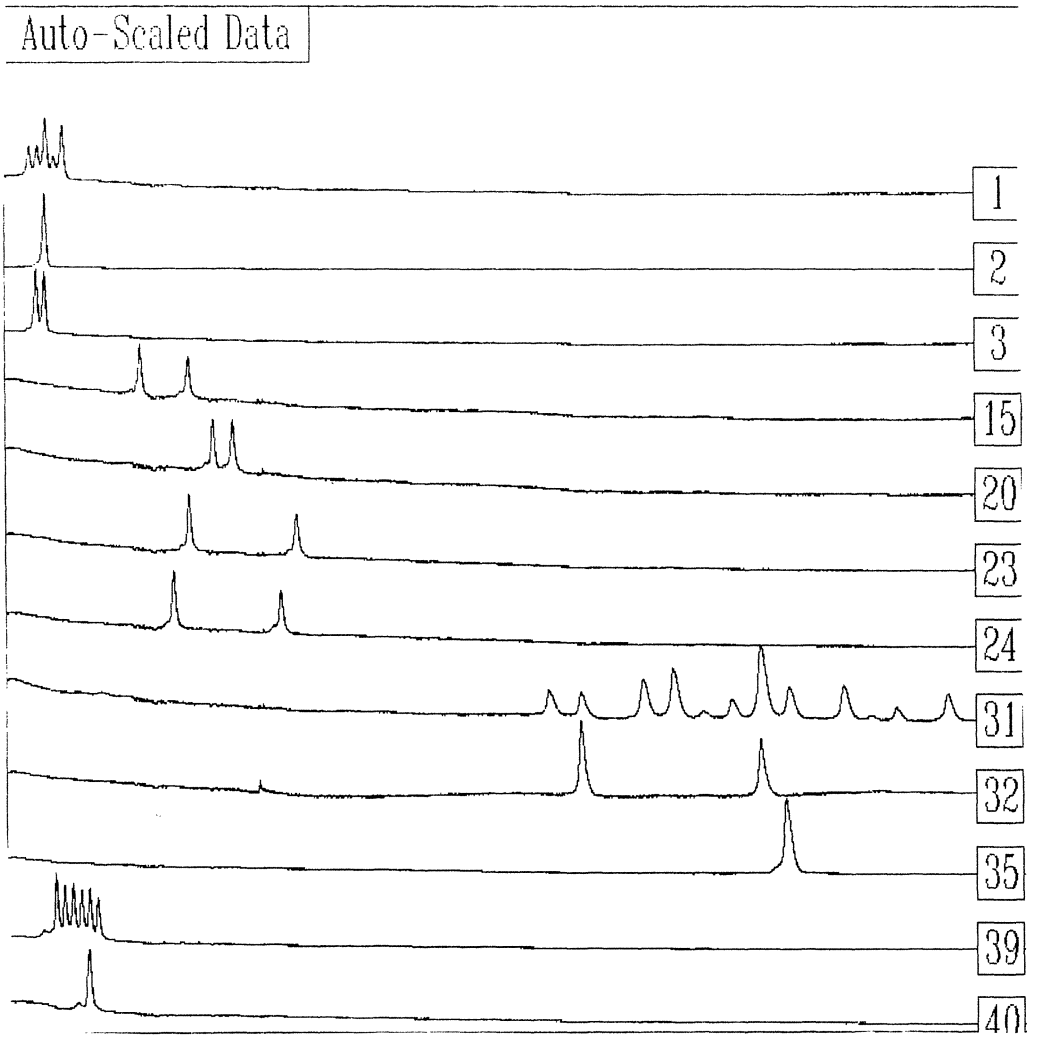

Figure 1
A typical chromatogram produced by the A.L.F. apparatus is shown. The simultaneous detection of four loci is achieved in a six hours run. In the first three lanes an examplification of the commonest allels of MITMH26 system is reported. In the subsequent lanes (n. 15, 20, 23 and 24) some HUMACTBP2 fragments are detected. A D1S80 fluorescent allelic ladder has been electrophoresed in lane number 31, while two amplified products for the same system are present in the adjacent lanes (n.32 and 35). The last two chromatograms show the allelic ladder and an homozygous individual for the HUMTHO1 system.

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alleles differing by 16 bp. For the other loci, an allelic difference of 4 bp is always met. Noteworthy, a correct separation and typing of all systems is possible in one standard experiment, irrespective of the basic difference in size and repeat unit. A comparison between the laser fluorograms and PAGE/silver procedures demonstrated the superior sensitivity and reliability of the automated procedure and its versatility. However, the use of a single fluorescent molecule made it possible "multiplexing" (i.e.: analysis in the same well) of only these systems with non-overlapping sizes. The A.L.F. resolving power clearly emerges by the fact that the so called 10-1 allele in the HUMTH01 system was easily typed (this fragment variant differs by a single base pair from the nearest allele). Experiments with age-degraded DNA samples showed that laser fluorence sensitivity is higher (by a factor of about 100) than the PAGE/silver procedure. The automated typing protocol is presently employed in our routine of paternity cases and its application to criminal analyses is planned.

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AUTOMATED LASER FLUORESCENT ANALYSIS OF AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS AND SHORT TANDEM REPEATS

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Genetic typing of Variable Number of Tandem Repeat (VNTR) loci is today feasible by the use of fluorescent primers in otherwise typical PCR reactions, and by subsequent laser-detection of amplified products during the electrophoretic run. These facilities, derived from procedures now currently used in automated DNA sequencing, allow for rapid and reliable typing of a variety of Amplified Fragment Lenght (AmpFLP) and Short Tandem Repeats (STRs) loci. Their adoption is having a remarkable role in speeding up procedures of forensic investigation (individual identification and paternity tests). We briefly report on our experience in typing VNTRs by a fixed argon laser-beam sequencer (A.L.F,Pharmacia) (1).

MATERIALS AND METHODS

Amplimers were synthesized on a 391 PCR-Mate (ABI) using a slightly modified cycle to label the 5'-end of one primer of any given pair by a fluorescein-amidite (FluorePrimeTM, Pharmacia). Genomic DNA was used to amplify the following loci: D1S80 (2), HUMACTBP2 (3), HUMTH01 (4), MIT-MH26 (5). Annealing temperatures were slightly adjusted to account for the presence of the fluorochrome molecule. Amplified products (0.5 to 3 microliter) were denaturated and dispensed in the vertical wells of the A.L.F. gels (6% T acrilamide, 7 M urea). Running conditions were 1500 V, 35 mA and 30 W and were administered for 6 hours to allow a simultaneous characterization of the four loci. Home-made DNA allelic ladders, composed of coamplified VNTR or STR alleles from different genotypes in pools, helped to control the gel resolving power and the final genetic typing. To identify alleles, comparison of adjacent lanes and occasional admixtures of PCR products were performed. Multiplexing of HUMTH01, MIT-MH26 and D1S80 was obtained by simultaneous gel separation of the three systems in the same lane. No attempt to amplify different systems in the same PCR cycle was performed.

RESULTS AND DISCUSSION

Figure 1 shows some typical chromatograms produced by the A.L.F. apparatus. For D1S80, a standard collection of 15 distinct fluorescent fractions was used, defining as many

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Peaks were identified as specific alleles by calibration curve of migration time vs fragment sizes using ϕX174 Hae III digested DNA.

RESULTS AND DISCUSSION

Complete and reproducible resolution of the APOB alleles in the range of 560 to 900 molecular weight was obtained without ambiguity. The electropherogram pattern was comparable to the profile obtained by agarose gel electrophoresis (Fig.1).

We have applied this technique to analyze retrospectively 10 cases of disputed paternity, previously shown to be fully informative for the APOB segregation. Paternity attribution was confirmed in 4 cases and excluded in 6. In three experiments in which DNA samples from two persons were co-injected, four distinct migration peaks were detected. Thus, the presence of two APOB genotypes, heterozygous for non-sharing alleles, was unambigously resolved. This result proves that non-paternity is readily and accurately detected using the present protocol.

Personal experience demonstrates high efficiency in separating the APOB amplified alleles using CE, which is proposed as a method for automatizing forensic DNA analysys.

Work supported in part by grants from MURST (Fondi 40%).

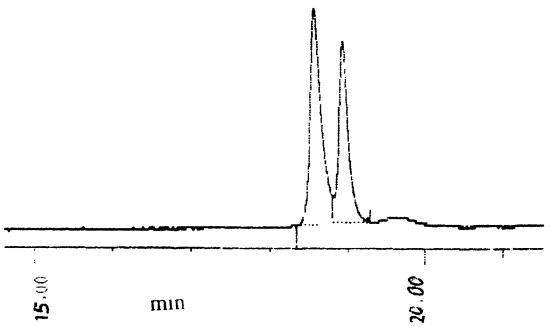

Fig.1. CE separation of APOB amplified fragments from an individual with alleles 35 and 39. Detection was by absorbance at 260 nm (0.05 full scale). Migration times (min) are indicated on the x-axis.

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APPLICATION OF THE CAPILLARY DNA CHROMATOGRAPHY IN THE PATERNITY TESTING USING APOB AMPLIFIED ALLELES

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Allele resolution and separation of "in vitro" amplified DNA is currently performed by gel electrophoresis followed by stain or probe detection (1,2). However, these are time-consuming procedures and require extensive manual operations.

Capillary electrophoresis (CE) has been successfully used as an alternative to standard gel electrophoresis for analysing DNA restriction fragments and PCR products of single viral and human sequences (3,4). The advantage of CE over other separation techniques is that only nanolitres of samples are required for each procedure, and rapid, efficient, sensitive and automated analyses can be achieved (5).

We have investigated the separation of amplified polymorphic alleles from the human APOB locus using CE with a coated fused silica column.

MATERIALS AND METHODS

Human genomic DNA was extracted by standard techniques and amplified "in vitro" using APOB specific amplimers (6). Amplified fragments were subjected to a phenol-chloroform (3:1 v/v) extraction and precipitated with cold ethanol and lyophilized. Samples were reconstituted in 10 μl of distillated water. CE was performed on the P/ACE™ system 2000 (Beckman, USA) in the reversed polarity mode. The temperature was set at 25°C and UV absorbance was monitored at 260 nm. A surface-modified fused silica capillary (100μm i.d.) was used for all analyses. Aliquots (1:100 diluted) of purified PCR products were run for 22 min at 12 KV.

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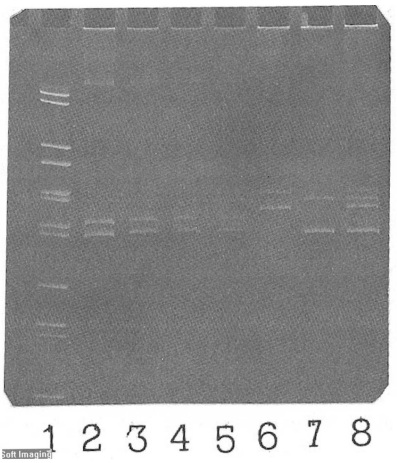

Fig. 2. Amplification of MCT 118 system. Lane 1: Marker VI (Boehringer); lane 2: control DNA from individual A; lanes 3, 4 and 5 : DNA extracted from semen stains of 50 µl, 25 µl and 10 µl; lane 6: DNA from individual B; lane 7: DNA from individual C; lane 8: extraction from stains made with a mixture B+C.

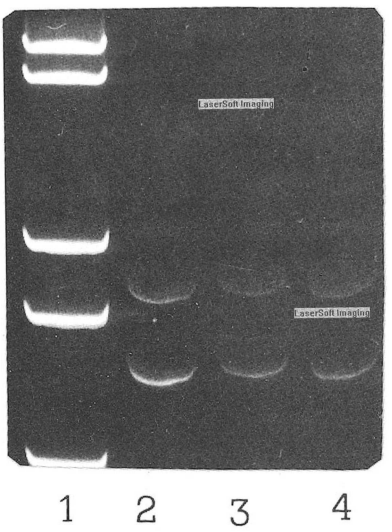

Fig.3. DNA extraction from cigarette butts. System Apo B. Lane 1: Marker VI (Boehringer); lane 2: blood DNA from individual A; lanes 3 and 4 : extractions from cigarette butts smoked from individual A.

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RESULTS AND DISCUSSION

Bloodstains: it was observed that DNA could be extracted from 1 to 2 day-old bloodstains made from at least 40 µl of blood by incubating the stain with 0.85% NaCl for 3 h at room temperature. However, effective DNA extraction with the smaller or older stains considered could only be achieved with the addition of Proteinase K and incubation at 65°C for 3 h.

The amount of distilled water to be added to the extracted DNA depended on the size of the bloodstains used: with stains containing up to 40 µl of blood 40 µl of dH_2O were enough, larger stains required up to 70 µl. The latter amount is sufficient for two amplifications (Fig.1).

Semen stains : with both buffers it was possible to extract DNA even from very small stains of semen (5-10 µl); furthermore, with the larger stains the incubation period could be safely shortened to 6 h.

The observations made regarding the quantity of dH_2O to be added to the DNA extracted from stains of blood are also applicable to DNA extracted from semen stains.

The above procedure was also found to be effective with mixtures of seminal and vaginal fluid, enabling differential extraction of seminal and vaginal DNA from a vaginal swab when amplifying AMP-FLP systems (Fig.2).

Saliva : it was found that good results could be achieved by incubation with 0.85% NaCl and Proteinase K, at 65°C for 3 h. The volume of distilled water added in the last step of the procedure was 40 µl (Fig.3).

Our results indicate that DNA can be extracted from the blood and saliva stains considered using 0.85% NaCl and Proteinase K by changing only the incubation time and temperature according to the nature and the age of the stain. However, semen stains required, as previous Authors have demonstrated (5), the addition of SDS and DTT to the incubation step.

Furthermore the possibility of using silver staining validating the reliability of these PCR markers for forensic application.

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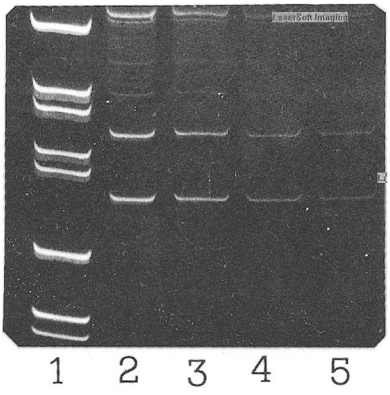

Fig. 1. Amplification of DNA extracted from bloodstains. SystemYNZ 22. Lane 1: Marker VI (Boehringer); lane 2: control DNA extracted from blood; lanes 3, 4 and 5 : extraction from 75 µl, 25 µl and 10 µl bloodstains respectively.

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THREE VNTR AND Y-CHROMOSOME IDENTIFICATION FROM BIOLOGICAL STAINS

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INTRODUCTION

The development of the PCR for amplifiyng DNA sequences has raised the possibility that variable number of tandem repeats (VNTR) and other DNA polymorphisms might be analyzed even in samples where either the sensitivity, the quantity, or the level of degradation would, have represented an unbrechable obstacle with other methods.

In this paper, we propose a rapid and efficent method for DNA extraction from limited amounts of human biological stains of blood, semen and saliva.

The purpose of the work was to identify a simple DNA extraction procedure suitable for subsequent PCR-based DNA profiling that could be applied to the different biological stains mentioned above.

With this procedure we were able to amplify MCT 118, Apo B, YNZ 22 and Y-Chromosome.

MATERIALS AND METHODS

Sample preparation

We considered blood and semen prepared from 5 to 100 µl of whole blood and seminal fluid of known genotype, respectively. The stains had been stored in a paper bag at room temperature prior to analysis.

The saliva stains were principally taken from cigarette butts (smoked by individuals with known genotype) and stored at room temperature in a paper bag up to 1 month prior to analysis.

Incubation

Bloodstains: a pair of stains (A and B) of equivalent age and size were first cut into small pieces and introduced into an eppendorf tube and then incubated as follows:

stain A with 1 ml 0.85% NaCl for 3 h at room temperature;

stain B with 1 µl 0.85% Nacl + 100 µl buffer K (25 mM MgCl2, 20mM Tris pH8.5, 0.5% Tween 20) + 10 µl Proteinase K (10 mg/ml) for 3 h in a water bath at 65°C.

Semen stains: the same preliminary procedure for bloodstains was adopted. The stains (A and B) were subsequently incubated overnight in a water bath at 65°C as follows:

stain A with 1 ml 0.85% NaCl + 0.5% SDS + 40mM DTT+ 10 µl Proteinase K +100 µl buffer K;

stain B with 1 ml lisys buffer (10 mM Tris-HCl pH 8, 10mM EDTA, 100mM NaCl).

Cigarette butts: the buccal end of the paper covering and filter were removed, cut into small pieces, and introduced into an eppendorf tube. Incubation was in a water bath for 3 h after the addition of:

1 ml 0.85% NaCl+ 100 µl buffer K + 10 µl Proteinase K.

DNA Extraction

Following the incubation, the same procedure was used for all the biological stains under investigation. The supernatant was removed and dispensed into another eppendorf tube, while the pieces of stains were rinsed with 500 µl of 0.85% NaCl before being added to the eppendorf containing the supernatant. The solution was centrifuged at 13000 rpm for 15 min., the supernatant discarded, and the eppendorf inverted to allow the pellet to dry. Between 40 and 70 µl of dH_2O were subsequently added to the eppendorf tube: the exact amount depending on the size of the original stain employed (see results).

The contents of the eppendorf tube were then boiled for 10 min. and the DNA amplified using PCR.

Amplification and typing

PCR amplification of the DNA was performed using previously published conditions and primers. The following systems were considered: MCT 118 (1), Apo B (2), YNZ 22 (3) and Y-Chromosome (4). In order to amplify each extraction, 30 µl of the final suspension were used in a total volume of 50 µl of PCR mix.

PCR products were separated by electrophoresis in 1x TBE Buffer using 6% polyacrylamide (29:1 Acrylamide/Bis) and for Apo B, 8% for MCT 118, YNZ 22 and Y-Chr. Electrophoresis was performed at 150 V in a C.B.S apparatus for about 2 h (depending on the genetic system). The separation distance was 10 cm. The marker used was Marker VI Boehringer.For each amplified sample the amount loaded was 20 µl.

The amplification products were visualized directly after staining the gel in ethidium bromide.

As the gels were difficult to read, the silver staining procedure described by Budowle et al.(1) was adopted.

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70 °C. Because light emission lasts for up to 3 days, re-exposures are easily obtained. The resulting images are sharper than those obtained with ^{32}P-labeled probes, therefore AP-probes give better resolution and greater power of exclusion.

4. Speed

Because of the fast and simple protocol it is possible to obtain at least one set of results per day. By doing daily rehybridizations with various AP-probes, it is easy to produce results with four different probes in a one week period.

5. Rehybridization

The AP-labeled probe is inactivated by heating at 65 °C for 20 min. Hybridization of membranes can be readily interchanged between AP-probes and radioactive probes. Therefore, rehybridization can be performed with ^{32}P-labeled or AP-labeled probes.

In summary, the use of AP-labeled probes is faster, safer, and easier than radiolabeled probes. They are ideal for laboratories that want to move away from the use of radioactive materials and/or without the resources for radiolabeled containment or for laboratories interested in fast turnaround time.

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Table 1. **Properties of AP-probes used for the analysis of VNTR loci.**

	SIZE RANGE OF DNA FRAGMENTS (KB)			
	Restriction Enzyme			%
Locus	Hae III	Hinf I	Pst I	Heterozygosity
D1S7	1.6 - 23	0.8 - 25		99
D1S339	0.6 - 12	1.2 - 13	1.2 - 13	95
D2S44	0.2 - 11	1.5 - 10	6.8 - 16	96
D2S92	2.4 - 13			94
D4S163	1.3 - 16	1.2 - 12	3.6 - 16	95
D6S132	1.0 - 8.5	4.3 - 12	1.7 - 9	92
D7S21		3.0 - 20	4.5 - 20	97
D7S467	1.6 - 7		3.5 - 15	94
D10S28	0.2 - 13			94
D12S11		3.0 - 18	4.7 - 25	95
D16S85	0.4 - 7	0.3 - 9	0.6 - 10	92
D17S79	0.6 - 2.4	1.0 - 4.3	2.5 - 5.7	88
D18S27		1.0 - 6.2	4.0 - 9	92
DNF24		1.0 - 23	2.0 - 25	multilocus

The use of AP labeled probes has a number of advantages over traditional radiolabeled probes. These advantages include:

1. Stability
   AP-labeled probes are stable for at least one year.

2. Safety
   AP-labeled probes do not require containment or monitoring because they are not radioactive. The reagents utilized do not require special handling.

3. Hybridization
   The hybridization conditions are generally milder (55 °C vs 65 °C) and shorter (20 min vs several hours) resulting in a decreased loss of DNA from filter. Exposures to the X-ray film are at room temperature or 37 °C instead of

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USE OF CHEMILUMINESCENT LABELED PROBES FOR FORENSIC AND PATERNITY DETERMINATIONS.

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Introduction.

Since the middle 1980's, issues involving human identification in forensic or paternity cases have been addressed by RFLP DNA analysis using probes radiolabeled with P^{32} (Kanter et al., 1986; Guisti et al., 1986; Baird et al., 1986). The main disadvantage of these types of probes are their short shelf life and the general inconvenience associated with working with radioactive materials. For the past several years our laboratory has been involved in the conversion of these probes to a non-isotopic format. This has been accomplished through the development of oligonucleotide probes labeled with alkaline phosphatase (AP-probe) that recognize VNTR loci (Baum et al. 1990, Neuweiler et al. 1992). The final detection of AP-probes is based on the detection of chemiluminescence with the substrate, adamantyl 1,2-dioxetane phenyl phosphate (Lumiphos™). This detection system offers equivalent sensitivity to P^{32} allowing detection of genomic DNA in the ng range.

Materials and Methods

Standard RFLP methodology was utilized to prepare, digest, size separate and transfer DNA. Starting with DNA bound to dry nylon membranes, the general procedure was as follows:

Wet Membranes (5 to 10 min at room temp.)
Hybridization (20 min at 55 °C)
Wash I (2 times for 10 min each at 55 °C)
Wash II (2 times for 10 min at 55 °C)
Rinse with Quick-Light™ Buffer (4 x 1 min dip)
Spray substrate (Lumi-Phos™)
Expose to X-ray film (O.N. at room temp. or 1-6 hrs at 37 °C)

Results and Discussion

Numerous VNTR loci were tested for their utility for DNA typing and for the properties of their respective AP-probes. Each AP-probe was synthesized as an oligonucleotide and directly conjugated with AP. Table 1 provides a list and brief description of the properties of 14 loci available in our laboratory for paternity and/or forensic DNA profiling. Where appropriate, databases have been developed with Hae III and/or Pst I digested DNA from unrelated North American Blacks and Caucasoids individuals. In addition, for some loci, databases have been generated from 14 different ethnic groups (Balazs, 1993).

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In summary, the method proposed here for those probes detecting minisatellite regions is reliable, reproducible and considerably facilitates its utilization because of a number of advantages including simplicity, operational advantages, speed, safety, and a higher resolution.

Acknowledgements. The authors wish to thank Ms. Milagros Portuondo, Ms. Cristina Otamendi and Mr. Gerard Mullins for their collaboration.

Table 1. Probabilities of sharing one band in every region between random pairs of individuals (p), mean probability of two unrelated individuals sharing all fragments (X) and heterozygosities (H) detected by multilocus DIG-probes 33.15 and 33.6.

DNA fragments size (kb)	Fragments per individual ± s.d.	p	H
Probe 33.15			
zone I (20-10 kb)	4.1±1.4 (2.9±1.0)	0.162	0.919
zone II (10-6 kb)	4.8±1.3 (5.1±1.1)	0.178	0.911
zone III (6-4 kb)	7.9±1.3 (6.7±1.2)	0.205	0.897
Mean nº of bands 16.4 ± 2.9, $X = 5.7 \times 10^{-13}$			
Probe 33.6			
zone I (20-10 kb)	4.6±1.7 (2.8±1.0)	0.168	0.916
zone II (10-6 kb)	5.6±1.5 (5.1±1.3)	0.190	0.905
zone III (6 -4 kb)	9.3±1.3 (6.7±1.2)	0.206	0.897
Mean nº of bands: 19.6 ± 3.0, $X = 1.1 \times 10^{-14}$			

Figures in parenthesis give results by (2)

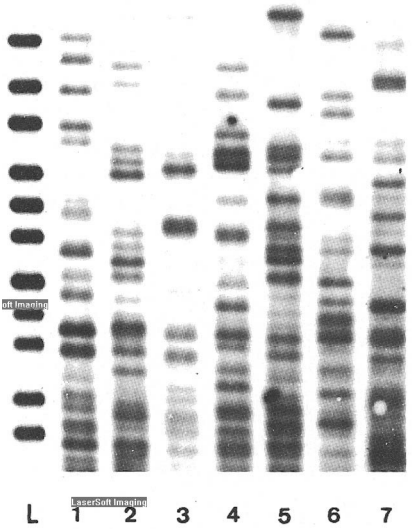

Figure 1.- Hybridization of the DIG-labeled probe 33.15 to Hinf I-digested genomic DNA samples of seven unrelated individuals (1-7) to the 4 kb cut off point. (L) ladder.

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analysis marker system) were treated in the same way as described above except that it was not denaturated and the hexanucleotide mixture was not added.
The membrane was prehybridized at 50 °C for 45 min in 0.5 M Na_2HPO_4 (pH 7.2) and 1% SDS. Hybridization was carried out at 62 °C overnight with 20 pg of denaturated DIG-labeled probe 33.15 or 33.6 and 0.1 µl of ladder DIG-labeled probe in 0.04 ml of hybridization solution/cm^2 of membrane. The hybridization solution used was the same composition as in the prehybridization but including a 10% v/v blocking reagent (casein in 0.1 M maleic acid buffer); after hybridization the solution was removed and stored at -20 °C to reuse. In order to remove the excess of the probe, the membrane was washed for 30 min in 0.2 M Na_2HPO_4 (pH 7.2) and 0.1% SDS at 62 °C.
The DIG Luminescent detection was carried out as in the Boehringer-Mannheim protocols with modifications: (1) the equilibration step was performed with 0.1 M maleic acid and 0.15 M NaCl pH 9.5; (2) the antibody incubation step was performed with 0.1 M maleic acid and 0.15 M NaCl pH 7.5 and without blocking reagent; (3) chemiluminescent detection was carried out with Lumiphos 530™ sprayed onto the membrane (0.01ml/cm^2); (4) an X-ray film was exposed to the membrane for 2-3 h at 37 °C using amplifying screens.
Statistical analysis of data was as described by Jeffreys et al. (2).

RESULTS and DISCUSSION

The labeling procedure does not require to remove the non-incorporated DIG-dUTP because it does not bind to membrane or DNA and does not create background. However, we have observed that the purification of the labeled probe with Sephadex G-50 column reduce the unspecific hybridization. This can be due to the elimination of the small DIG-fragments generated during the labeling procedure of these probes, formed by unions head-and-tail of the core sequences.
DNA fingerprints were obtained from a sample of 30 unrelated individuals from the Basque Country. The bands of every sample were analysed in the range within 20-4 kb (figure 1). The average number of bands per individual obtained with DIG-labeled probe 33.15 is 16.4 ± 2.87 in the 20-4 kb size range and probe 33.6 detecs 19.6 ± 3.03 bands in the same range (table 1). Previous data (2,3,4) show a lower average number of bands when probes 33.15 and 33.6 are radiolabeled. The comparison between the number of fragments resolved by DIG-probes and ^{32}P probes shows that DIG-probes detect more fragments in every region being the highest increase in the region 20-10 studied with the probe 33.15.
The bandsharing probabilities between two unrelated individuals are also lower than those for ^{32}P probes in every studied region and therefore the mean probability of sharing one band is also lower for both DIG-probes (0,187 for probe 33.15 and 0.192 for probe 33.6). The bandsharing distribution between two individuals fits a binomial distribution for both probes individually (X^2_{3df} = 0.830 for 33.15 and X^2_{3df} = 1.176 for 33.6, $P > 0.05$). Both probes show very high heterozygosities (table 1), which points out that they are extremely resolutive for identity purposes, since the mean probability of all bands matching between two unrelated individuals is 5.695×10^{-13} for probe 33.15 and 1.115×10^{-14} for probe 33.6.

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MULTILOCUS PROFILES OF THE BASQUE COUNTRY POPULATION WITH DIG-LABELLED PROBES 33.15 AND 33.6

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INTRODUCTION

Probes 33.15 and 33.6 are frequently used due to their high discrimination power. Usually methods for labeling these multilocus probes are based on radioactive isotopes. The main disadvantages of this labeling-technique are the short half-life of the isotopes, the requirement of safety facilities, and the manipulation risk. For these reasons, probes labeled with molecules such as biotin or digoxigenin (DIG) have emerged as practical alternatives to radiolabeled probes (1).

We have developed a method to obtain DNA fingerprints with multilocus probes 33.15 and 33.6 employing the combination of non-isotopic labeling techniques by enzimatic incorporation of DIG-11-dUTP and chemiluminescent developing. These so labeled probes were also applied to a population sample of 30 unrelated individuals from the Basque Country in order to compare the discriminative power with ^{32}P labeled probes.

MATERIALS and METHODS

DNA from individuals were extracted by the phenol-chloroform method. Human DNA (8 µg) was digested with 30 units of Hinf I and the fragments were separated in 0.7% agarose gels with TBE 1x buffer at 3 V/cm until the 2.3 kb λ Hind III fragment migrated 18 cm. Also, in order to achieve accurate DNA fragments sizing, a ladder was loaded within each gel. Fragments were transferred to nylon membrane (Boehringer-Mannheim) using Southern's method.

Probes 33.15 or 33.6 (DNA inserts) were labeled as follows: 8 ng of probe made up to 20 µl with H_2O-MilliQ and denaturated, 4 µl of 10x Hexanucleotide mixture, 4µl of dNTPs (containing the Boehringer-Mannheim DIG-labeled dUTP), 4 µl of 2M Hepes buffer pH 6.6, 4 µl of labeling buffer (0.05M $MgCl_2$, 1M Tris-HCl pH 8.0) and 4 µl of 2U/µl Klenow enzyme were mixed and incubated at 37 °C o/n. The probe was precipitated with 4 µl of 8M LiCl and 110 µl of prechilled (-20 °C) ethanol, and left at -80 °C for 1 h. After centrifugation for 20 min at 15000 g, the pellet was washed with 110 µl of prechilled 70% ethanol, centrifugated for 5 min at 13000 g and dissolved in 40 µl of TE buffer o/n at room temperature. The labeled probe was further purified using a Sephadex G-50 column. The eluate was collected in Eppendorf tubes (3 drops in each one). Five µl of each eluted fraction were removed to prepare a slot-blot. Those fractions showing the maximum intensity (which contain the purified probe) by means of the DIG-luminescent detection described below, were mixed. The purified probe may be used directly for hybridization or stored at -20°C.

In order to label the ladder probe, 22 µl of 0.4 µM DNA marker probe (BRL DNA

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the DNA fragments appeared, in most cases, distorted and diffuse on the silver stained gels. The same results were obtained even when a stacking gel was used (data not shown). It was also observed in these experiments that the glycine-chloride moving boundary formed at the interface of the upper buffer and the top of the gel was distorted when it passes through the sample zone.
By simply filling the sample wells with a cap gel containing the leading ion and underlayering the samples into the well bottoms, the moving boundary was prevented from distortion while passing through the sample zone. This resulted in sharp DNA fragments in the silver stained gels (Fig. 1).

Resolution and reproducibility

As can be seen in Figure 1 the resolution obtained with this electrophoretic system (using a separation distance of only 12-13 cm) could clearly distinguish VNTR alleles ranging in size from 400 to 900 bp and differing by only 16 bp (D1S80) or 15 bp (ApoB) as well as STR alleles of aproximately 200 bp and differing by only 4 bp (HUMTH01) or 3 bp (HUMFABP).
Maximal resolution was achieved when the electrophoresis was carried out at constant current (30-45 mA) until the moving boundary had migrated 1 cm from the bottom of the sample wells and then at constant voltage (50-75 V) overnight.
A comparison of the relative migration of the D1S80 alleles separated at constant current (30 mA) or at constant voltage (55 V) is shown in Figure 2.

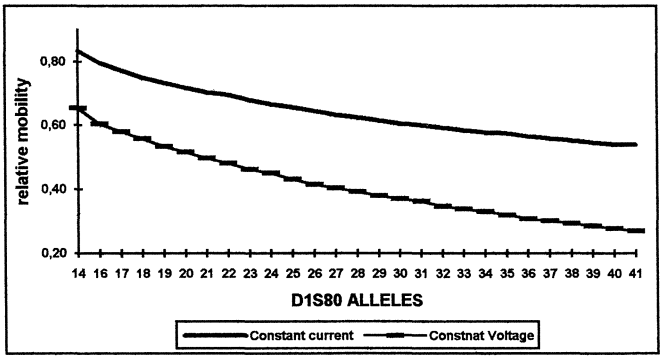

Figure 2. Relative migration vs size of the D1S80 alleles separated at constant current (30 mA) or at constant voltage (55 V).

On the other hand, this vertical electrophoretic system provides a high inter-gel reproducibility and is free from band smiling or waving across the gel. This make very easy the classification of the different alleles by comparison with the control ladders.

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vertical electrophoresis unit at constant current (30 mA) during the first hour and then at constant voltage (55 V) until the bromophenol blue reaches the bottom of the gel. The electrode buffer was 0.025 M Tris-glycine, pH 8.8. After electrophoresis the DNA fragments were silver stained [8]. The composition of the cap gel and the separating gel depending of the marker analyzed were as follows:

	D1S80	ApoB	HUMTH01 and HUMFABP
CAP GEL	7% T, 3% C	6% T, 3% C	8% T, 3% C
SEPARATING GEL	7.5% T, 3%C	6% T, 3% C	11% T, 3% C

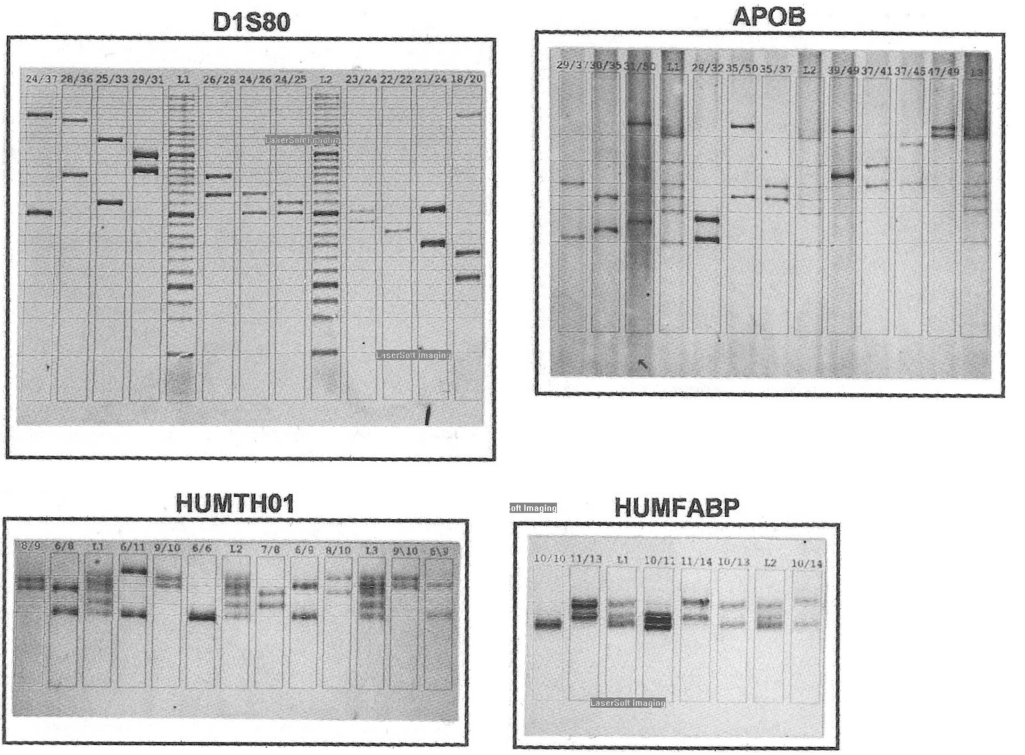

Figure 1. Analysis of two VNTR loci (D1S80 and ApoB) and two STR loci (HUMTH01 and HUMFABP) by vertical discontinuous polyacrylamide gel electrophoresis using the Tris-glycine/Tris-Chloride buffer system.

RESULTS AND DISCUSSION

The cap gel: a simple strategy to assure the initial sharpening of DNA fragments.

The first attempts to separate DNA fragments by vertical discontinuous polyacrylamide gel electrophoresis using the Tris-glycine/Tris-Chloride buffer system were carried out in our laboratory by using homogeneous polyacrylamide gels without stacking gel and loading the samples into the wells filled with the gel buffer (0.375 M Tris-Chloride). Under these conditions

EVALUATION OF THE TRIS-GLYCINE/TRIS-CHLORIDE DISCONTINUOUS BUFFER SYSTEM FOR THE ELECTROPHORETIC ANALYSIS OF VNTR AND STR LOCI.

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INTRODUCTION

The analysis of PCR-amplified products by discontinuous polyacrylamide gel electrophoresis followed by silver stain provides high resolution and increased sensitivity when compared with continuous zone electrophoresis in agarose or polyacrylamide gel and ethidium bromide staining [1]. A simple and high resolution, horizontal discontinuous (Tris-borate/Tris-sulfate buffer system) polyacrylamide gel electrophoresis method has been described for AMP-FLP analysis of VNTR loci [2]. More recently, it has been shown that the use of a stacking gel, a wide pH difference between the gel and running buffer, and a low cross-linking agent concentration in vertical discontinuous polyacrylamide gel electrophoresis yields good resolution and sharp DNA fragments in the AMP-FLP analysis [3].
In this article we present a vertical discontinuous polyacrylamide gel electrophoresis method based on the Tris-glycine/Tris-chloride buffer system [4, 5]. The initial zone sharpening of the DNA fragments produced by the glycine-chloride moving boundary is assured by simply using a cap gel above the sample rather than using a stacking gel and an operational shift in the pH. The resolution and reproducibility of this electrophoretic method in the separation of AMP-FLP alleles of two VNTR loci (D1S80 and ApoB) and two STR loci (HUMTH01 and HUMFABP) is evaluated.

MATERIALS AND METHODS

Sample preparation

DNA was extracted from EDTA blood samples by the standard phenol-chloroform extraction procedure.

Amplification

PCR amplification was performed for two VNTR loci (D1S80 and ApoB) and two STR loci (HUMTH01 and HUMFAPB). The primer sequences and PCR time-temperature profiles were as previously described [2, 6, 7].

Electrophoresis

Vertical polyacrylamide gels (16-24 X 14 X 0.075 cm) were prepared by mixing the required amounts of acrylamide and piperazine diacrylamide as cross-linker in a solution containing 0.375 M Tris-Chloride, pH 8.8. A 20 teeth comb (25 mm deep) was used to form the sample wells. After polymerization, wells were washed with 0.375 M Tris-Chloride and filled with the cap gel (0.04% amonium persulfate). The samples (5µl of PCR product mixed with 1 µl of 80% sucrose, 0.2% bromophenol blue in 0.375 M Tris-Chloride) were immediately loaded with a microsyringe into the well bottoms. Electrophoresis was carried out in a HOEFER SE600

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Fig.1.- Different mobilities of pBR322/MspI (——) and the ACTBP2 heterozygote 16-19 (---)

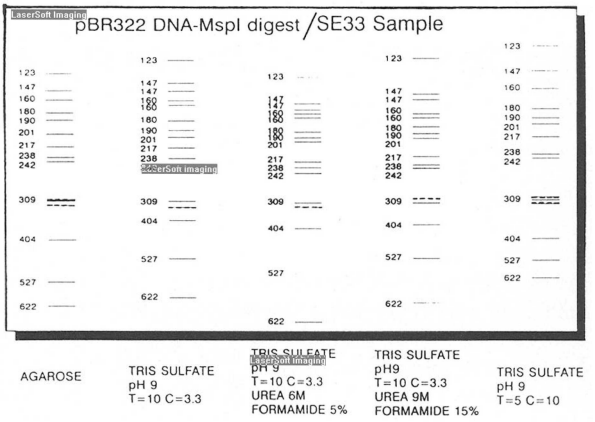
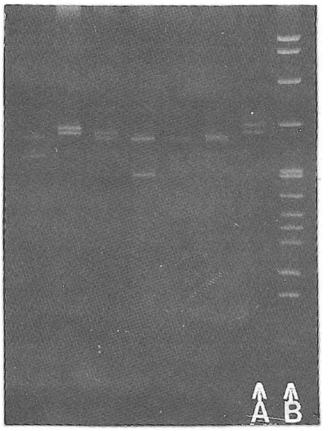


Fig.2.-
Metaphor 4.5%
A.- ACTBP2 control 16-19
B.- pBR322/MspI digest



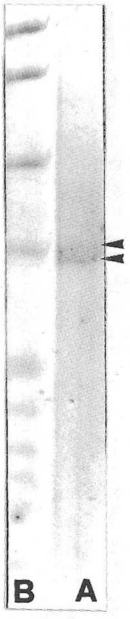


Fig.3

Fig.4

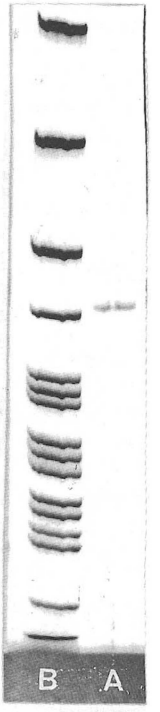


Fig.5

Fig.3.- PAGE T:5 C:10. A.- ACTBP2 control 16-19. B.- pBR322/MspI
Fig.4.- PAGE T:10 C:3.3. A.- ACTBP2 control 16-19. B.- pBR322/MspI
Fig.5.- PAGE T:10 C:3.3+Urea 6M+Formamide 5%. A.- ACTBP2 control 16-19. B.- pBR322/MspI

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(Fig.3) and a pseudohomozy gote in T:10 C:3.3 gels (Fig.4) even when different denaturing conditions were used (Fig.5).
The pBR 322/MspI digest ladder shows different patterns at different T and C values, and has an electrophoretic behaviour in polyacrylamide at high C values similar to that observed in agarose gels. The use of denaturing conditions has only a minimal influence on the mobility of the pBR 322/MspI ladder. Using as reference the normal electrophoretic behaviour of the 123 bp repeat ladder, at least 5 regions of the pBR 322/MspI digest ladder are responsible for that electrophoretic behaviour.
As much as for SE33 fragments as for the pBR322/MspI ladder, different mobilities can be observed for the same DNA fragment in gels with different compositions having the same effective median pore radious. Therefore absolute mobility does not correlate with gel pore size unless the physical properties and composition of the gel are held constant.
From the forensic point of view the more interesting finding was the anomalous migration in the SE33 fragments which is probably due to differences in DNA gel interations which are affected by variation in gel compositions and by a DNA bending phenomenum (to which AT rich sequences are more suceptibles). Anomalous migration in AT rich DNA sequences has been reported for AT rich VNTRs and other DNA sequences (Marini et al. 1982, Ulanovsky et al. 1986).
These results are in complete agreement with the last EDNAP results and support the conclusions of the EDNAP report.
We believed that the use of AT rich STRs showing anomalous electrophoretic behaviour should be avoided, but if they are to be used, common electrophoretic systems should be employed.
Each STR should be tested in order to investigate the possible electrophoretic problems before validation.

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Table 1.- Electrophoretic conditions.

PAGE.-

* TRIS-Sulfate pH 9.0 (gel),
* TRIS-Borate pH 8.5 + AGE 2% (strips)
* 600 V, 15°C, 3 hours. Silver staining.

T/C	Pore radii	Denaturing conditions
T:5 C:3.3	99 nm	T:5 C:3.3 + UREA 9M
T:8 C:3.3	80 nm	T:10 C:3.3 + UREA 9M + FORMAMIDE 5%
T:10 C:3.3	70 nm	T:10 C:3.3 + UREA 6M + FORMAMIDE 15%
T:5 C:10	27 nm	T:10 C:3.3 + UREA 6M + FORMAMIDE 15%
		T:10 C:3.3 + UREA 9M + FORMAMIDE 15%

AGE.-

* TAE pH 7.8/ 80 V, 5 hours./ EtBr staining
* METAPHOR 4.5%. Pore radii.- 62 nm.

ANOMALOUS ELECTROPHORETIC BEHAVIOUR OF HUMACTBP2 (SE33)

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INTRODUCTION

The microsatellite tetrameric tandem repeat sequence in the 5'flanking region of the human ACTBP2 (SE33) locus (Polymeropoulos et al. 1982) was one of the first STRs evaluated for forensic applications. Because it is highly polimorphic, population studies in caucasians and forensic validation studies have recently been carried out (Polymeropoulos et al. 1992, Weigand et al. 1993).
The sequence variability of this tetranucleotide repeat has recently been reported (Urquhart et al. In Press) and it has been found that it is an AT rich sequence, with at least 30 alleles, some of which may vary by as litle as 1 base.
In the course of the last EDNAP exercise (Gill et al. submitted for publication) anomalous electrophoretic mobility was observed in some samples which showed different SE33 patterns in agarose and polyacrylamide. Because of this, a study of the electrophoretic mobility in SE33 fragments was carried out using agarose and polyacrylamide at various T and C values- -in some of which denaturing conditions were employed.

MATERIAL AND METHODS

The primers and amplification conditions for HUMACTBP2 were made as described by Polymeropoulos et al. (1982). The sizes of the ACTBP2 control alleles, determined by sequencing, ranged from 234 to 318 bp.
The electrophoretic conditions are shown in table I. As for PAGE, various T and C values were used including the additions of different concentrations of denaturing agents.
Horizontal discontinous polyacrylamide electrophoresis was performed using 33mM TRIS-Sulfate pH 9.0 as a gel buffer and 0.52M TRIS-Borate pH 8.5 as an electrode buffer in 2% agarose strips. The gels were subsequently silver stained.
The agarose gel consisted of 4.5% Metaphor in TAE, and were run in TAE at 80 V for 5 hours. ACTBP2 fragments were made visible using EtBr.
Msp I digest of pBR322 and a conventional 123 bp were used as ladders.
Polyacrylamide and agarose gel pore sizes were calculated from Ferguson plots of the ladders as described by Holmes et al.(1991).

RESULTS AND DISCUSSION

Fig.1 shows the different mobilities of PBR322/MspI and the ACTBP2 heterozygote (16-19) used as the control. This ACTBP2 sample was a heterozygote in agarose (Fig.2) and T:5 C:10 gels

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Table 1

PROBE	REFERENCE	POSITIVE CLONES
33.15	(10)	#2.25 38 (A); #2.2 89 (B)
33.6	(10)	#2.18 27 (C;A); #2.23 70 (C;C); #2.2 19 (B)
g3	(2)	#2.2 78 (C)
MS1	(2)	none
MS43	(2)	none
MS8	(2)	none
3'HVR	(9)	none
pMLJ14	(3)	none
M13	(8)	none

The letters in brackets refer to the pattern detected by the probe (see text for more details).

4. DISCUSSION

A Y specific cosmid library was screened with nine highly polymorphic probes. All positive cosmids gave invariably a monomorphic, although different, pattern. The screening of whole genome library failed in finding polymorphic minisatellites (12), possibly because the Y chromosome is only 1 % of the human genome. We were expecting that the screening of a selected Y chromosome library could lead to the identification of some polymorphic sequence of the chromosome but none of the 800 ordered array cosmid detected a polymorphism. This result can be explained by the absence of recombination events in this portion of the chromosome. Furthermore our results do not support the hypothesis (13) that others mechanisms as insertion, deletion, sister chromatid exchange, play a major role in generating new VNTR alleles, at least for this chromosome.

In conclusion, this work provides further evidence that the degree of polymorphism of the Y chromosome minisatellite sequences is very low or null.

ACKNOWLEDGMENTS

We thank J. Wolfe and A.J. Jeffreys for providing us with the cosmid library and some of the minisatellite probes.

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fragments were recovered from the gel, subcloned in Bluescript and then used to probe human DNA.

3. RESULTS

We identified six positive clones (Tab. 1): three clones were positive for probe 33.6, two clones were positive for probe 33.15 and one clone was detected by g3. The six positive cosmids were hybridized to male and female human DNA and cosmids #2.25 38, #2.2 89 and #2.2 78 gave specific monomorphic pattern. The Y chromosome specific bands were obtained when the male DNA was digested with HaeIII, Sau3AI (Fig. 1) but not when digesting with Hinf I. The clones #2.18 27, #2.23 70 and #2.2 19 gave a "smear" and were digested and screened with 33.15, 33.6, g3 and MS1. The fragments obtained from each of the cosmid insert proved to be positive to more than one of the minisatellite probes (fig.2). Most probably the cosmid inserts were composed of different families of repeated sequences. Five of the fragments obtained from the cosmid inserts were used to probe male and female DNA.

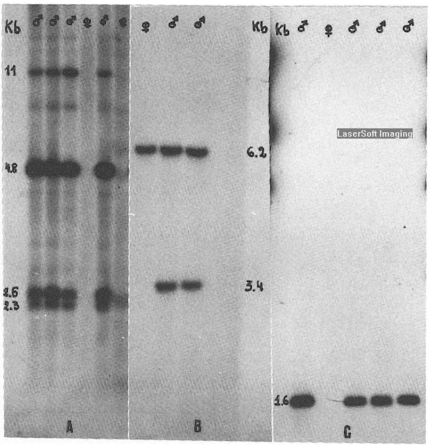

Fig.1 Y-specific monomorphisms detected by #2.25 38/Sau3AI (A), #2.2 89/HaeIII(B) and #2.2 78/HaeIII (C) digests.

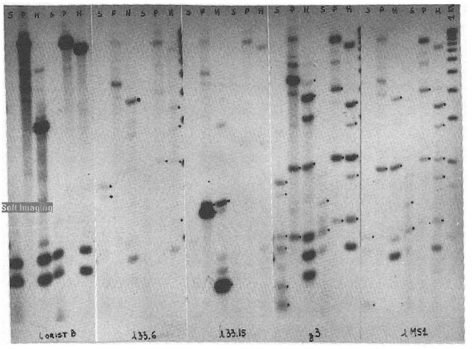

Fig. 2 Cosmids #2.23 70 and #2.2 19 digested by Sau3AI (S), PvuII (P) and HindIII (H) and hybridized with the probes shown below. Marked bands are minisatellite specific fragments.

The three positive cosmids and the 5 bands subcloned from the other three cosmids gave the following patterns:

A) - chromosome Y specific multi-band pattern: clone #2.25 38 and the 1.2 kb Pvu II fragment of clone #2.18 27

B) - chromosome Y specific single band plus one or more band in common with the X chromosome: clone 2.2 89 and the 2.1 kb Hind III fragment of clone #2.2 19.

C) - chromosome Y specific single band: clone #2.2 78; the 1.4 kb Hind III fragment of #2.18 27 and two Pvu II fragments (1.3 and 1.1 kb) of #2.23 70.

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MINISATELLITES OF THE Y CHROMOSOME

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1. INTRODUCTION

In recent years, the use of VNTR polymorphisms has became one of the most successful changes in the field of forensic medicine for personal identification and paternity testing. VNTR sequences have been identified on all human chromosomes and usually they map at or close to the telomers (1). VNTR have been usually identified by screening human DNA cosmid or charomid libraries by multi locus probes, single locus probes at very low stringency conditions or by synthetic oligonucleotides (2,3,4).

The human Y chromosome, by contrast, shows a very low degree of polymorphism (5) with the exception of the pseudoautosomal region (4,6). VNTRs of this region show the same allelic pattern of autosomal VNTRs and are not linked to a specific sex. On the contrary, a Y-specific marker would be of great practical use for cases of sexual assaults or paternity testing when the alleged father is not available but male relatives are available.

Here we report the results of the screening of a Y-chromosome library and the identification of some minisatellite sequences of the Y chromosome.

2. MATERIALS AND METHODS

2.1 Library screening. About 800 ordered array cosmids, carrying most of the euchromatic region of the human Y chromosome (7), were replicated with a "hedgehog" onto Hybond-N filters and hybridized with the following probes: 33.15 (10), 33.6 (10), g3 (2), MS1 (2), MS43 (2), MS8 (2), 3'HVR (9), pMLJ14 (3), M13 (8). Hybridizations were carried out at 58°C in 7% SDS, 0,5 M phosphate buffer pH 7,2, 1 mM EDTA and 2 ug/ml sonicated and denatured E. coli CB767 DNA as competitor. The filters were washed at 58°C in 1xSSC and 1% SDS and exposed to X-Omat AR films.

2.2 Characterizing Y-specific patterns. The P32 labeled positive clones were used to probe Southern blots of four male and two female DNAs digested by following restriction enzymes: Hinf I, Bam HI, Eco RI, Mbo I, Sau3AI, Hind III, Pvu II, Hae III, Msp I, Alu I, Rsa I and Pst I. In this case the hybridizations and washes were carried out under high stringency conditions (2) without any competitor DNA.

2.3 Identification of minisatellite specific fragments.

Some positive clones, probed on human DNA, gave a "smear" and were subcloned. Each of these clones was digested with three restriction enzymes (Sau3AI, HindIII and PvuII), electrophorezed through a 1% agarose gel in TBE buffer, Southern blotted and hybridized with probes 33.15, 33.6, g3 and MS1. The cloning vector Lorist B (11) was used as negative control. The hybridization conditions were the same adopted for the screening of the library. Some of the minisatellite

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of the gel. Individuals can therefore easily be separated by MVR-mapping.

The photo was primarily presented to demonstrate that the MVR-typing can be achieved nonradioactively. We are now about to do population genetic studies by nonradioactive MVR-mapping. The MVR will be performed on unrelated persons, to determine the variability of MVR patterns within a population. Additionally related persons will be screened, so that the power of the method, e. g. for paternity tests, can be estimated.

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for 9 h at 80 V and 22 h at 100 V. The gel was denatured in NaOH 0.5 M/NaCl 1.5 M for 30' and passively southern blotted onto a nylon membrane (Oncor) over night. After UV cross-link (0.257 J/cm^2), the membrane was blocked (blocking buffer: 0.5 M Na_2HPO_4; 1% sodium dodecyl sulfate; 1% casein) for 1 h at 50°C and hybridized with 500 pmol digoxigenated MS32 probe in blocking buffer at 50°C over night. By use of an anti-digoxigenin antibody coupled to alkaline phosphatase, a nonradioactive immunological detection was performed according to Boehringer's manual for lumigen or colour development.

RESULTS AND DISCUSSION

We altered Jeffreys MVR-PCR protocol from 1991 slightly and used a digoxigenated MS32 probe (DIG-MS32) to circumvent the radioactivity. The immunological detection of the DIG-MS32 was achieved by an anti-digoxigenin antibody coupled to an alkaline phosphatase (AP). The AP catalysed a chemiluminescent reaction or colour development using lumigen or NBT/BCIP respectively. Ternary MVR codes were produced using genomic DNA for the MVR-PCR.

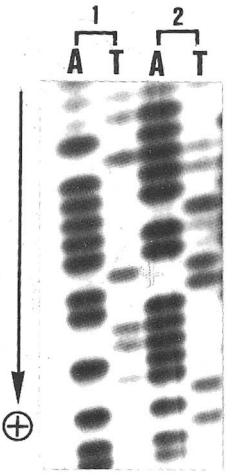

Fig. 1: Nonradioactive MVR of 2 different individuals. A: A-type MVR; T: T-type MVR.
Lanes (1): A- and T-type MVR of individual 1.
Lanes (2): A- and T-type MVR of individual 2.

The photo shows a small segment of a MVR-typing gel, after nonradioactive detection. The A- and T-type MVR products were electrophoresed adjacent to each other for each person. A ternary code for the individuals was determined as follows:

The homozygous A-type MVR was coded 1, the homozygous T-type MVR was coded 2 and the heterozygous A- plus T-type MVR was coded 3. So this small segment reads (anode to cathode):

Lanes 1: 11212 21221 12111 11213 33
Lanes 2: 11212 11111 22332 13311 33

Even though the two individuals show anodally many similarities, they exhibit a highly polymorphic pattern in the cathodal region

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NONRADIOACTIVE APPROACH TO TYPE DNA BY MINISATELLITE VARIANT REPEATS

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INTRODUCTION

A nonradioactive approach was established to type DNA by minisatellite variant repeats (MVR). Sequence variation in the repeat units of minisatellites were described by Jeffreys et al. 1990. The human minisatellite clone MS32 detects the hypervariable locus D1S8. The core sequence of this minisatellite is a 29 bp repeat unit. Approximately half of the repeat units share an A to G transition that creates a *Hae*III cleavage site (*Hae*III'units). 1991 Jeffreys et al. developed a simple MVR mapping system amenable to any length of minisatellite.

Two different MVR specific primers (TAG-A and TAG-T) primed off the *Hae*III' (A-type) or (T-type) units. Amplification using one of these primers together with a fixed primer (32D) flanking the minisatellite at the 5'end generated two complementary sets of PCR products from the ultravariable end of any MS32 allele. To prevent a shortening of the PCR products, an artificial sequence ('TAG') was added to the allele specific primers. The fixed primer for the 5'end and the artificial TAG site flanked the different repeat units and could amplify them whithout internal priming that would shorten the PCR products.

When genomic DNA was used, Jeffreys generated ternary MVR maps by hybridysation of southern blots with a radioactively labeled MS32 probe. Here we describe a nonradioactive approach of the MVR technique. We exemplarily show MVR patterns of 2 different individuals.

MATERIALS AND METHODS

DNA was extracted from peripheral blood leucocytes according to Miller et. al. (1988). The MVR-PCR mixture contained in 100 µl: 2 µg DNA; 10 pmol each of 32D and TAG; 0.01 pmol of TAG-A or 0.02 pmol TAG-T (sequences of 32D, TAG and TAG-T/TAG-T were according to Jeffreys et al., 1991); 200 µM each of dATP, dCTP, dGTP, dTTP; $MgCl_2$ 1.5 mM; KCl 50 mM; Tris-HCl 10 mM; Triton X-100 0.1%; and were layered with 60 µl mineral oil. DNA was denatured at 94°C-5' ; 2.5 U *Taq* Polymerase were added, 25 cycles of amplification at: 94°C-90''; 68°C-60''; 70°C-5' in a thermocycler (Crocodile II, Appligene); were followed by 2 cycles at: 67°C-1'; 70°C-10'. MVR-PCR products were electrophoresed through a 35 cm long 1% agarose (BRL) gel in TBE (Tris-borate 89 mM; EDTA 2 mM)

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2. DNA fractionation.

Gel electrophoresis		16.0

Day 2.

3. DNA transfer.

Denaturation	0.25	
Southern Transfer	0.5	
Drying	0.1	
	Sub-total:	0.85

4. DNA hybridization to AP-probes.

Hybridization	0.25	
Washing	1.0	
Exposure at 37 °C	3.0	
	Sub-total:	4.25
	TOTAL:	~ 28 hrs

The main objective of this manuscript was to summarize our efforts to shorten and simplify the procedures required to produce DNA profiles for paternity cases using VNTR loci. The procedure outlined above uses several simple changes to the general methods used for RFLP analysis that shortens the time required to complete routine paternity cases to less than 2 days.

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DNA transfer. DNA was transferred to a nylon membrane (Pall Biodyne B or MSI Magna) by capillary transfer (30 min). The nylon membranes were dried in an oven at 80 °C for 1 hr or for 2 to 3 min in a microwave oven.

DNA hybridization. Hybridization (15 to 20 min) was done using alkaline phosphatase labeled probes (Lifecodes Corp). Membranes were washed and incubated with Lumi-Phos™ 480 (Lumigen, Inc) as substrate. The light emission was detected with X-ray film by exposing the membranes for 2 to 3 hrs at 37 °C or overnight at room temperature.

Results and Discussion.

The process of DNA typing VNTR loci can be divided into the following four major sections: isolation and digestion of DNA, fractionation by electrophoresis, transfer to membrane and detection of alleles. The procedure routinely used in our laboratory for the isolation and digestion of DNA has been described by Grimberg et al. (1). We have also examined the use of a chaotropic agent, such as guanidinium thiocyanate, for DNA isolation. Both procedures produce DNA suitable for digestion with restriction enzyme. The method using the chaotropic agent required less time to complete than the Proteinase K method. However, the DNA recovered was not as pure as with Proteinase K and the DNA fragments migrated somewhat slower than highly purified DNA indicating that the procedure may need additional changes. Fractionation of DNA fragments was done overnight (16 to 18 hrs). DNA from the thin gels were transferred to the nylon membrane over which the gel had been casted. Both neutral and positively charged membranes have been used successfully. The only difference in the procedure was that for the amphoteric membrane the gel was soaked in neutralizing solution prior to transfer, while for the charged membrane the transfer was done with alkaline solution. A comparison of various transfer times showed transfer was complete in approximately 30 minutes. Using 6 mm thick gels the equivalent transfer procedure required 3 hours. After transfer the membrane was quickly dried for a 2 to 3 minutes in a microwave oven. The non-isotopic method used for the detection of the polymorphic loci was based on the use of chemiluminescence. The initial procedure developed in our lab (3,4) has been further simplified and the quality of the results improved by the elimination of several steps and changes in formulation. A flow chart summarizing the steps used in this process and the duration of each step is summarized in Table 1.

Table 1. Procedure for DNA profiling with VNTR loci.

Day 1.	**Time (hr)**
1. DNA Isolation and digestion with restriction enzyme.	
Cell Lysis	0.5
Nuclear lysis	2.0
DNA quantitation (yield gel)	1.0
Restriction enzyme digestion	2.0
Salting out	0.25
DNA precipitation	0.5
Sub-total:	6.25

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STREAMLINING VNTR ANALYSIS. A FAST PROCEDURE FOR NON ISOTOPIC DNA PROFILING.

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Introduction.

Several modifications to the general procedure used for RFLP analysis of VNTR loci have been developed through the years. These modifications include the use of simple protocols for DNA isolation, fast capillary transfer of DNA, fast hybridization procedures and the use of non-isotopic detection. We have selected or developed a combination of methods that have resulted in a significant increase in speed and efficiency of VNTR based DNA profiling. The protocol described below allows the laboratory to obtain consistent results within a period of 2 days or less.

Materials and Methods.

DNA isolation. DNA was prepared from blood samples by Proteinase K treatment and selective precipitations (non-organic procedure) as described by Grimberg et al., or using guanidinium salts and chloroform (non-phenol extraction method) by a modification of the method of Chomczynski and Sacchi (2). The general process consisted of: washing the blood cells (0.5 to 1 ml of blood) with buffer containing Triton X-100 and collecting nuclei by centrifugation.

In the non-organic procedure, nuclei were lysed by treatment with Proteinase K (1). The amount of DNA in each sample was estimated with a yield gel by comparing the fluorescence of the sample against known amounts of DNA. Approximately 3 μg of DNA was digested with 20 fold excess of the restriction enzyme PstI or HaeIII. Following digestion, residual proteins or peptides were removed with LiCl. DNA was precipitated with 2 volumes of ethanol, washed with 70% ethanol and dissolved after drying in 10 μl of H_2O.

In the non-phenol extraction method, nuclei were lysed by addition of guanidinium thiocyanate (2) and extraction with chloroform. DNA was precipitated with a half volume of isopropanol, washed with ethanol and dissolved after drying in 50 μl of H_2O.

DNA quantitation. The amount of DNA in each sample was estimated with a yield gel. Approximately 3 μg of DNA was digested with restriction enzyme. DNA (0.5 to 1 ug) was fractionated by electrophoresis in trays (12 x 21.5 cm or 12 x 28 cm) containing a 2 mm thick, 1.0% or 0.8% IDna agarose gel (FMC Bioproducts) casted directly on to a nylon membrane.

Fractionation of DNA. Electrophoresis was done in an horizontal apparatus for 16 to 18 hrs. After electrophoresis, the membrane/gel combination was soaked (15 min) in an alkaline solution to denature the DNA.

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The basepair difference between alleles 5 and 6 is 3 basepairs and there is only 1 basepair between alleles 6 and 7.
The ACTBP2 system is more complex and was more complicated to analyse due to the alleles of intermediate sizes. The difference between two close alleles can be as small as 1 basepair.
After having classified the alleles according to their basepair lengths, they were given allele numbers. We have found 35 alleles including the intermediates distributed all over the size range. The alleles range from 223 to 316 bp containing an AAAG repeat unit (fig. 2). For ACTBP2 the most common allele was number 17 (289 bp) with a frequency of 0.086.
No mutations were found for the loci after having analysed mother–child combinations. All markers were in genetic equilibrium according to Hardy Weinberg. The combined exclusion capacity is 0.98 for these loci.

Table 2.

LOCUS	NO. OF SAMPLES	EXCL. CAPACITY
D1S80	455	0.62
ACTBP2	302	0.88
HUMTH01	249	0.54
		0.98

CONCLUSION

The alleles of the D1S80 and HUMTH01 loci are distinct and are readily identified as number of repeats and as allele numbers. For D1S80 the allele frequencies are similar to those found in a Finnish and a North American population (5). For ACTBP2 the identification of alleles is difficult due to the close intermediate allele sizes. Under the present conditions we have had some difficulties with the reproducibility for these alleles. This problem may be solved by using internal size markers of accurate sizes close to the ACTBP2 size range.

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respectively. This mixture was added into one lane of a 4 % hydrolink gel. In this way 36 samples and 4 external allelic ladders for each marker could be analysed in one run (fig. 1). The gel was used two or three times. The total running time was 4.5 hours at 1500 V. The fragment sizes were automatically determined by the software in basepairs or in number of repeats.

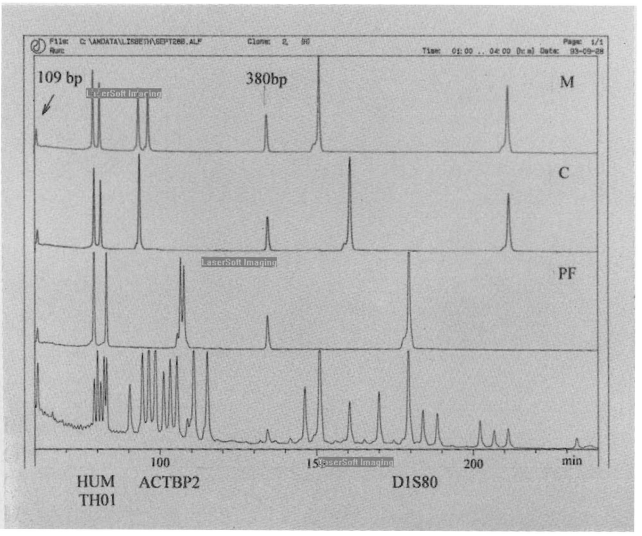

Fig. 1. Simultaneously analysed PCR fragments from mother (M), child (C) and putative father (PF) for HUMTH01 (79–83 min), ACTBP2 (90–115 min) and D1S80 (145–234 min.). Two internal size markers at 109 bp and 380 bp are marked with arrows.

RESULTS

For D1S80 21 alleles were found ranging from approximately 400 to 765 bp. The core sequence is 16 bp and the alleles were named after their number of repeats. The alleles number 18 and 24 are the most common ones with frequency values of 0.25 and 0.37 respectively (fig. 2).

HUMTH01 contained 8 alleles named in allele numbers 1–8. This locus ranged from 178 to 202 bp and the repetitive unit is AATG. Allele number 5 was the most common one with a frequency of 0.35 (fig. 2).

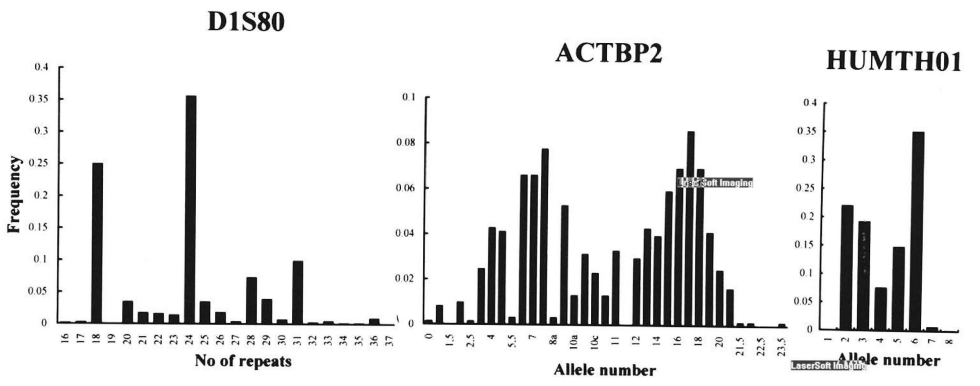

Fig. 2. Frequency diagrams for D1S80, ACTBP2 and HUMTH01.

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Simultaneous analysis of STR and VNTR polymorphisms.

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One Variable Number of Tandem Repeat (VNTR) polymorphism, D1S80(1), and two Short Tandem Repeat (STR) systems, ACTBP2 (2,3) and HUMTH01 (4) were simultaneously analysed on an Automatic Laser Fluorescent (A.L.F.) DNA Sequencer.
Allelic ladders were used as external standards and two internal size markers were added into each lane. For D1S80 and HUMTH01 21 and 8 distinct alleles were found respectively. ACTBP2 contained 35 alleles including alleles with intermediate sizes. These intermediate alleles are distributed all over the size range. The difference between two alleles can be as small as 1 basepair. Frequencies were established for the three loci and no mutations were found by analysing mother-child combinations. All three loci are in genetic equilibrium and give a combined exclusion capacity of 0.98.

MATERIALS AND METHODS

Amplification

DNA from randomly selected blood donors was amplified separately for D1S80, ACTBP2 and HUMTH01 (tab. 2). Additionally DNA from 212 mother-child combinations was amplified for all the systems.
For all amplifications the 50 ul reaction volume contained 0.5 uM of each primer, of which one was labelled with fluorescein, 200 uM of each dNTP and 1.5 uM of $MgCl_2$. For the D1S80 locus 2U of Taq poymerase was added and for the two STR loci 1U each. Amplification conditions are presented in table 1.

Table 1.

Locus	Temperatures (°C)	Times (sec.)	No of cycles
D1S80	94, 66, 70	42, 42, 470	25
ACTBP2	94, 60, 72 *	15, 30, 1+1+1.....	30
HUMTH01	94, 61, 72	45, 30, 1+1+1.....	34

* Prior to the original program a hot start at 94^0 C for 15 sec. was used.

A.L.F. analysis.

The amplified products were analysed on an Automatic Laser Fluorescent, A.L.F. DNA sequencer (Pharmacia LKB). Up to 1ul from the three different amplification products were mixed with approximately 5 fmol of two internal sizemarkers, 109 bp and 380 bp

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mutations; it is readily automated in a way that enables many samples and loci to be analysed simultaneously; powerful statistical methods can be used to assign confidence limits to the data.

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oligonucleotides over the region of the sequence difference. Such arrays could be used in forensic applications by analysing sequences where there are may base variants over a short distance, such as the mitochondrial D–loop or the HLA region. We are using these arrays to develop methods for analysing disease genes which show a high frequency of different mutation; for example the more than 300 known mutations causing cystic fibrosis.

A third types of array provides a tool that can be used to fingerprint any nucleic acid, whether its sequences is known or not. Oligonucleotides of a given length are made as a complete set on the surface of the array [Southern, Maskos and Elder, 1992].

Ideally, the length of oligonucleotides should be octamers or longer, to achieve reasonable yields of hybridisation at room temperature. However, a complete set of octanucleotides is 65,536 in number. Smaller sets can provide a great deal of information. We have made arrays of the type $N_3X_2N_3$ where N represents a defined base and X a mixture of all four. This set contains all 65,536 octanucleotides sequences but the size of the array is reduced to 4096 cells [Bains and Smith, 1988; Bains, 1991]. An array that could be particularly appropriate for analysing individual "fingerprints" comprises all sequences of the formula N_3CGN_3. CG is a rare doublet in mammalian DNA, but is known to have a high rate of mutation to TG. The reason for this is that the C in CG is often methylated and 5-methyl cytosine deaminates to T, which is not recognised as an abnormal base by the repair systems. The "general" arrays analyse every constituent oligonucleotide of the target sequence, whereas arrays of the type N_3CGN_3 will "see" only those oligonucleotides conforming to this pattern. However, this will permit longer target sequences to be analysed.

After hybridisation with a radioactive or fluorescently tagged target sequence, an image is collected, using the PhosphorImager for radioactivity or a CCD camera for fluorescence. Both devices produce a digitised image. Such images can be displayed and manipulated on the VDU screen, but most importantly for the present discussion, they can be compared by numerical methods. Thus the image produced by one sequence can be subtracted from that produced by another. Oligonucleotides held in common in the two sequences disappear leaving only those which are different. Thus two sequences each one kilobase in length would give an image with almost a thousand "spots". If they differed by a single base change, the difference would affect only eight spots in an octamere array.

Model experiments using small arrays of octanucleotides have shown the power of this approach: subtraction did indeed simplify the images and produce a pattern that was easy to interpret. A most important feature of the overall approach was that the final assignment could be assessed quantitatively and the probability of its correctness readily estimated [Southern, Maskos and Elder, 1992].

In conclusion, hybridisation to oligonucleotides offers a range of opportunities in the field of forensic analysis of DNA sequences. It has sensitive discrimination of point

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found. For example, around 300 point mutations have been found in the CFTR gene, causing cystic fibrosis [Zielenski *et al.*, 1991]. The need to analyse point mutation is obvious and many methods have been developed for this purpose [Cotton, 1993]. The methods can be divided into two main types. One type depends on gel electrophoresis to detect differences in sequence which in one way or another affect the mobility of fragments. The others depend on molecular hybridisation with oligonucleotide probes. An advantage of this approach is that it is possible to see both mutant and wild type in a single, simple analysis. The technique most commonly used is to amplify the relevant gene and fix the PCR product to a nylon membrane, which is then hybridised separately with the allele specific oligonucleotides (ASOs)[Conner *et al.*, 1983]. A homozygous wild type or mutants will test positive with only one probe, whereas a heterozygote will be positive with both. An advantage of the latter method is that large numbers of samples may be analysed together. However, each probe must be used separately, so that many analyses would be required to give an individual fingerprint. We and others have been developing a system in which multiple oligonucleotides can be used simultaneously. The oligonucleotides are made *in situ* or applied to a solid flat surface, of glass or plastic, so that they are covalently bound and available for molecular hybridisation. We have developed three radically different types of array. They are all made by the same basic procedure. We first apply a twenty atom linker to the surface of the glass plate. Oligonucleotides are synthesised using phosphoramidite chemistry, the first residue being coupled to a primary hydrxyl group at the end of the linker.

We normally apply the coupling reagents through channels formed by clamping a template to the surface of the glass plate. As the reagents pass through the channels coupling takes place, creating rectangular patches or lines of coupled bases on the surface. In one kind of array we make lines, each line comprising an allele specific oligonucleotide. These arrays can be used to test several samples simultaneously by applying the samples as lines orthogonal to the lines of oligonucleotides [Maskos and Southern, 1993]. Hybridisation takes place where the lines cross. This method can be scaled up to analyse many different alleles – up to 200 on a 200 mm plate – using multiple samples. For forensic purposes, a twenty allele system, each with two variants, would provide $2^{20} \simeq 10^6$ different genotypes. However, the discriminating power of the system would depend on the frequency of each allele in the population.

A second type of array can be used to "scan" a length, reading a short window of bases at each point in the sequence [Southern *et al.*, in preparation]. Such arrays are made by applying the first base to be synthesised (the complement of the first base in the target sequence) in a circular patch on the glass surface. The template is then offset by a fraction, say one tenth, of a diameter and the second base applied. The process is repeated. After 10 steps, the first ten bases of the sequence are represented as a decanucleotide. A further step produces a decamer representing bases 2–11 and so on to the other end of the sequence. Hybridisation of the target sequence then shows every position down the sequence. Variants are seen as lost signal in set of

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of a short motif. These sequences are of two main types. The major satellites occurs as large blocks in particular regions of chromosome, most notably in the heterochromatic regions close to the centromeres. The basic unit repeated in the major satellites varies from two base pairs up to quite long sequences.

The minisatellites and microsatellites (also known as VNTRs) used in fingerprinting, are scattered around the genome. They comprise repeats of 2–30 base pairs. The feature which makes these sequences so useful as genetic markers and in fingerprinting is the sequences variability which is found in a high proportion [Jeffreys *et al.*, 1985]. Variability is caused by shrinkage and expansion by losing or gaining repeat units. Such sequences and their variations are easy to analyse. Sequence specific restriction endonucleases cut the DNA flanking the repeats, but not the repeat itself. Gel electrophoresis to separate the fragments by size, followed by molecular hybridisation to show the position of the bands reveals the pattern of fragment sizes characteristic of the individual. Such multilocus probes give a pattern rich in individual information. However, in some cases, it is preferable to use single locus probes, particularly for the analysis of small samples which must be amplified by the PCR to provide enough DNA for analysis. For this purpose it is necessary to isolate individual examples of a mini – or microsatellite and sequence the flanking DNA to obtain the information needed to make primers for the PCR. It is easy to find many individual examples by probing a collection of clones of genomic DNA with a mini – or microsatellite sequence. Several methods can be used to analyse the products after amplification by the PCR, but most commonly, for the micro and mini–satellites, gel electrophoresis is used to estimate the size. Many examples of the application of minisatellite analysis are cited in this symposium.

In addition to the micro – and minisatellites, the other major source of variation in the DNA is that due to point mutation – base substitution deletions and insertions. This kind of variability is also very great; between chromosome there is roughly one base difference for every 100–1000 bases, so that there will be roughly ten million differences between distantly related individuals. As compared with the micro and minisatellites, however, the number of types at any given site (the number of alleles) is relatively small [Botstein *et al.*, 1980]. Most often there are just two types in the population, and it is usually the case that one is more frequent than the other. Nevertheless, there is much interest in analysing point mutations because they are a major cause of genetic disease. Many cancers and inherited diseases are now known to be caused by point mutations in critical genes [Cooper and Krawczak, 1989]. Analysis is important first for finding the gene causing the disease. This may involved analysing many kilobases of DNA to compare wild type with mutant genes in many different individuals. Once the identity of the gene has been confirmed, methods for detecting mutation in individuals must be developed. The nature of the analysis used to detect mutation varies from one gene to another, as each has its own spectrum of mutation. Some diseases, such as sickle cell disease, are caused by only one mutation in one gene, a T to A transversion in the β-globin gene in this case. In others, very many different point mutations are

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DNA VARIATION AND ITS ANALYSIS BY HYBRIDISATION TO OLIGONUCLEOTIDE ARRAYS.

EM SOUTHERN

Introduction

DNA analysis is now widely used in medical and forensic science. As an indicator for the presence of an infectious agent, as a means of detecting mutation associated with an inherited disease gene, or as an individual fingerprint, DNA analysis surpasses all other known tests. The reasons for this are severalfold. Unlike specific proteins, such as the blood group or leucocyte antigens, DNA is found in almost all cells. So that DNA analysis can be carried out on blood, semen and even spittle and hair. In recent years methods have been developed which allow analysis to be carried out on DNA from just a few cells, giving detection sensitivities not available for most molecules. Furthermore, the same reagents and methods can be used with any DNA sample, simplifying laboratory procedures. Finally individual variation in DNA is far greater than in any single phenotypic character.

DNA variability

Bacteria, viruses and simple eukaryotes such as yeasts have small compact genomes – most of the nuclear DNA codes for proteins. By contrast, in man it is estimated that less than 5% of the DNA codes for protein. The rest comprises a mixture of sequences, most of which appear to have no function relevant to fitness and hence are collectively known as "junk" DNA.

Much of non–coding DNA is found in the introns which interrupt the coding sequences. It has been speculated that these introns are the sites of recombination which, over evolutionary time, lead to new combinations of exons, providing new proteins with new functions, a process known as exon shuffling. Although they may be functional in this sense, there is much evidence to suggest that the base sequence of the DNA in the introns is not important. There is also a good deal of DNA between the genes. Apart from short sequences which bind proteins used to regulate gene activity there are no functions associated with this large fraction of the DNA. The non–coding DNA contains many sequences which are repeated in many copies throughout the genome. Among the most abundant are the so–called Alu sequences. These make up around 6% of the DNA by weight and in some regions of the genome occur once every 1–2Kb. The sequence of the Alu repeat is related to that of a small RNA which is present in all cell types. Many other dispersed repeats are related to transcribed sequences, suggesting that they were introduced into the genome by retrotransposition – a process in which RNA is copied to DNA which is then inserted into the genome. Although each family of repeated sequences has a characteristic consensus sequence, there is a great deal of variation between members, indicating that mutation is not selected out at the same rate as it is in the coding sequences. Other repeated sequences are made up of tandem repeats

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4. DNA methodology: present and future trends

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membrane and UV fixed for 30sec. Prehybridisation in Promega blocking reagent was for 45min at RT in sandwich boxes. Hybridisation was carried out in a rotisserie oven for 45min at 55°C. To 10ml hybridisation buffer (1% ficoll, 1% PVP, 1% BSA, 0.75M NaCl, 0.75M NaCit, 1% N-lauryl-sarcosine, 0.2% NaAzide) per membrane was added 0.25µl/ml MW100 probe (Cellmark) and 0.5µl/ml "NICE" MS43a (Cellmark) or 4µl/ml YNH24 or 8µl/ml TBQ7 (Promega). Washing was performed in sandwich boxes (2x10min 0.5xSSC 1% SDS 65°C, 1x10min 0.5xSSC RT, 1x10min equilibration buffer (Promega) pH9.6 RT). All washing solutions were filtered through 0.4µ filters prior to use.

Results

The results shown in fig.2 show marked differences in estimated fragment sizes using the two methodologies.

For fragments <6kb there is no significant trend away from zero difference ($r=0.115$, $p=0.232$), but at higher molecular weights the ^{32}P/Amersham ladder size estimates are significantly larger than the chemiluminescent/BRL ladder size estimates.

Despite this systematic size difference, Fig.3 shows that the overall magnitude of the differences is small (3sd = 2.5%)

Discussion

Size differences observed using various different ladders have been observed previously (1,2). It has been suggested that the species origin of the ladder DNA, and the use of different protocols may both contribute to variations in different ladders' mobilities. A study comparing the two ladders used here, but with the same detection system for each showed no significant differences between them.

The reasons for the differences observed in this study are not clear. Both ladders are composed primarily of lambda DNA and would be expected to behave similarly under electrophoresis. A possible factor which may contribute to this effect is the characteristic slight distortion of the high molecular weight ^{35}S ladder bands (see fig.1a), which could cause problems with band position assignment during measuring. It is also possible that the incorporation of ^{35}S in the Amersham ladder may slightly alter its mobility. It is noted that a linear shift of 0.4mm would produce a difference of about 200bp at 12kb (1.7%) but just 18bp at 2kb (0.9%) (data not shown).

Overall, the differences presented here are relatively small and well within this laboratory's +/- 5% window used to extract frequencies from databases. It is concluded that it is thus reasonable and safe to use our existing databases with this new methodology.

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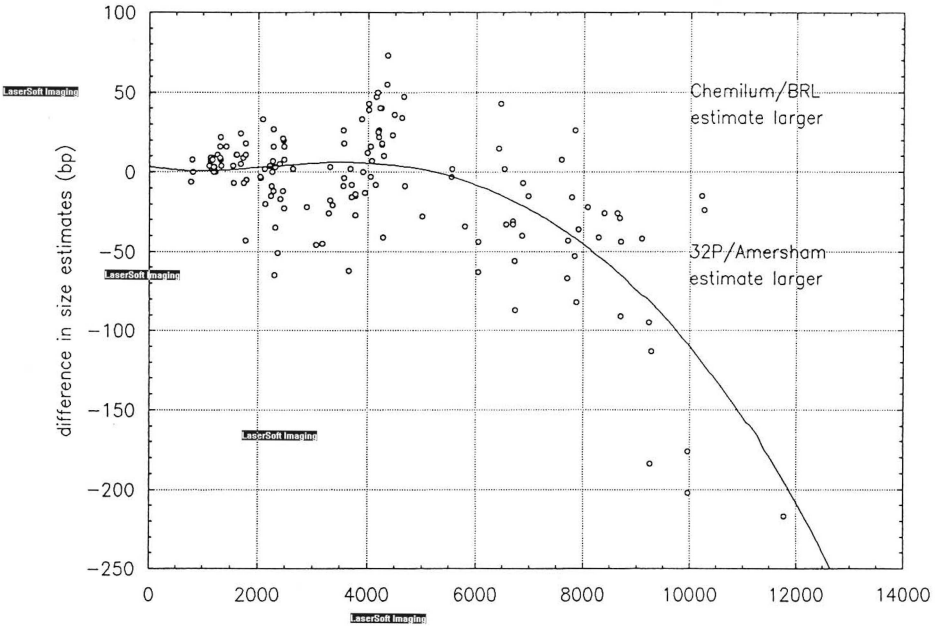

Fig 2

Size differences observed in measurements made after radiolabelling, using the Amersham size ladder, and after chemiluminescent detection of fragments using the BRL sizing ladder

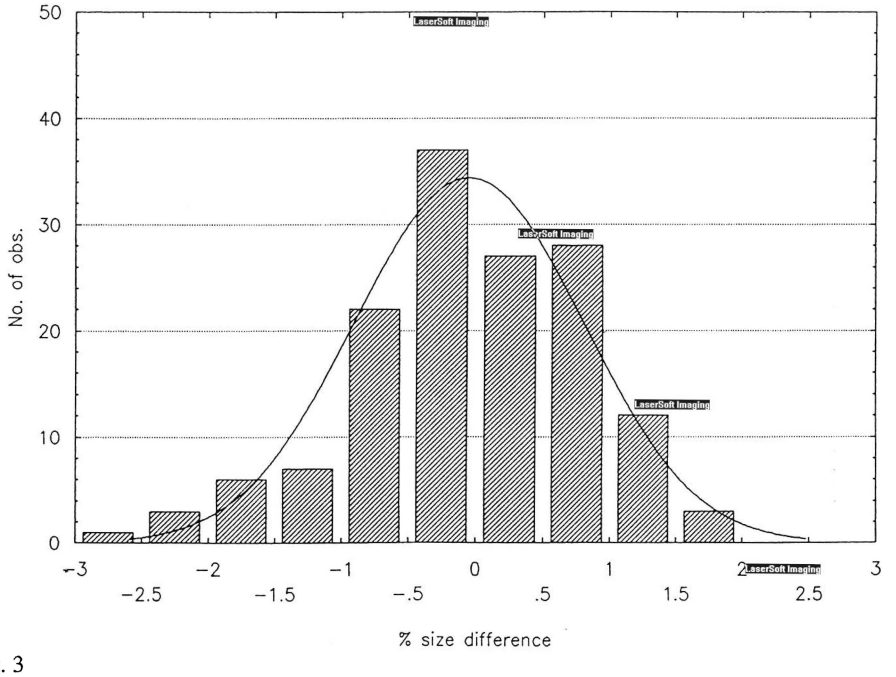

Fig. 3

Percentage size differences observed between the radio labelled and chemiluminescent labelled fragments

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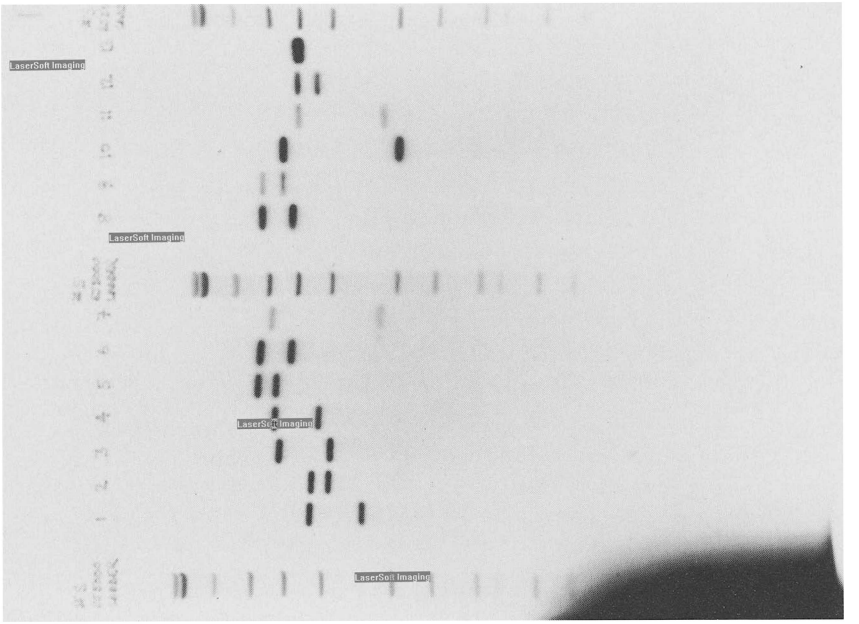

Fig 1a
Autoradiograph of blot probed with a ^{32}P-labelled probe, showing the ^{35}S-Amersham sizing ladder.

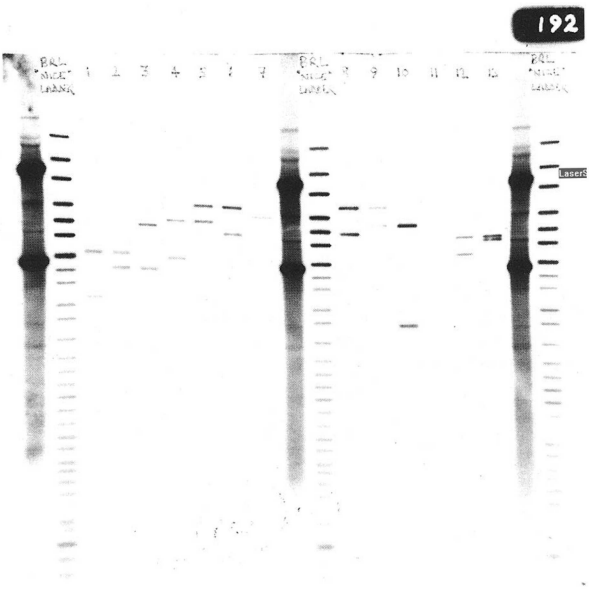

Fig 1b
Autoradiograph of blot reprobed with alkaline phosphatase conjugated oligonucleotide probe, showing the BRL size ladder

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AN OPTIMISED CHEMILUMINESCENT DETECTION SYSTEM: IS IT COMPATIBLE WITH DATA GENERATED USING ^{32}P AND A DIFFERENT LADDER?

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Introduction

Chemiluminescent detection systems are rapidly becoming the method of choice in VNTR analysis. Such systems offer considerable advantages over ^{32}P labelled probes in terms of speed and safety. However, many laboratories have constructed large population databases using ^{32}P techniques. It is important to verify that the results obtained using the chemiluminescent detection systems are directly compatible with those using ^{32}P. This is particularly relevant if, as in this laboratory, a different molecular weight ladder has been used with each methodology.

Previous studies comparing different ladders have shown some significant differences, notably where the protocol has also changed (1). A study involving the two ladders compared here (2) suggested no significant differences between them, but did not compare the effects of different methodologies.

This investigation attempts to clarify the situation using the combinations of ladder and methodology relevant to the changing protocols in many laboratories.

Methods

DNA samples from 26 individuals were analysed, Each gel was loaded with both ^{35}S Amersham SJ5000 ladder and BRL "NICE" ladder. The resulting blots were first probed with ^{32}P labelled probes (MS43a (Cellmark), YNH24, TBQ7 (Promega)) and sized with the ^{35}S Amersham ladder (Fig.1a). The blots were then reprobed for the same loci using alkaline phosphatase (AP) conjugated oligonucleotide probes ("NICE", Cellmark or GPL, Promega) and sized using the BRL "NICE" ladder. A total of 146 fragments were identified and sized using each method. Sizing was by manual measurement and a local reciprocal fit algorithm.

Detection using ^{32}P labelled probes was carried out as previously described (3).

The following protocol was used for generation of the Southern blots and chemiluminescent detection of AP labelled probes.

1μg samples of *Alu 1* digested DNA were electrophoresed in 25cm 1% agarose TAE gels (40V, 20hrs). 3 lanes of each ladder were loaded (2.5μl/lane BRL "NICE" ladder, 8μl/lane Amersham SJ5000 ladder). Gels were neutralised and blotted onto Promega GPL

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rors of ± 1 mm (± 1.4 SD). For the remaining profiles the coordinates for both probes fell either within the upper accept area (31 %) or within the lower (16 %) accept area (Fig. 2). This means that all deviations were correlated which is in agreement with the finding of Evett *et al.* [6].

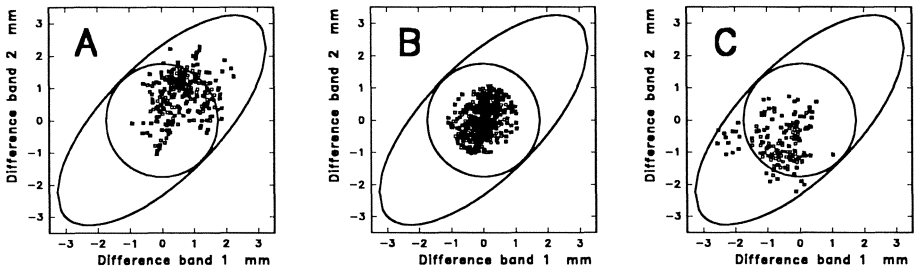

FIG. 2. CORRELATION OF TRANSFORMED DEVIATIONS.
For each set of determinations of 3 samples with MS43a and YNH24 all size differences were calculated. The difference between the HMW bands was plotted against the difference between the LMW bands. A: positive differences. B: insignificant differences. C: negative differences. SD = 0.7 and ρ = 0.7.

CONCLUSION

Interlaboratory comparisons cannot be performed on the basis of the coefficient of variation in terms of basepairs (bp%). Deviations will be underestimated for small fragments and overestimated for larger fragments. However, an 'accept-corridor' [3] based on fragment length dependent SD-values can be used. We suggest the use of transformed [4] fragment lengths, or of normalized migration distances corrected for transversal skewness. The data should be tested for correlation.

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The match plot used in the EDNAP report is identical with the cumulative frequency distribution of the absolute differences between all possible combinations of fragment lengths. With a normal distribution of the differences this means that 99.7 % of the differences will be less than 3 SD, or that 99.7 % match will be expected with a window of 3 SD.

When the differences were measured in terms of bp% - as in the EDNAP report - the SD and the window width increase with increasing fragment length. For the 14 kb fragment the 99.7 % window will be approximately 12 bp%, for the 9 - 10 kb fragments it will be 6 - 8 bp% and for smaller fragments it will be approximately 2.5 bp%. This was confirmed by testing 4 fragments of different size (Fig. 1A). The width of the window increased with increasing fragment length which strongly indicates that the test is inadequate. In the German study [3] the interlaboratory comparison was performed on the basis of SD values which increased with increasing fragment length.

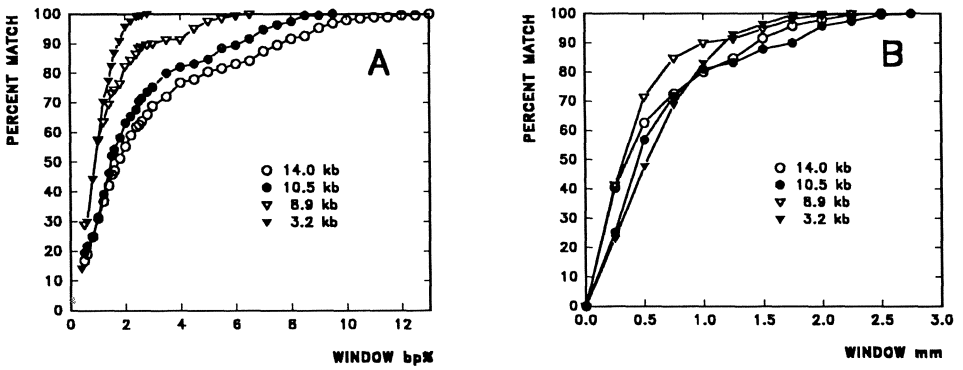

FIG. 1. MATCH TEST WITH 4 FRAGMENTS OF DIFFERENT LENGTH
Fragment lengths: 3.2 - 14 kb.
A. Window width in bp%. B. Window width in mm

When the differences were measured in terms of normalized migration distance they were normally distributed with an SD of 0.7 mm ($0.5 \times \sqrt{2}$) and a 99.7% window of 2.1 mm. This was confirmed by a test performed with all determinations (number of comparisons: 3184). When the analysis was performed on single fragments, *e.g.* those analysed in Fig. 1A, minor deviations were seen (Fig. 1B). The maximum deviation was 2.6 mm.

In the EDNAP report the test involved both bands of a bandpair. This, however, is of no significance because the smaller fragment always will be accepted by the window size determined by the larger fragment. It would be more relevant to investigate whether the deviations were correlated. To this purpose the data from all 4-band profiles obtained with the two probes were analysed as described previously [5]. For 53 % of the comparisons the deviations were insignificant, *i.e.* their coordinates fell within the central part of the accept area which accepts measurement er-

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QUALITY CONTROL: INTERLABORATORY COMPARISON OF RFLP RESULTS.

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INTRODUCTION

Interlaboratory quality control programmes for RFLP analysis have been established by some European forensic laboratories. Two such programmes (EDNAP 1-2) and 3 German programmes were published [1-3]. The evaluation of proficiency testing represents an important part of such programmes, and it is the aim of this report to draw attention to weaknesses in the methods of comparison applied and to suggest a better approach.

METHODS AND DATA

Data from the second EDNAP exercise were used as published in appendices to the report [2]. The data comprise analyses from 12 laboratories of 4 different samples of DNA using 2 probes, YNH24 and MS43a. Each sample was supplied as a bulk restricted (*Hin*fI) sample and as an unrestricted sample. There was no significant difference between these two series of determinations which consequently were combined in this study.

It should be well known that the measurement error in terms of basepairs (bp) and also in terms of bp% increases with increasing fragment length. To circumvent this obvious inconvenience we have transformed all fragment lengths into normalized migration distance. After transformation the measurement errors were normally distributed and independent of the fragment length [4].

In the second EDNAP exercise the results of the different laboratories were compared by a special test for match. For each sample and for each probe all possible combinations of bandpairs were tested for match using windows ranging from 2 to 8 bp%. A few determinations of high molecular weight fragments from one of the laboratories were not accepted by an 8 bp% window, and all the results from this laboratory were omitted from the statistical treatment in the report. In the present study all results are included.

RESULTS AND DISCUSSION

The fragment lengths varied from 1,935 to 14,018 bp. The coefficient of variance in terms of basepairs increased with increasing fragment length (range: 0.47-2.85) and it does not make sense to calculate an average. After transformation the standard deviation (SD) in terms of mm was independent of the fragment length. The SD ranged from 0.33 to 0.68 mm and the average was 0.49 mm. In our laboratory the SD for determinations on different plates is 0.5 mm.

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relationship between migration and molecular weight very precise gained by using only marker fragments close to the unknown bands. The only condition for calculating with this algorithm is the use of a molecular weight marker covering the whole blot with 20-30 well-defined and distinctly separated fragments.

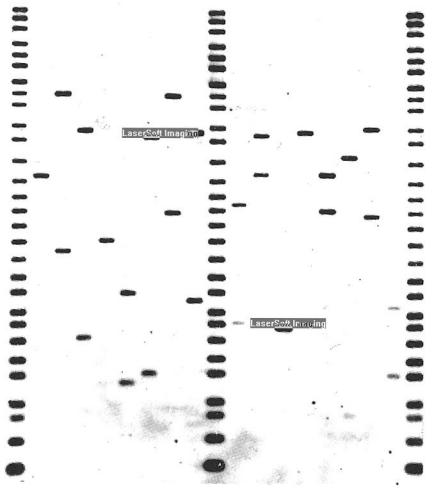

Fig.1: Hae III restricted DNA from 16 individuals, detected by TBQ7, molecular weight marker MW100 (1,10,19)

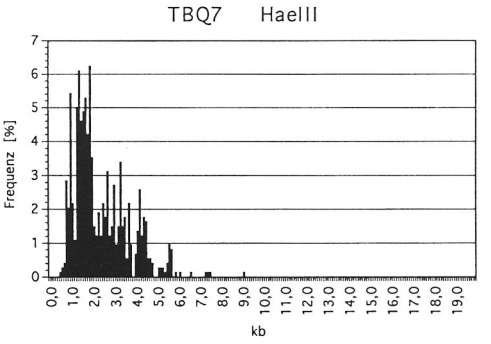

Fig. 2: Allele frequency distribution at the locus D10S28 (TBQ7/Hae III), n = 380 unrelated individuals

4.3. STATISTICAL EVALUATION

Tab. 1 summarizes the statistical results. It is apparently that low SD values correspond to precise analysis methods.

TBQ7	intergel V.	
	f1	*f2*
AM (kb)	1,742	1,173
SD	0,010	0,006
CV (%)	0,574	0,480

Table 1: Analysis of intergel variance (n=20) for molecular weight calculations of two K562 DNA fragments, detected by TBQ7 (HaeIII), (AM=arithmetic mean, SD= standard deviation, CV=coefficient of variation (%), f1,f2 =fragment 1,2)

Fig. 2 presents the allele size and frequency distribution at the locus D10S28 generated by Hae III restriction enzyme digest of DNA from 380 unrelated individuals detected by the TBQ7 DNA probe.
Summarizing the data revealed in this study using the RFLP-MAC system answer all requirements claimed by an accurate and reproducible analysis method.

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3.1. IMAGE-ACQUISITION

Hardware: RFLP patterns are scanned via a high resolution, 8-bit gray-scale, 300 dpi; *OneScanner*, (Apple Computer Inc.) to a *Macintosh IIci* computer with 20 MB RAM and 80 MB harddisk. Complete digital images are stored as an image file (50 KB size, PICT-Format) on the internal harddisk or external 88 MB removable cartridge.
Software: Special automatic scanning software enables to pick up even subtle changes in film or blot density and allows the user to optimize the background area.

3.2. IMAGE-ANALYSIS

Software: *Image* 1.45 (National Health Institute, Bethesda/USA). Efficient image-processing options: morphology, minimum height and area of the bands compared to the image background level, noise reduction, two-dimensional integration of the optical density within each band; this densitometric center defines each single band and is therefore the reference point for exact determination of x- and y-location measured in 0.1 millimeter-units

3.3. MOLECULAR WEIGHT CALCULATION

Software: Self-developed, running within the multi-tasking *4th Dimension* 3.0 relational database multi-user environment.
Mathematics: a) Correction of gel distortions (intragel variation): 3rd order polynomial regression algorithm, fits a curve through each corresponding triplet of the molecu-lar weight marker fragments. b) Correlation of migration and molecular weight: selection between linear, logarithmic, exponential and spline models over the full range of the molecular weight standard (global method) and between an additional interpolation model over a limited range of the standard (local method), (2-4); we use the local version of the reciprocal method.
Database management: All data are stored in a relational database system.

3.4. STATISTICAL EVALUATION

Descriptive statistics: Selection of common statistical parameters: arithmetic mean (AM), standard deviation (SD), coefficient of variation (CV), variance and confidence interval. To check these paramters we have made serial intergel measurements of the two K562 DNA fragments separated on 20 gels and hybridized to TBQ7 (HaeIII).
Population- and formal genetics: Allele frequencies of the investigated probe systems can be analysed by three methods: classical rounding, "fixed bin" (5) and "sliding window" (6) fit. All data are available in tabulated and graphical form. We investigated 380 unrelated individuals from SW Germany.

4. RESULTS AND DISCUSSION

4.1. GEL IMAGE

Fig. 1 shows the fragment patterns of Hae III restricted DNA from 16 unrelated individuals detected by TBQ7. Correction of gel ditortions by 3rd order polynomial regression algorithm requires the use of 3 tracks with molecular weight markers, running in lanes 1, 10, 19.

4.2. RFLP-MAC

The image-aquisition, image-analysis, and molecular weight calculation method described above insure consistent results from gel to gel. Determining the optical density distribution of each band results in a precise calculation of its center compared with other methods localizing bands with a ruler, micrometer or digitizing tablet. For the calculation of the molecular weight we prefer the local version of the reciprocal method, because this algorithm describes the

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RESTRICTION FRAGMENT LENGTH POLYMORPHISM : IMAGE ANALYSIS AND MOLECULAR WEIGHT CALCULATION WITH A SCANNER-BASED COMPUTER SYSTEM

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SUMMARY

Image analysis and calculation programs are essential to enable precise DNA profiling and to reduce the subjective aspects of band measurements. We present a new system, called *RFLP-MAC*, **R**estriction **F**ragment **L**ength **P**olymorphism - **M**olecular weight **A**nalysis and **C**alculation. The system consists of a scanner unit and an Apple Macintosh computer supported by a combination of a shareware and self-developed software package. Molecular weights are calculated profiling the optical density distribution of each band. The migration distances of known marker fragments can be determined by the use of different mathematical models and gel distortions are corrected by a polynomial regression algorithm. All data are stored in a relational database system. Descriptive statistics can be performed as well as allele frequencies.
Serial intergel measurements are investigated in order to check the precision of the RFLP-MAC system; these data are subjected to a standard analysis of variance (maximal 3 SD =0.030 =1.723%). Allele size distribution of 380 unrelated individuals at the locus D10S28 (TBQ7, Hae III) are presented.

1. INTRODUCTION

Highly polymorphic variable number of tandem repeat (VNTR) loci are proving increasingly profitable for genetic analysis in man, i. e. in population genetics, linkage studies, forensic medicine and paternity testing, when used in restriction fragment length polymorphism (RFLP) analysis. One problem of major concern is the accuracy and precision of fragment length calculations being obligate for further biostatistical procedures and for comparability of results.
We present a new system, specifically developed to meet these demands, called RFLP-MAC, **R**estriction **F**ragment **L**ength **P**olymorphism - **M**olecular weight **A**nalysis and **C**alculation.

2. EXPERIMENTAL TECHNIQUE

DNA-Isolation: 5 ug human genomic DNA and cell-line K562 DNA according to the protocol of Miller et al. (1).
RE-Digest: 50 U Hae III (Boehringer Mannheim / FRG).
Molecular weight Marker: NICE™ DNA Analysis Ladder (GIBCO BRL, Eggenstein/FRG) with fragment sizes ranging from 22.621 to 0.526 kb.
Electrophoresis: 0.8% agarose gel (TBE buffer), 40V, 30hrs.
Transfer: Vacuum blotting with 50 mbar
Hybridization: Alkaline phosphatase-conjugated single-locus probe TBQ7 (Promega,Heidelberg/ FRG) and the molecular weight marker probe MW100 (Zeneca Bioproducts, Frankfurt / FRG)
Detection: Chemiluminescent with Lumi-Phos 530 (Boehringer) or CSPD (Promega)

3. RFLP-MAC

The observed frequencies of heterozygotes was 0.78 at HUMTHO1 and 0.96 at HUMACTBP2. The heterozygosity and allele frequencies at HUMTHO1 and the fragment size distribution at HUMACTBP2 appears to be in good agreement with what Kimpton *et al* and Wiegand *et al* reports from Caucasian populations in England and Germany. The observed and expected genotype frequencies did not deviate significantly in any of the two loci. At HUMACTBP2 the alleles were pooled in nine groupes (227-243, 247-251, 255-258, 260-264, 266-273, 275-283, 287-291, 295-299 and 303-315).

In this study we have showed that the standard deviation when fragments are estimated on the same gel is less than 0.1 bp for both HUMTHO1 and HUMACTBP2, see Table 1. The variation in size estimates at HUMTHO1 is also lower than 0.1 bp when fragments are estimated on different gels. Typing can therefore rely solely on fragment size estimates. The use of an allelic ladder is therefore only recommended but appears not to be necessary.

Typing of HUMACTBP2 is more complicated and the number of alleles is most likely larger than the number of allelic groups that can be discerned by a size estimate. Kimpton *et al* has recently reported the same level of variation at HUMTHO1 and HUMACTBP2 both within and between gels as in our study. An allelic ladder run in at least one lane on every gel is necessary to confirm the allele designation derived from the size estimation using the internal size standard.

	HUM THO1		HUM ACTBP2	
	Allele 1	Allele 2	Allele 1	Allele 2
n	58	57	59	59
Mean	157.8 bp	173.5 bp	258.8 bp	291.8 bp
Within std.dev. (bp)	0.06 bp	0.04 bp	0.07 bp	0.07 bp
Within std.dev. (%)	0.04 %	0.02 %	0.03 %	0.02 %
Between std.dev (bp)	0.07 bp	0.03 bp	0.31 bp	0.41 bp
Between std.dev. (%)	0.04 %	0.02 %	0.12 %	0.14 %

Table 1. Reproducibility of size estimates for fragments run on the same gel and between gels.

In conclusion, further genetic loci to be introduced as genetic marker in forensic casework should have a low inter-gel S.D., in order to determine alleles automatically without comparison against an allelic ladder. In addition, the nomenclature should be easy to standardise, which means that they should be moderatly polymorphic and alleles should differ be full length repeats. Eventually, a DNA profile will be generated in a few days using PCR, fluorescent tagged primers and an automated sequencer.

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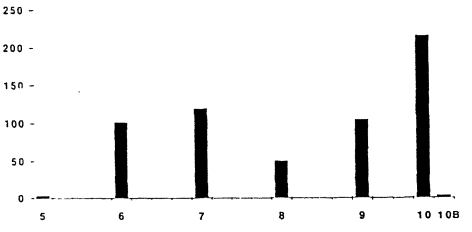
Primer sequences for HUMTH01 were 5'-L GTG GGC TGA AAA GCT CCC GAT TAT-3' (L, aminolink) and 5'-GTG ATT CCC ATT GGC CTG TTC CTC-3'. Primer sequences for HUMACTBP2 were 5'-L AAT CTG GGC GAC AAG AGT GA-3' and 5'-ACA TCT CCC CTA CCG CTA TA-3'. The left primer in each pair was prepared for labelling with a fluorescent dye by an aminolink. Amplification was performed with a Perkin Elmer 9600 in 0.2 ml tubes. The same PCR cycle was used for both loci and consisted of a 15 seconds predenaturation at 94°C, thirty cycles with 15 seconds denaturation at 94°C, 15 seconds annealing at 60°C and extension for n seconds, where n equals cycle number, at 72°C. The amplification reaction mixture was the same for the two genes and consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatine, 200 mM of each dNTP, 250 nM of each primer, 1 unit AmpliTaq and 10 ng template DNA per 50 µl reaction.

RESULTS AND DISCUSSION

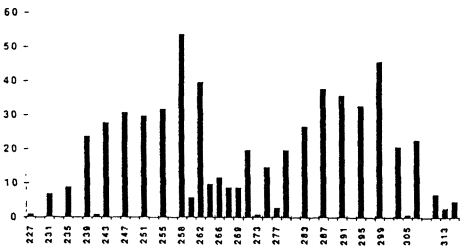
The nomenclature for alleles at the tetrameric HUMTHO1 is based on the number of repeats of $(TCAT)_{5-10}$. HUMACTBP2 is a complex tetrameric locus and it is not possible to base the nomenclature on number of repeats of the sequence $(AAAG)_n$. We choose to base the nomenclature on the estimated fragment size. The vast majority of fragments fell into discrete groupes of fragments and we use the mean fragment size in each group as allele name. An allelic ladder for HUMTHO1 and HUMACTBP2 respectively was constructed, using amplified samples from the database.

The genetic variation we found in the Swedish population sample at HUMTHO1 and HUMACTBP2 are summarised in Figure 1 and Figure 2.



Allele	Size (bp)	Frequency
5	154	0.007 (4)
6	158	0.0169 (102)
7	162	0.199 (120)
8	166	0.085 (51)
9	170	0.174 (105)
10	173	0.359 (216)
10B	174	0.007 (4)
		602 chromosomes

Fig1. Allele frequencies at HUMTHO1



Allele	Size interval	Allele frequency	Allele	Size interval	Allele frequency
227	225.86-227.46	0.002 (1)	271	270.67-272.27	0.033 (20)
231	230.05-231.65	0.012 (7)	273	272.50-274.10	0.002 (1)
235	234.11-235.71	0.015 (9)	275	274.56-276.16	0.025 (15)
239	238.30-239.90	0.040 (24)	277	276.17-277.77	0.005 (3)
241	240.10-241.70	0.002 (1)	279	278.49-280.09	0.033 (20)
243	242.19-243.79	0.046 (28)	283	282.25-283.85	0.045 (27)
247	246.16-247.76	0.052 (31)	287	286.18-287.78	0.063 (38)
251	249.92-251.51	0.050 (30)	291	290.06-291.66	0.060 (36)
255	253.80-255.40	0.053 (32)	295	293.99-295.59	0.055 (33)
258	257.58-259.18	0.090 (54)	299	297.87-299.47	0.076 (46)
260	259.35-260.95	0.010 (6)	303	301.95-303.55	0.035 (21)
262	261.28-262.88	0.066 (40)	305	303.87-305.47	0.002 (1)
264	263.14-264.74	0.017 (10)	307	305.84-307.44	0.038 (23)
266	265.19-266.79	0.020 (12)	311	310.00-311.60	0.012 (7)
268	266.79-268.39	0.015 (9)	313	311.82-313.42	0.005 (3)
269	268.55-270.15	0.015 (9)	315	314.80-316.40	0.008 (5)

Fig 2. Allele frequencies at HUMACTBP2

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FLUORESCENT BASED TYPING OF TWO SHORT TANDEM REPEAT LOCI IN A SWEDISH POPULATION SAMPLE AND REPRODUCIBILITY OF FRAGMENT SIZE ESTIMATES

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SUMMARY

The objective of this study was to compile a population database for two tetrameric short tandem repeat loci (STR) and investigate the reproducibility of fragment size estimates. An Applied Biosystems 373A Sequencer with Genescan 672 collection and analysis software was used for typing of HUMTH01 (intron 1 in human tyrosine hydrolase gene) and HUMACTBP2 (also named SE33, human cytoplasmic beta-actin related pseudogene). PCR products from both loci were sized in one lane using an internal size standard.

The amount of genetic variation was investigated in the Swedish population (n=301). Seven alleles were found at HUMTH01 and the observed heterozygosity was 0.78. At HUMACTBP2 locus more than thirty alleles were found and the observed heterozygosity was 0.96. The observed genotype frequencies at HUMTH01 and HUMACTBP2 did not deviate significantly from Hardy-Weinberg expectation.

In order to compare within and between gel reproduciblity of fragment size estimates a control sample was typed three times on each of 20 gels. Within gel, the standard deviation (S.D.) was less than 0.1 bp for four fragment sizes between 158-291 bp. Standard deviations between gels were slightly higher for the two shorter fragment sizes (HUMTHO1), while the larger fragments gave a 0.3 and 0.4 bp S.D (HUMACTBP2).

We conclude that both HUMTHO1 and HUMACTBP2 can be reliably typed. However the latter require an allelic ladder to be run on each gel. The combined chance of a random match between two unrelated individuals from the Swedish population is approximately 1 in a 1000.

INTRODUCTION

PCR-based typing of VNTR/STR loci has become a technique of growing importance in paternity and crime investigations. It is likely that compilation of population databases will be generated faster than for the VNTR markers used in DNA-profiling with RFLP-technique. Also, the simplicity of many STRs make the standardisation of nomenclature and comparison of allele frequencies in different populations much easier. The result of this investigation will influence the choice of further STRs to be introduced in forensic casework.

MATERIALS AND METHODS

Blood samples were collected from 301 blood donors with Swedish names from three cities in Sweden. One individual, with one short and one long allele at both loci, was selected for repeated analysis in order to test the reproducibility of the estimates of fragment sizes. A total of 20 gels were run, each with three replicas randomly distributed among a total of 24 samples.

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TABLE 3 - SUCCESS RATE OF DNA-PROFILING AS A FUNCTION OF THE NUMBER OF CLASSICAL ANALYSES WITH CONCLUSIVE RESULTS.

Conclusive classical systems	BLOOD Number	Success rate Percent	SEMEN/VAGINAL SECRETION Number	Success rate Percent
Not carried out	1	100 %	25	40 %
0	17	29 %	107	40 %
1	20	20 %	49	65 %
2	31	45 %	242	71 %
3	23	48 %	74	75 %
4	40	53 %	14	86 %
5	43	65 %	-	-
6	67	72 %	-	-
7	79	77 %	-	-

The correlation of the count of sperm cells and the success rate of the DNA-analysis was high. Also the score of the phosphatase screening was correlated with the count of cells (Table 4). In 3 cases DNA-profiles were obtained with stains without sperm cells. These stains exhibited positive phosphatase tests.

TABLE 4 - CORRELATION BETWEEN COUNTS OF SPERM CELLS AND DNA RESULTS
Sperm cells were scored on a scale from 0 to 4 and phosphatase on a scale from 0 to 3.

Sperm cell scores	Number of stains	Number of DNA-profiles	Success rate DNA-analysis	Phosphatase scores
4	104	89	86 %	2.8
3	96	79	82 %	2.8
2	125	75	60 %	2.5
1	99	35	35 %	1.9
0	30	3	10 %	1.5

The consistency of DNA-analysis and classical typing

For 467 stains results were obtained with DNA-analysis as well as with classical typing. For 394 of these stains a match with a person in the case was recorded. With these stains 1245 classical typings were performed. In 32 of these typing results a disagreement in relation to the corresponding blood sample was observed: In 18 blood stains GM3 (16) or KM (2) could not be detected. In 6 semen/secretion stains A or B were unexpectedly detected, and in 7 stains the presence of A, B, O or Lea were expected but not detected. In one stain the GC subtype 1F and 1S disagreed in the stain and in the corresponding blood sample. In one case a GC variant could not be detected in the stain.

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Classical systems

The rate of success of the classical blood-grouping systems with blood stains and stains with semen/vaginal secretions is given in Table 2.

TABLE 2 - SUCCESS RATE OF CLASSICAL SYSTEMS

	BLOOD			SEMEN/VAGINAL SECRETION		
System	Stains	Conclusive results	Percent	Stains	Conclusive results	Percent
ABO	286	165	58	483	358	74
SECRETOR	-	-	-	481	328	68
PGM1	297	252	86	279	96	34
ACP1	271	213	79	-	-	-
GC	291	237	81	144	31	21
HP	256	203	79			
GM	222	208	94			
KM	206	189	92			

The frequencies q of the classical type profiles comprising up to 7 markers varied from 10^{-6} to 1 (inconclusive) for blood stains. For stains of semen/vaginal secretion where only 4 markers were used the frequencies ranged from 10^{-4} to 1. The distributions of the frequencies are given in Fig. 1.

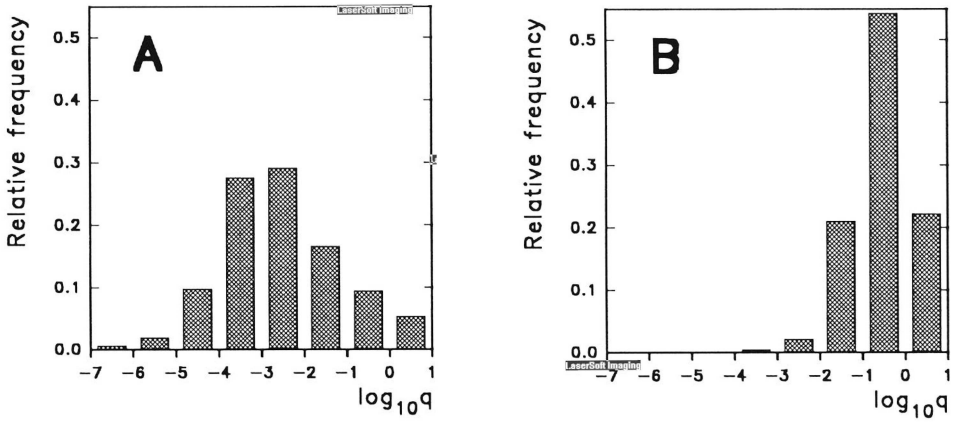

FIG. 1 - DISTRIBUTIONS OF THE $\log_{10}$ FREQUENCIES OBTAINED WITH CLASSICAL SYSTEMS
A. Blood stains. B. Stains with semen/vaginal secretions.
Inconclusive results were scored in the interval 0 - 1.

DNA-Profiling

The number of conclusive classical analyses was correlated to the success rate of the DNA-analysis. For blood stains and stains of semen/vaginal secretion the success rate of the DNA analysis was recorded according to the number of conclusive classical systems (Table 3). On the average 60 - 80 % of the DNA-profiles were complete with bands for 4 probes.

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COMPARISON OF DNA-PROFILING AND CLASSICAL BLOOD GROUPING IN CRIMINAL CASES

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INTRODUCTION

It was the purpose of this study to compare data from DNA-profiling and classical blood grouping applied to stains and other biological specimens related to criminal cases. Data relating to more than 850 stains were reviewed. Correlations and discrepancies of the classical analyses and the DNA analyses will be discussed.

METHODS AND DATA

DNA-profiling was carried out on *Hinf* I restricted DNA using the VNTR probes MS1, MS31, MS43a and YNH24 [1,2]. With blood stains and tissues the classical markers were: ABO, PGM1, ACP1, GC, HP, GM and KM. With stains with semen/vaginal secretion the markers were ABO, secretor (Lewis a and b), PGM1 and GC, with saliva ABO and secretor and with hairroots PGM1. The analyses were carried out according to the routine procedures of the laboratory. The counts of sperm cells were recorded on a scale ranging from 0 to 4. The results of the screening for phosphatase were recorded on a scale from 0 to 3. The study comprises 863 stains and biological specimens related to 399 criminal cases (Table 1).

RESULTS AND DISCUSSION

DNA-profiles were obtained from approximately two thirds of the stains - except for saliva stains (Table 1).

TABLE 1 - NUMBER OF STAINS AND DNA-PROFILES

STAIN	Number of stains	Number of DNA-profiles	Percent
Blood	321	193	60
Semen/vaginal secretion	511	340	67
Tissues	9	7	78
Saliva	20	5	25
Hair roots	2	1	50
Total	863	546	63

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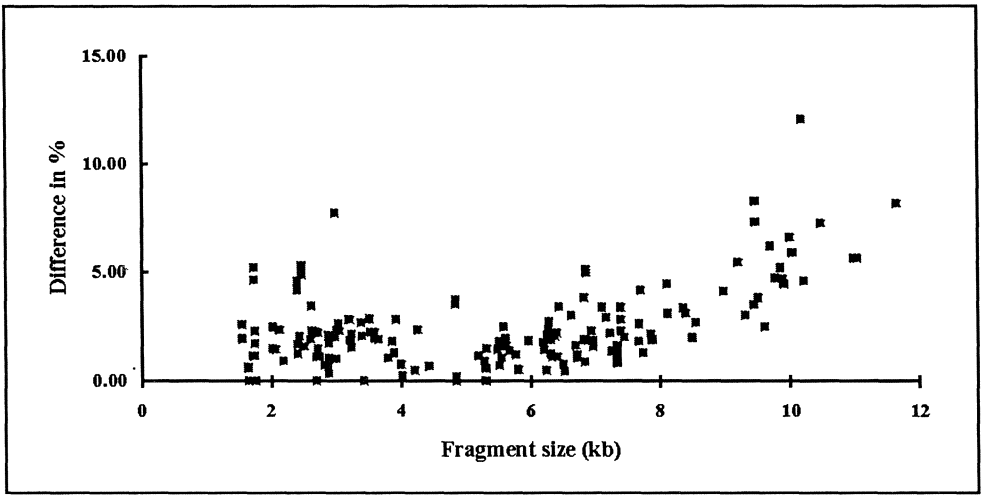
As shown in figure 1 exclusions and no exclusions were in concordance for case 5 and 6. In case 7 two differences were found for the D7S21 and D14S13 systems respectively. These discrepancies were found among a total of 16 excluding combinations.

The reports from Norway included an analysis of close relatives. In case 5, 6 and 7 the mother and the putative father had a common fragment in 4/5, 7/7 and 4/5 systems respectively, which indicates a close relationship between the persons. This was also the case; as for example in case 7 in which the mother and the "putative father" were two sisters.

The differences in fragment size estimation, as shown in figure 2, were below 5 % for most of the fragments up to 10 kb. In 56 out of 80 comparisons the lowest estimated fragment sizes were reported by Norway. This difference in size estimation could be explained by the different size markers used.

Figure 2. Differences in fragment size estimation



Conclusion

Even with differences in the estimated fragment size and the matching criteria used the final conclusions in each paternity case are in concordance. That is the most important result. However, the discrepancies found in one of the cases indicates that there might be a possibility to increase the sensitivity in the exclusions with a more narrow tolerance interval. This has to be done with great care not to introduce false exclusions. A question that has arisen during this work is also whether a more significant conclusion could be drawn from paternity investigations when no exclusion is found. When is paternity confirmed?

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The methods for DNA–typing have been reported elsewhere. The technical procedure is mainly the same in all three laboratories. Different size markers are however used: in Denmark and Sweden Amersham S 5000 and in Norway Gibco/BRL 4401SA. Size estimation is done with different image processing systems. In Denmark and Sweden band matching is obtained by using an interval of tolerance (sliding window) and in Norway by visual comparison.

Results

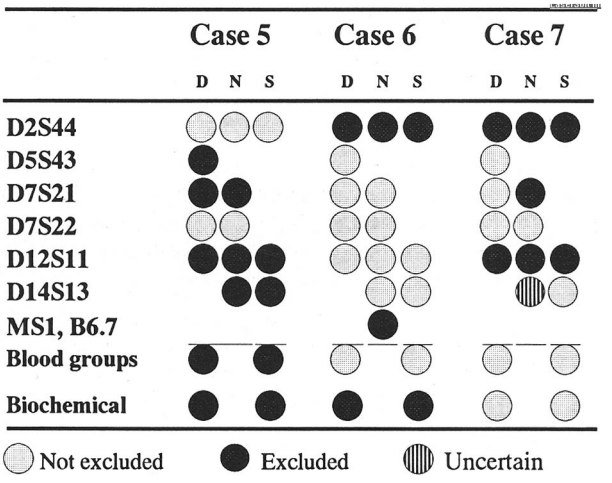
Concordant conclusions were obtained in all cases as shown in table 2. In four cases no exclusions were found. In the conclusions from Denmark and Sweden this was indicated as "No Exclusion". The paternity likelihoods calculated in these cases were greater than 0.9999. These cases were signed with "Paternity Confirmed" in Norway. This conclusion was drawn when no exclusion was found in the five DNA–systems used.

Table 2. The conclusions in the seven cases.

Cases	Denmark	Norway	Sweden
#1	No Exclusion	Paternity Confirmed	No Exclusion
#2	No Exclusion	Paternity Confirmed	No Exclusion
#3	No Exclusion	Paternity Confirmed	No Exclusion
#4	No Exclusion	Paternity Confirmed	No Exclusion
#5	Excluded	Excluded	Excluded
#6	Excluded	Excluded	Excluded
#7	Excluded	Excluded	Excluded

In cases 5, 6 and 7 the putative father was excluded. The exclusions were based on the results obtained in more than one DNA– or conventional marker system.

Figure 1. Exclusions in DNA– and conventional systems.



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Quality control of paternity investigations in Scandinavia.

Bertil Lindblom, Niels Morling and Brita Teige

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Paternity investigations in Denmark, Norway and Sweden are performed at the Institute of Forensic Medicine in Oslo, the Institute of Forensic Genetics in Copenhagen, and the State Institute of Forensic Serology in Linköping. These three institutes have a joint program to increase the collaboration and to give support in quality improvement. This program is referred to as the Scandinavian Quality Program for Paternity Testing.

The basic concept for the program is that one fictitious paternity case from each of the three institutes should be shipped to and analysed in all three participating laboratories. This should be repeated four times a year. The samples should be analysed according to the local procedures and with the marker systems ordinarily used.

The results are distributed to the participating laboratories. The conclusions and the results of each system are compared and both the intra and the inter laboratory variations are to be focused upon. The results will be the basis for a further discussion to improve the quality of the tests.

Material and Methods

Samples from six fictitious cases, two from each institute, have been analysed. One case included two putative fathers. The results from this case have been reported as two separate cases thus giving a total of seven cases.

Blood was taken in vacutainer tubes containing EDTA. A set of samples from each case was sent to the laboratories. The samples were included in the routine procedures at each laboratory. The analysis include the systems given in table 1.

Table 1. Marker systems used in routine and extended paternity testing

Laboratories	DNA-systems	Other systems
Denmark	D2S44, D5S43, D7S21, D7S22, D12S11	AB0, Rh, MNSs, HP, GC, PGM[1], ACP[1], GPT, ESD,GLO
Norway	D2S44, D7S21, D7S22, D12S11, D14S13	
Sweden	D2S44, D12S11, D14S13	AB0, Rh, MNSs, HP, GC, PI, PGM[1], ACP[1], ESD, GLO, F13B

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CONCLUSION

The author recently came across the following statement which clearly enunciates the true meaning of quality in stark terms and should be understood without further comment:

"Quality . . . was a concept that was not rigorously taught or a principle to which we consecrated our hearts and souls. It was simply understood that patients could die if we made mistakes."

In this commitment to quality, it is clear that Quality Assessment plays a very important role as a tool both for monitoring and improving quality.

Acknowledgements

The author wishes to express his appreciation of the wholehearted support given by the staff of NLBTC towards advancing the Quality culture. In particular, Dr M Contreras (Chief Executive-Medical Director), Dr B Brozovic (Consultant Haematologist), Dr J Seghatchian (Head of Quality Laboratory), and Mrs M Vickers, (Senior Clinical Scientist-LEQAS).

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of the same six selected and pooled blood samples at monthly intervals; these were also assessed by two cell counters and a standardised haemoglobinometer by the Quality Department. Each team tested these samples by both copper sulphate and haemoglobinometer techniques and returned results for analysis at NLBTC. The pilot was run for 10 sample distributions and the results were most promising as shown in Figure 1. The scheme permitted the identification of poor performers in the use of the haemoglobinometers.

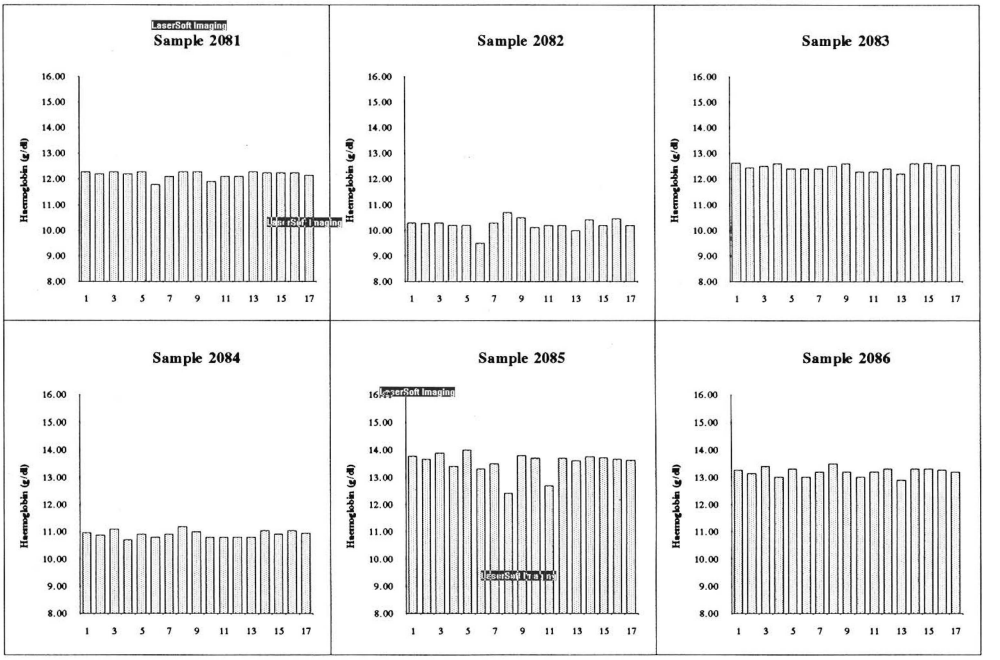

Fig 1. Haemoglobin LEQAS - Samples measured by haemoglobinometer at different sites

By further manipulation of the data accumulated over the all the monthly returns, it was also possible to compare the qualitative results by copper sulphate against the quantitative results obtained from the cell counter, as shown in Figure 2.

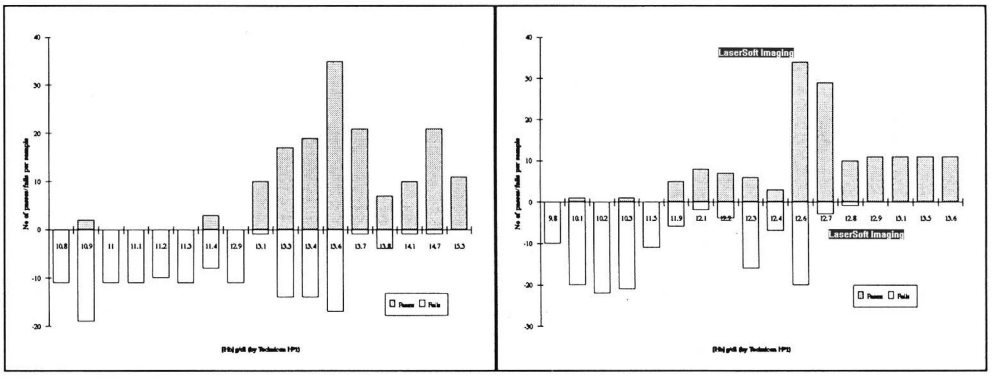

Fig 2. Haemoglobin Screen of Male (left) and Female (right) Donors by Copper Sulphate

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EQAS IN THE UK (NEQAS)

EQAS in the UK has been in existence for some 25 years, and has grown consistently over this time in serving the needs of clinical laboratories. At the present time there are some 58 schemes registered with the NEQAS Executive:

Schemes	Number
Autoimmune serology & special immunochemistry	12
Blood coagulation	1
Blood group serology	1
Clinical cytogenetics	1
Drug assays	6
General clinical chemistry	7
Haematology	2
Histopathology	5
Human leucocyte antigens	5
Hormones	8
In vitro allergy testing	2
Leucocyte immunophenotyping	3
Medical microbiology	3
Trace elements	2

A LOCAL EXTERNAL QUALITY ASSESSMENT SCHEME 'LEQAS' FOR HAEMOGLOBIN SCREENING OF BLOOD DONORS AT THE NORTH LONDON BLOOD TRANSFUSION CENTRE (NLBTC)[4]

In 1991, it was perceived at NLBTC that there was a need to assess the performance of each blood collection team at screening donors' haemoglobin (Hb) levels, prior to donation. According to established guidelines, men with Hb levels below 13.5 and women below 12.5 g/dL cannot donate whole blood. The screening of donors' Hb levels by the copper sulphate method, where a drop of blood from the donor's finger is observed sinking through the solution, is in routine use. Ambiguous or low results lead to a retest by HemoCue haemoglobinometer in order to obtain a quantitative result on site.

A pilot local quality assessment scheme was introduced in order to assess the machine/operator performance as well as influencing motivation. All teams were sent aliquots

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- Changes in reagents/instruments: may show a change in reagent batch although this is more usefully identified from a laboratories own internal QC.

- Changes in environment: some methods may be temperature sensitive, and differences may show in an uncontrolled working environment.

- Changes in personnel: this can be a useful indicator in the EQAS testing policies of some laboratories where the best person may be allocated the duties of testing EQAS samples. This then is tantamount to being a proficiency test of the worker not the laboratory, certainly not in keeping with the ethos of EQAS.

- Changes in methods: often but not always the case when a new method is introduced. Unfortunately, the nature of EQAS is such that if a new more accurate method is introduced and used only by few participants, it will show a bias because of the weighting effect of the many using other methods.

CRITERIA FOR EQAS

The following are some of the criteria that should be considered in using an EQAS fully an to best advantage:

- The parameter being measured must be appropriate to the method.

- Degree of quantitation and frequency of assessment must be compatible with work carried out in the laboratory. Thus a scheme distributing samples at two-weekly intervals would be less useful to a laboratory carrying out the test every three months.

- Variables associated with assessment must be known and understood.

- Presentation and communication of results must be clear, informative and timely. Late or irregular dispatch of results often bring discredit to the scheme and appear to diminish its authority.

- The scheme's limitations must be known and understood.

- The extent of coverage (whether international, national or local) may have an important influence on its usefulness. Truly international schemes tend to be commercial in origin and should analyse data not only in method groups, but also in national groups. National schemes predominate and an example is the UK NEQAS which has a very good and consistent track record and includes participants from outside the UK. Local schemes are certainly the most variable in quality and reliability, but they also provide the means of tackling situations that often cannot be covered by the bigger schemes.

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QUALITY ASSESSMENT

The approach that we know as Quality Assessment first developed out of a need for peer comparison of similar procedures carried in geographically disparate locations. As such, this technique lent itself well to the monitoring of performance of analytical, and later clinical laboratories

External Quality Assessment (EQA) is concerned with examining and reporting the differences between different sites testing the same analyte. Typically, one or more identical specimens are distributed to participants at regular intervals for assessment under the normal test conditions used by each particular test site. Results are returned to the Organiser by the due date and are then processed to evaluate a consensus result, spread, and method differences. Reports are sent to all participants and poor performers are highlighted and notified. Persistent poor performers may have further action taken in an effort to effect improvements.

The purpose of this paper is not to examine the schemes in detail, but to examine the basis and principles of EQA in the context of Total Quality. EQAS Organisers often publish detailed annual reports for their schemes and these should be consulted for more detailed information.[3]

INFLUENCES ON EQAS PARTICIPATION

As in most endeavours to improve quality, there are positive and negative reasons for progressive adoption of new practices.

On the positive side, one can cite that the past few decades have seen a significant increase in the level of quality awareness in laboratories. This has been driven by the need to demonstrate competence both to external bodies and customers. The development and imposition of Good Manufacturing/Laboratory Practices and the later introduction of laboratory accreditation schemes have assisted in this trend.

On the more negative side, the introduction of product liability legislation and the general global increase in litigation have established the need to continually assess performance against an independent benchmark and to have such data available for any eventuality.

WHAT EQAS MAY MEASURE

By their very nature, not all EQAS offer the same level of quantitation or analysis. However they will normally measure/detect the following:

- Accuracy and precision of method: some methods or procedures may show bias or be inherently less precise.

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- Quality is about inter-relationships not only outside an organisation but also within the organisation; hence the concept of the *internal customer.*

QUALITY TOOLS

A wide variety of tools is used in order to accomplish quality related tasks; each has its place and its particular relevance for one or more of the quality disciplines. The particular selection that follows is comprehensive but not necessarily exclusive:

Observation is the most basic of the tools available for Inspection and QC. Much can be learnt about the quality of a product by simple but informed observation of characteristics. Similarly in a service environment, observation can often be the first indicator of potential problems.

Monitoring is an important generic function when related to process quality. In its widest sense it can be used to describe any surveillance procedures, but there are instances where it has a more specific application. One such is in the Transfusion Service where quality monitoring is an activity akin to QC, but not carried out as a pre-release procedure.

Measurement is the fundamental tool of QC activities that are based on quantitation. This tool provides an admirable basis for the application of various appropriate numerical techniques for further interpretation.

Analysis is another fundamental tool of QC, applicable particularly on less quantifiable procedures, such as the testing of raw materials. The development of a more defined quality culture saw many Analytical Departments converted into QC Departments.

Trend Analysis is one of the newer tools that uses established mathematical techniques to monitor product or service quality over time, provided that quantitative data are available. Typical parameters used are means, medians, $\pm$nSD, maxima and minima. A particular development of this tool is **Statistical Quality Control** (SQC) which seeks to refine the technique by linking data collected with particular knowledge of a process.

Quality Audit is a means of monitoring compliance with a particular standard or guideline. Current variants include GMP audits, certification audits to ISO 9001, accreditation audits, etc. By their very nature, these are qualitative tools, but particularly powerful and influential if carried out properly by able auditors.

Quality Assessment in its widest sense includes any activity that elucidates the quality status of a product or service. It has sometimes been used in the past to indicate less than precise procedures that were not easily quantified, but nevertheless provided an accurate assessment. Nowadays, the term as used in the laboratory sciences is largely synonymous with External Quality Assessment Schemes, which form the remainder of this paper.

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observation of product, e.g. visual inspection, whilst the latter is normally based on a more quantitative procedure. These somewhat disparate and independent activities were later incorporated into **Quality Control** which ISO defines as *'the operational techniques and activities that are used to fulfil requirements for quality'*. Thus QC Units were set up in many organisations with specific responsibilities for ascertaining product quality through appropriate inspection and testing regimes. It should be noted however that all such initiatives were product related, not service related. Where laboratory services were concerned, the practice of using controls was established, in order to monitor variations within a particular laboratory. These practices come within the scope **Internal Quality Control** which assesses the performance of a laboratory or testing site, over real time, usually through the use of control samples.

All the quality related activities referred to so far have been reactive in nature and hence, while preventing customers from receiving a defective product or poor service, they did not address the root cause. This aspect was to be covered by **Quality Assurance**, defined as *'all those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality'*. For the first time, a proactive approach was being put forward whose main objective was to tackle the cause of defects rather than the effect. This approach has proved to be so successful that most organisations nowadays possess a QA unit of some sort.

With increasing regulation and legislation, the promulgation of various codes of Good Manufacturing Practice (GMP), the laboratories' equivalent Good Laboratory Practice (GLP), and various certification and accreditation schemes, as well as the rapid growth in quality concepts based on management and scientific principles, the QA concept has developed into **Quality Systems** which consider the organisational structure, responsibilities, processes and resources for implementing quality management. These developments are now covered by such international certification schemes as those based on the ISO 9000 series of broadly based quality systems standards and the more specific accreditation schemes, e.g. NAMAS and Clinical Pathology Accreditation in the UK.

The culmination of the development of the quality concept is **Total Quality Management** (TQM). The fundamental principles of TQM may be summarised as[2]:

- Quality is not the monopoly of particular persons or units within an organisation, but is the responsibility of everyone within it.
- Quality must be an intrinsic part of all processes and procedures from the start rather than inspecting, testing, or controlling it out at the end.
- The quality ethos is subscribed to by everyone within an organisation, including top management who must declare its commitment and provide appropriate resources.

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QUALITY ASSESSMENT AS AN ESSENTIAL TOOL FOR THE ACHIEVEMENT OF TOTAL QUALITY

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INTRODUCTION

The pursuit of the highest quality is one of mankind's most noble activities in that, in so doing, we are seeking to approach perfection in our endeavours. Though the explicit concept of quality is of recent rigorous definition, the original concept must have originated from beyond the mists of prehistory. Who can view the relics of antiquity or the artistic masterpieces of the Renaissance and not appreciate these products of artisans, craftsmen and artists for their intrinsic quality?

Whilst in many fields, this pursuit could be related mainly to personal satisfaction, peer acclaim and success in general, quality in healthcare-associated activities has all to do with people's lives and well-being. In this paper, the subject of Quality Assessment is examined as an overview by reference, firstly, to the fundamental and global concepts of quality and, secondly, to the variety of tools available for Quality Assurance. Finally, an example of a local Quality Assessment Scheme for haemoglobin levels in blood donors is then reviewed.

QUALITY CONCEPTS

One of the inherent difficulties in developing and implementing quality-related activities is that of definition of the commonly used, and often misused, terms used when discussing the various aspects of quality. For example, it is common to find the terms Quality Control (QC) and Quality Assurance (QA) used interchangeably, when in reality they refer to different but complementary activities. Similarly, modern use of language has resulted in the misuse of the word **quality** as referring to the best attainable, not as it should be, a mere description of the property of an object.

According to the International Standard ISO 8402[1], **Quality** is defined as *'the totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs'*. This definition has an important bearing on the formulation of quality policy as it injects realism and attainability and puts quality in the context of the needs of a product or service.

In tracing the development of the formalised concept of quality, one finds that the earliest forms of investigation consisted largely of Inspection and/or Testing; the former refers to

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3. Quality control and quality assurance

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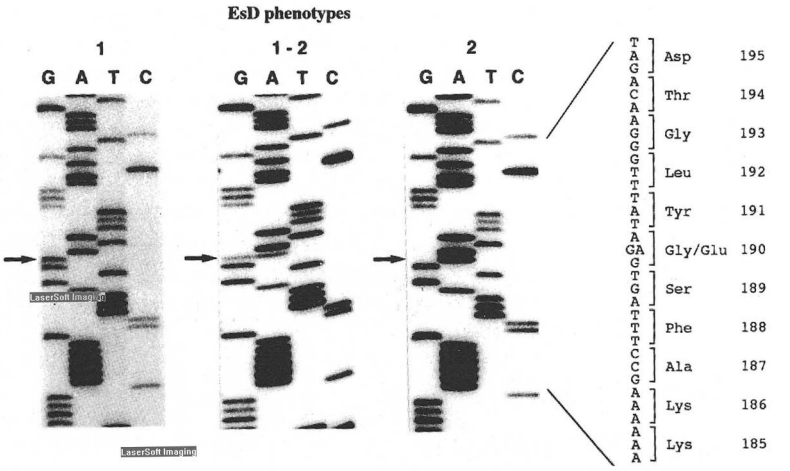

Fig. 1 Representation of the characteristic sequence difference among EsD phenotypes EsD 1, EsD 2-1 and EsD 2. Arrow heads indicate the polymorphic positions. A G-to-A point mutation is observed among EsD 1 and EsD 2 phenotypes. There are two bands(G and A) at the same level of the autoradiogram, which indicate that EsD 2-1 phenotype is heterozygous for the G-to-A mutation. The codon numbers and the corresponding amino acids are listed to the right.

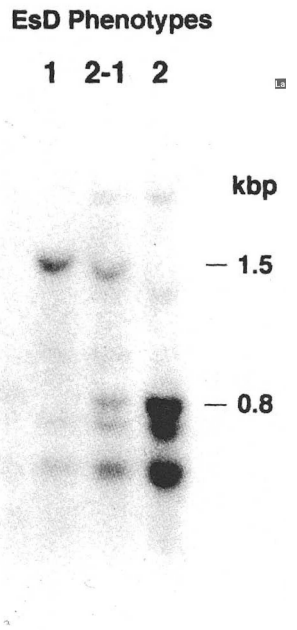

Fig. 2 Southern blot analysis of the polymorphic *Ssp* I digestion site on the genomic DNA caused by the G-to-A point mutation by the amplified EsD cDNA as a probe. Total DNAs from individuals exhibiting EsD 1 show a 1.5 kbp-band and lack a 0.8 kbp-band. Those from EsD 2 phenotype show a 0.8 kbp-band and lack a 1.5 kbp-band, indicating that a *Ssp* I restriction site occurs in the 1.5 kbp-band caused by G-to-A point mutation. Both of them are observed in EsD 2-1 phenotype. Approximately size is shown in the right.

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acid(Glu) in the cDNAs from EsD 1 and EsD 2 phenotypes, respectively. The G-to-A substitution between the cDNAs exhibiting EsD 1 and EsD 2 phenotypes led to the change of the digestion site with a restriction enzyme *Ssp* I. A new digestion site occurred in the cause of a point mutation in EsD 2-type cDNA. The cDNA from EsD 2-1 phenotype was heterozygote both of EsD 1 and EsD 2-type cDNAs. When genomic DNA samples from EsD 1, EsD 2-1 and EsD 2 phenotypes were digested with *Ssp* I and applied to southern hybridization analysis using the amplified EsD cDNA as a probe, two polymorphic fragments, which were approximately 1.5kbp and 0.8kbp in size, were observed(Fig. 2). In the individuals exhibiting EsD 1 phenotype, only the 1.5kbp-polymorphic fragment was detected. On the other hand, the samples from EsD 2 phenotype showed the 0.8kbp-polymorphic fragment instead of the 1.5kbp-fragment. Both of them were detectable in the individuals exhibiting EsD 2-1 phenotype.

DISCUSSION

We present here the sequences of EsD cDNAs from common three phenotypes and made clear the differences of EsD cDNA and the predicted amino acid sequences among different EsD phenotypes. When the pI values of EsD proteins predicted by cDNA sequences were calculated, although there were some differences between pI values observed by IEF and estimated by the predicted amino acid sequences, EsD 1 protein had higher pI value than EsD 2 protein, that was in accordance to that observed by IEF. The G-to-A substitution between cDNAs from EsD 1 and EsD 2 phenotypes makes the change of not only a predicted animo acid sequence but also a digestion site with restriction enzyme *Ssp* I. The difference of the digestion site makes it possibly and easily to determine the EsD phenotypes using DNA samples. EsD is present abundantly and ubiquitously in almost human tissues. EsD typing has been carried out using the lysate of red blood cells by some kinds of electrophoresis and enzyme activity using methylunbelliferyl esters as a substance. In the samples except red blood cells and in the specific condition, for instance, old samples which has lost EsD activity or the tiny samples which have no detectable EsD activity, EsD typing is difficult. However, using the RFLP of EsD gene with *Ssp* I , it is possible to determine the EsD phenotype from the samples without EsD activity.

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Molecular Analysis of Esterase D Polymorphism

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INTRODUCTION

Since the polymorphism of Esterase D(EsD) was described initially by Hopkinson et al. (1973), a number of population studies were reported. EsD polymorphism has been one of the most useful markers in genetic studies. Three common phenotypes, EsD 1, EsD 2-1 and EsD 2, are identified and determined by two autosomal codominant alleles, EsD^1 and EsD^2. Although the complete EsD cDNA have been sequenced, there are no information about the phenotypes of EsD. In this study, we make clear the relationship between EsD isozyme phenotypes detected by electrophoresis and DNA sequences. Moreover, a RFLP related to EsD polymorphism is presented and applied to the EsD typing by DNA samples.

MATERIALS AND METHODS

EsD phenotypes were determined by the method described by Hopkinson et al (1973) and isoelectric focussing electrophoresis. Peripheral blood samples were obtained from each three healthy individuals exhibiting three common EsD phenotypes, EsD 1, 2-1 and 2. Total RNA was extracted from the peripheral blood samples by guanidinium / hot phenol method (Maniatis et al.1982). Genomic DNA was isolated from peripheral blood samples using SDS-proteinase K treatment and phenol / chloroform extraction. Primers for the amplification and sequencing were constructed according to the DNA sequence presented by Young et al.(1988). Reverse transcription was carried out using Gene Amp™ RNA PCR Kit (Perkin Elmer-Cetus). The amplification of a DNA fragment including the coding region of EsD cDNA was carried out under the following conditions : denaturation, 1 min at 94 ℃ ; annealing, 1 min at 55 ℃ and extension, 3 min at 72 ℃; 30 cycles. After the generation of single-strand DNA fragments by an asymmetric PCR using inner primers, direct sequencing was carried out by employing a Sequenase™ Kit version 2.0 (United State Biochemical Co.). RFLP analysis was carried out using restriction enzyme *Ssp* I. An amplified EsD cDNA was used as a probe for detecting the RFLP patterns from genomic DNA samples.

RESULTS

The DNA fragments with a same length were amplified using total RNA samples from individuals exhibiting the different EsD phenotypes. Sequence analysis of the amplified EsD cDNA fragments demonstrated a single difference among three common EsD phenotypes. A guanine(G)-adenine(A) substitution was observed in the position of nucleotide 698 of EsD cDNA described previously (Young et al.1988)(This position was 569th nucleotide from the first nucleotide at starting codon)(Fig. 1). The nucleotides in the cDNAs of EsD 1 and EsD 2 phenotypes were G and A, respectively. In EsD 2-1 phenotype, there were two bands, G and A, at the same position of the autoradiogram. The G-to-A point mutation led to a substitution of the amino acid at residues 190. The predicted amino acids were glycine(Gly) and glutamic

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The *A, *B, and *C sequences were found to differ at three sites as indicated in the table below:

	*A	*B	*C
Exon 3F base 15	C	C	T
Exon 3S base 12	T	C	C
Exon 5 base 24	G	A	A

The first two substitutions are silent but may play a role in regulating f:s ratios. The substitution in exon 5 is in the second base of codon 105 and accounts for the Glu->Arg difference that distinguishes the *A type from the *B and *C types.

DETECTION OF *A, *B, AND *C ALLELES

The base substitution in exon 5 creates a TaqI restriction site in the *A allelic sequence not present in the *B and *C allelic sequences; this has been exploited in a typing system to distinguish the *A allele from the *B and *C alleles [15]. Amplification using primers ACP38 (5'GGATGTTTCAGAAGACCCTAGCAG3', sense strand) and ACP18 (5'GCTCCCAAGTAGTTCAATTTTAGC'3) yields a 149 bp product for each of the *A, *B, and *C alleles. Restriction with TaqI cleaves the *A allele product into 106 bp and 43 bp fragments; the *B and *C allele products are not cut.

The base substitution in exon 3F creates a CfoI restriction site in the *A and *B allelic sequences not present in the *C allelic sequence; this can be exploited in a typing system to distinguish the *C allele from the *A and *B alleles. Amplification using primers ACP10 (5'GTCGATCACCCATTGCAGAAGC3', sense strand) and ACP44 (5'CACACAACTTCAAGTCCAAGGACG3') yields a 303 bp product for each of the *A, *B, and *C alleles. Restriction with CfoI cleaves the *A and *B allele products into 177 bp and 126 bp fragments; the *C allele product is not cut.

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HUMAN RED CELL ACID PHOSPHATASE (ACP1): GENETIC TYPING AT THE DNA LEVEL

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Human red cell acid phosphatase (ACP1) is one of the best known of the forensically useful genetic polymorphisms. Two common alleles - ACP1*A and ACP1*B - are found in all major population groups. A third allele - ACP1*C - is common in Caucasians and a fourth - ACP1*R - is common in Blacks of African origin. A number of rare alleles have also been noted.

Each of the common alleles is expressed as a pair of electrophoretically distinct isozymes, termed fast (**f**) and slow (**s**) according to their anodal mobility. The **f:s** isozyme ratios for the *A, *B, and *C alleles are 2:1, 4:1, and 1:4 respectively [1]. This allelic variation in **f:s** ratio accounts for observed phenotypic variation in activity modulation by purines and folates, and in phosphotransferase activity [2-9]. The allele dependent variation in the amount of enzyme produced in red cells is independently regulated.

Recent protein and DNA sequencing studies show that the **f** and **s** isozymes are identical over the protein sequence regions spanning residues 1-39 and 74-157 but differ significantly (59%) in sequence in the middle region, residues 40-73 [10-12]. The sequence differences in this middle "signature" region presumably account for the differences in the catalytic and molecular properties of the **f** and **s** isozymes. The **f** and **s** isozyme pairs have been postulated to be generated by an alternative splicing mechanism in which the **f** and **s** signature sequences are encoded in mutually exclusive exons [10,13]. The gene structure of the ACP1 locus is consistent with this hypothesis [14].

Gene sequence analysis of the *A, *B, and*C alleles was undertaken to identify base substitutions that distinguish between them. A base substitution at codon position 105 should account for the glu-arg amino acid substitution that distinguishes *A from *B and *C [11,15]. Other base differences should account for the allele dependent variation in **f:s** isozyme ratio and in red cell activity level. Base differences between the alleles can also be used for general typing purposes.

ACP1 SEQUENCE AND GENE STRUCTURE

Genomic sequence spanning approximately 6 Kb of the ACP1 locus has been determined. Six linearly positioned exons have been identified; these account for all the coding sequence except for amino acid residues 1-13 at the N-terminus. The missing coding sequence appears to be located in an exon or exons at least 4 Kb upstream of the first sequenced exon. Genomic sequences of homozygous *A, *B, and *C types show the same gene structure.

As previously predicted, the **f** and **s** signature sequences are encoded in different exons, exons 3F and 3S; the two exons are of equal length. The intervening sequence is 41 bp, too short to serve as a functional intron. This gene structure is consistent with the mutually exclusive alternative splicing hypothesis.

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Further research will be needed to disclose the precise biological function and a detailed analysis of the ACP1 locus combined with deliberate manipulations of the sequence are required to determine the mechanisms that control the splicing pathway.

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DNA-based genotyping:
The allelic sequence differences generate restriction sites that can be used for PCR based genotyping as recently described [36]. However, the sequence differences can also be detected conveniently using the single-strand conformation polymorphism technique introduced by Orita [37]. This is based on the observation that single base substitutions when positioned within a relatively short DNA fragment (e.g. 100-300 bp PCR products) in many cases leads to a change in the electrophoretic mobility of the separated native strands. The reason for this is probably a change in the tri-dimensional conformation of the refolded single strands as caused be the base substitution. Using this technique on 240 bp and 190 bp genomic PCR products encompassing the mutational sites in exons 3F and 3S, respectively, it is possible to determine the common 6 ACP1 phenotypes within a few hours (Fig. 6).

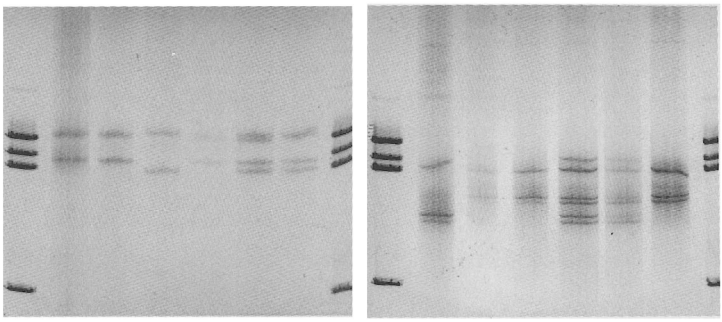

Fig. 6. Determination of classical ACP1 genotypes using PCR and SSCP. The electrophoretic separation was performed in precast 20% polyacrylamide gels (Pharmacia) for 300 Volt hours at 10^0C using the PhastSystem (Pharmacia). Anode at bottom. Lane 1,8,9, 16: Φx174 HAEIII ds size markers (603, 872, 1,078 and 1,353 bp); Lanes 2-7: 240 base ss-DNA encompassing the C-specific mutation at codon 43 in the 3F exon amplified from genomic DNA from individuals of ACP1 phenotype A, B, C, AB, AC and BC, respectively. Lanes 9-15: 190 ss-DNA encompassing the A-specific mutation at codon 41 in the 3S exon amplified from genomic DNA from the same individuals as in lanes 2-7.

Occurrence and physiological importance:
The "red cell" acid phosphatase has been detected in all tissues tested [38,39] and is identical to the cytosolic low-molecular-weight acid phosphatase which has been isolated from diverse vertebrate species [22,40-45]. Thus a high degree of sequence homology has been found between the human, bovine and rat enzymes [28,44,46]. The generation of two isoforms appears to be a general property of low-molecular-weight acid phosphatase genes [29 and references cited therein].

The physiological function (or functions) of the low-molecular-acid phosphatase has yet to be determined. However, certain phosphotyrosyl proteins are hydrolysed by the enzyme and recent results suggest it to play a role in the important control of protein phosphotyrosine levels, which affects cellular processes such as proliferation, differentiation and transformation [47-51]. On the other hand, flavin mononucleotide (FMN) is also a naturally occurring substrate [14,52,53]. By influencing the level of FMN and thereby FAD the low-molecular-weight acid phosphatase may thus affect the activity of flavoenzymes, which are of importance for a number of cellular processes. Given the different properties of **f** and **s** isozymes it is likely that they serve different cellular functions.

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generated from each allele, the messengers consisting of the N,F,C and the N,S,C exons, respectively (Fig. 4) [26,28]. Mutational events in the N or C exons common to **f**-mRNA and **s**-mRNA would alter the **f** and **s** isozymes to the same extent, precisely as is seen with the Gln-105 to Arg substitution in the **A** isozymes. This offers a simple mechanism for the pairwise variation in the electrophoretic mobility of the **f** and **s** isozymes as observed for the various ACP1 alleles.

Although the splicing model has been questioned by Wo et al. [32] it is strongly supported by recent DNA-sequencing results [33]. Genomic sequence spanning approximately 6 kb of the ACP1 locus was determined and it was found that the **f** and **s** specific sequences are indeed encoded by two distinct exons (E3F and E3S) interspaced by a 41 bp non-coding segment. The E3F and E3S exons are flanked on either side by linearly arranged exons (E2, E4, E5, E6) encoding the amino- and carboxy-terminal sequences identical in the **f** and **s** isozymes (Fig. 5). The six exons account for all the coding sequence except that for amino acid residues 1-13. Genomic sequence 3' to E2 as well as the interior portions of the 3Kb and 1.8Kb introns have yet to be determined. Interestingly the 41 bp intervening sequence between exons 3F and 3S is too short to act as a functional intron [34]; this is consistent with a mechanistic model for mutually exclusive spliced exons in which a transcript segment containing either E3F-I3F or I3F-E3S is spliced out in the formation of the

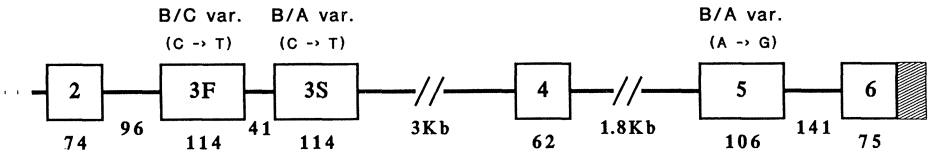

Fig. 5. Scheme of the ACP1 gene locus of human low-molecular-weight acid phosphatase. Boxes represent exons, lines represent introns, and the hatched box represents 3' non-coding sequence. Exon and intron sizes (bp) are indicated below. Allelic base transitions are indicated above the respective exons.

messengers for the **s** and **f** isozymes, respectively. Only three base substitutions, all located internally in exons, have so far been found to distinguish the ACP1*A, ACP1*B and ACP1*C alleles. An A-G transition in codon 105 of E5 accounts for the Gln-105 to Arg substitution that distinguishes the A isozymes from the B and C isozymes. The other two substitutions, both silent C-T transitions, are interesting in that they are located 15 and 12 bases into the 3F and 3S exons, respectively. The former distinguishes the ACP1*C alleles from the ACP1*A and ACP1*B alleles and the latter the ACP1*A alleles from the ACP1*B and ACP1*C alleles. Exon based substitutions located in the vicinity of a splice site may influence splice site recognition [35]. They may therefore provide the basis for allele specific splicing pathways, which result in the quantitative differences of **f** and **s** isozymes as expressed by the ACP1*A, ACP1*B and ACP1*C alleles [23].

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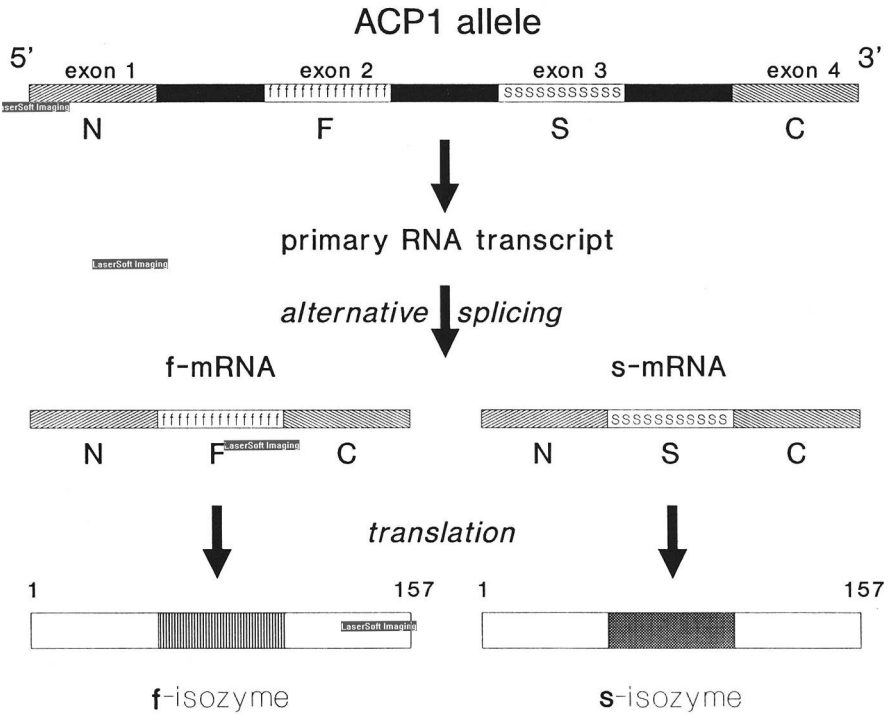

Fig. 4. Proposed mechanism for the generation of **f** and **s** isozymes by ACP1 alleles.

The specific **f** and **s** sequence segments must account for the differences in catalytic and molecular properties between the **f** and **s** isozymes. The **s** specific sequence contains an extra basic residue (His-69) which explains the lesser anodal mobility of the **s** isozymes compared to the corresponding **f** isozymes (Fig. 3). His-69 may also be identical to the histidine residue which appears to participate in the catalytic activity of the **s** isozymes [22]. The **f** specific sequence exhibits a lesser capacity for hydrophobic and a higher capacity for hydrophillic interaction than the **s** specific sequence [28]. This may account for the lower stability of the **f** isozymes relative to the **s** isozymes.

Structure of the ACP1 locus:

Based on the results obtained by peptide mapping of the **Bf** and **Bs** isozymes it has been hypothesized that the generation of **f** and **s** isozymes are the result of alternative splicing of the primary RNA transcript [26]. This hypothesis was further supported by the subsequent determination of the primary structure of the 6 common **f** and **s** isozymes [27-29]. The hypothesis predicted that the ACP1 locus is composed of at least 4 coding sequences, exons (A, F, S, C), interspaced with non-coding sequences, introns. The exons code for the amino-terminal region, the **f** specific region, the **s** specific region and the carboxy-terminal region of the isozymes, respectively. By alternative splicing of the primary RNA transcript two different mRNA molecules, a **f**-mRNA and a **s**-mRNA, are

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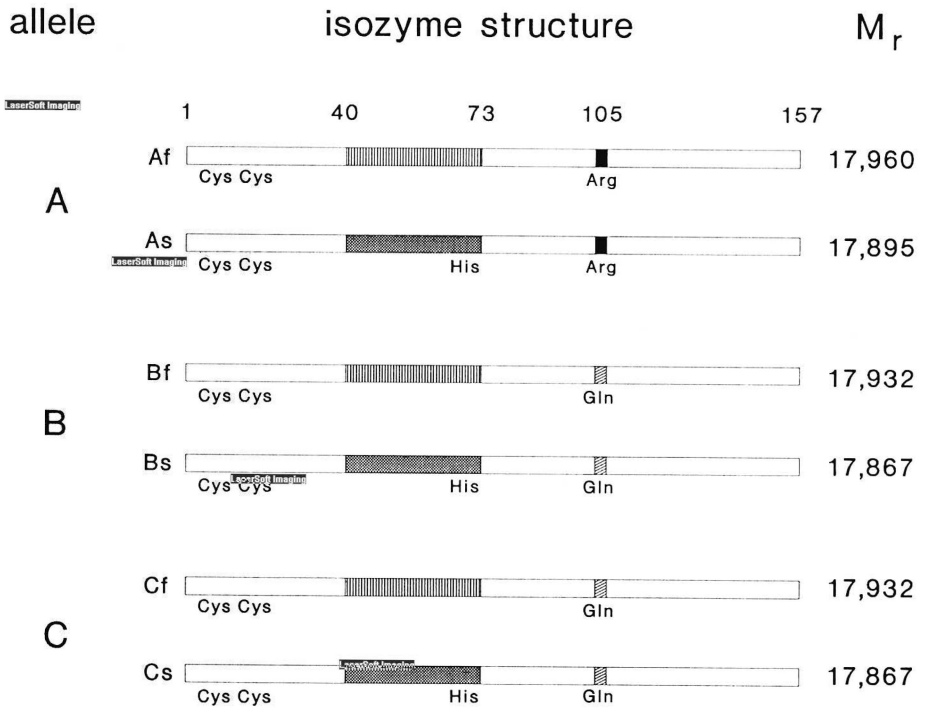

Fig. 3. Diagram of the 6 common isozymes of human "red cell" acid phosphatase.(||||||) **f** specific segment, (xxxxxx) **s** specific segment. Cys-12, Cys-17 and His-69 which all may be at the active site are indicated by Cys and His.

With the determination of the complete amino acid sequence of the **Bf** and **Bs** isozymes and subsequently of the **Af, As, Cf** and **Cs** isozymes a surprising structural relationship between these isozymes was revealed [27-29]. Each isozyme consists of a single non-glycosylated peptide chain of 157 amino acid residues which is acetylated at the amino-terminal alanine. The most striking feature is the existence of a specific **f** or **s** sequence segment, residue 40-73, over which the **f** and **s** isozymes differ substantially (Fig. 3). **f** and **s** isozymes of the same genetic type are identical over the remaining four-fifth of the sequence. Another interesting feature is that the **Bf/Bs** pair of isozymes is identical in sequence with the **Cf/Cs** pair of isozymes. Thus the ACP1*B and ACP1*C alleles encode exactly the same isozymes, the only difference at the protein level being the proportion of **f** and **s** isozyme and the quantity of enzyme protein in the cell [23,29]. The **Af/As** isozyme pair differs from the **Bf/Bs** isozyme pair at a single amino acid residue due to a Glutamine-105 to Arginine substitution. The substitution of a neutral residue with a basic residue in the **A** isozymes explains the higher isoelectric points of the **A** isozymes relative to those of the corresponding **B** and **C** isozymes [30]. Both **f** and **s** isozymes contain 8 cysteine residues at identical positions, all occurring as free sulphydryls. Two of the cysteines appear to be located at the active site [31] (Fig. 3).

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Table 1. Catalytic parameters for the 6 common acid phosphatase isozymes as determined at pH 6.0, 30^{0}C, using *p*-nitrophenylphosphate as substrate.

				activity modulation (%)[2]	
isozyme	sp.act.[1]	Km (mM)	Ki (mM)[3]	adenine	hypoxanthine
Af	34	0.14	0.47	61	501
Bf	33	0.14	0,44	60	483
Cf	33	0.14	0,43	62	491
As	46	0.53	0,81	383	100
Bs	49	0.51	0,86	407	100
Cs	49	0.52	0,73	407	100

[1]Specific activity is expressed as μmol of *p*-nitrophenol liberated per min per mg of enzyme-protein.
[2]The maximal or minimal activity at saturating concentrations of the modulator expressed as percent of the activity without the modulator.
[3]Inhibition constants of the competitive inhibitor and substrate analogue *p*-aminobenzylphosphonic acid.

of the phenotypes [25]. The genotypic activity variation (ACP1*A < ACP1*B < ACP1*C) is mainly a consequence of the similar variation in the enzyme protein level (but also of the different specific activity of the **f** and **s** isozymes and their relative distribution). The genotypic orders with respect to activity modulation by purines (ACP1*B < ACP1*A < ACP1*C or ACP1*C < ACP1*A < ACP1*B) are accounted for by the different proportions of **f** and **s** isozyme and by the activation or inhibition of these two isozymes exerted by the modulators [24]. Similarly the phenotypic differences in phosphotransferase activity appears to be caused by different properties of **f** and **s** isozymes, as indicated by the higher transferase activity of **f** isozyme relative to **s** isozyme [15]. The higher relative stability of **s** isozymes explains the higher stability of the BC and AC phenotypes as compared to the A, AB and B phenotypes [16,17], since the former phenotypes have a higher content of **s** isozyme [23].

Structure of the isozymes.

A decisive proof against the conformational isomer hypothesis came from unfolding/refolding experiments with the **Bf** and **Bs** isozymes [26]. Unfolding of differently folded isomers of the same polypeptide chain should reduce these to a common molecule, and renaturation of either of the unfolded isomers should result in a mixture of the isomeric forms; however, neither was found to be the case. Peptide mapping revealed that significant structural differences distinguish the two isozymes, but also that large parts of the molecules are identical. These findings were also incompatible with one isozyme being the product of simple posttranslational modifications of the other [26].

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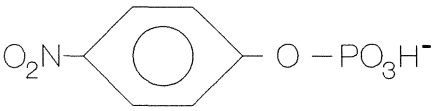
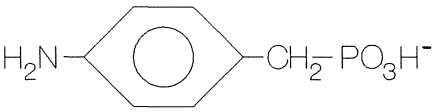
ment of an efficient purification method by which **f** and **s** isozymes were purified for the first time [21,22]. The procedure involves affinity chromatography on a medium containing the substrate analogue p-aminobenzylphosphonic acid (Fig. 2) and allows the isolation of the acid phosphatase isozymes in mg quantities from red cells with a purification factor of 30,000.



p-nitrophenylphosphate



p-aminobenzylphosphonic acid

Fig. 2. Substrate (top) and substrate analogue of "red cell" acid phosphatase.

Using antibodies produced against homogeneous isozymes it was found that **f** and **s** isozymes of each genetic type contain different as well as common epitopes. No immunochemical differences were observed between the **Af, Bf** and **Cf** isozymes or between the **As, Bs** and **Cs** isozymes [23]. The isozymes were quantitated in red cells from individuals of the various phenotypes using crossed immunoelectrophoresis and it was found that the quantity of enzyme protein paralleled the enzyme activity level, viz. ACP1*A < ACP1*B < ACP1*C ≈ 2:3:4 [23]. Further, it was found that the proportion of **f** and **s** isozyme is allele dependent, the **f:s** ratio being 2:1, 4:1 and 1:4 for the ACP1*A, *B and *C alleles, respectively. The order of this allelic effect (ACP1*C < ACP1*A < ACP1*B) is thus the same as that found at activity modulation. Analysis of the thermostability and the sensitivity to denaturation with urea showed that the **Af, Bf** and **Cf** isozymes have identical stability as have the corresponding three **s** isozymes, whereas the **s** isozymes are significantly more stable than the **f** isozymes [22]. Also in size exclusion chromatography and in SDS polyacrylamide gel electrophoresis differences were observed between **f** and **s** isozymes, whereas identical properties were found within each of these two species of isozymes [22].

A similar pattern was observed with respect to the catalytic properties of the isozymes. The three **f** isozymes (**Af, Bf, Cf**) were indistinguishable in all the measured parameters (specific activity, Michaelis constants, inhibition constants, activity modulation) and the the same was the case for the three **s** isozymes, whereas the **f** and **s** isozymes differed significantly in all the parameters [22,24]. Estimation of the phosphotransferase activity has shown the **Bf** isozyme to be more than twice as active as the **Bs** isozyme [15]. The most striking finding was the diametrically opposite response of **f** and **s** isozymes to modulators; the activity of the **f** isozymes was increased 5.1 fold by hypoxanthine and decreased 40% by adenine, while the activity of the **s** isozymes was unaffected by hypoxanthine but increased 4.6 fold by adenine. Examples of the catalytic properties are given in Table 1. The enzyme kinetic results indicated the presence of a histidine residue in the **s** isozymes, but not in the **f** isozymes, which is of importance for the catalytic function [22].

The important conclusion, therefore, is that the **f** and **s** isozymes differ in all the properties tested, while the **f** isozymes (**Af, Bf, Cf**) are identical in these properties (except electrophoretic mobility) as are the corresponding **s** isozymes, despite the different genetic types. It therefore follows, that from a functional point of view the common phenotypes of acid phosphatase consist of only two different isozymes, **f** and **s**. The allelic differences in quantity and relative proportion of these two isozymes result in the different properties

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THE MOLECULAR BASIS OF THE "RED CELL" ACID PHOSPHATASE POLYMORPHISM

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Introduction.

The "classical" genetic markers, poetically referred to as "old friends" by Dr. Sensabaugh, include the fascinating enzyme "red cell" acid phosphatase (ACP1). This polymorphism was discovered in 1963 by Hopkinson et al. [1] who used the powerful new starch gel electrophoretic technique introduced by Smithies [2] on human hemolysates. ACP1 was thus the first genetical enzyme polymorphism to be discovered. To date at least 17 allelic genes have been observed at the ACP1 locus located to a distal region (2p25) on the short arm of chromosome 2. The ACP1*A and ACP1*B alleles prevail in most populations, ACP1*C occurs in polymorphic frequencies in European populations and ACP1*R in certain populations in Southern Africa [3-11].

Acid phosphatase was found to possess several puzzling characteristics. These include genotypic variations of enzyme activity levels [12], activity modulation by purines and pteridines [13,14], phosphotransferase activity [15] and stability [16,17]. Furthermore, the allelic ordering of these variations were found to differ. Thus, ACP1*A < ACP1*B < ACP1*C was found for activity levels, while ACP1*B < ACP1*A < ACP1*C was observed at modulation by pteridines and certain purines (e.g. adenine) or ACP1*C < ACP1*A < ACP1*B for modulation by certain other purines (e.g. hypoxanthine). The order ACP1*A, ACP1*B < ACP1*C (viz. phenotype A, AB, B < AC and BC) was observed with respect to heat stability. Another characteristic property is the expression of each allele as two different isozymes [1], termed "fast" (f) and "slow" (s) in accordance with their electrophoretic mobility [18](Fig. 1). These two isozymes were long believed to be conformational isomers [19,20].

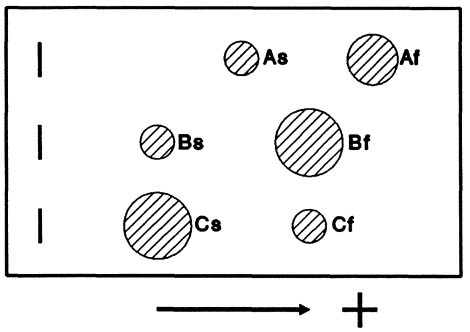

Fig. 1. Electrophoretic patterns of the homozygous ACP1 phenotypes A (top), B and C (citrate buffer, pH 5.9). The allelic **f** and **s** isozymes are denoted **Af** and **As** etc. Application at vertical lines.

Results obtained mainly over the last few years have provided a considerable insight as to the properties and function of the acid phosphatase isozymes and to the structure of the gene. The purpose of the present paper is to review these results and to relate them to the early findings.

Properties of the isozymes.

Of major importance for the analysis of the acid phosphatase isozymes was the develop-

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In the interests of brevity, the references listed above tend to be survey articles; a complete reference list would have been much longer. I apologize to any that may not have been included.

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ADENYLATE KINASE

Due to its small size and important role in energy metabolism, adenylate kinase (AK) has been extensively studied [28]; protein sequences are known for several species and the 3-D structure has been solved for the pig enzyme. This allows interpretation of the sequence data generated for the human allelic products.

Human AK is a monomeric enzyme, 194 amino acids in length. The AK1*1 and AK1*2 allelic proteins differ in a glu->gln substitution at residue position 123. This substitution accounts for the different electrophoretic mobilities of the allelic products. Residue position 123 sits at the end of an alpha helix which arches over the top of the catalytic site and which contains two args which interact with substrate. Glu at this position forms an ion pair with arg-127 further on in the helix, contributing to the stability of the helix and the catalytic site. Substitution of a gln at this position potentially destabilizes both. Thus, the substitution can account also for allelic differences in activity and stability.

CONCLUSION

This excursion into the molecular biology of the classical markers provides some insights into their behavior. Although these markers are fading from use, we are now in a position to understand at a fundamental level the nature of our experience with their past use.

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The first protein and cDNA sequence analyses of Gc indicated homology to two other plasma proteins, albumin and alpha-fetoprotein; Gc is a somewhat truncated form of the other two [24]. This homology allows a 3-D structure for Gc to be modeled on the 3-D structure of albumin; since albumin is also a ligand binding protein, the ligand binding domains of Gc can also be posited. The vitamin D and G-actin binding regions appear to be situated at the opposite ends of the protein, in segments spanning residues 14-58 and 350-403 respectively [23]; these correspond to binding domain IA and III in albumin.

The homology of Gc with albumin and alpha-fetoprotein allowed primers to be designed for sequencing Gc exons without interruption by introns. The sequence substitutions for the common *1F, *1S, *2, and *1A1 variants were located in exon 11 at codons 416, 420, and 429. The substitutions distinguishing the *1F, *1S, and *2 alleles are at 416 and 420 and are both transversions, T->G at 416 and C->A at 420; a G->A transition at 429 defines the 1A1 allele [25]. Interestingly, the 1A1 polymorphism appears identical in Australian aborigines and American blacks. The corresponding amino acid substitutions are indicated in the table below and account for the observed electrophoretic variation among the types.

	416	420	429	net charge pH7
Gc*1F	asp	thr	arg	0
Gc*1S	glu	thr	arg	0
Gc*2	asp	lys	arg	+1
Gc*1A1	asp	thr	his	-1

This pattern of substitution suggests that Gc*1F is the ancestral form of the gene. None of these residues are located within the identified ligand binding regions although they are found in the same domain as the G-actin binding region. Their biological significance, if any, remains to be determined.

HAPTOGLOBIN

Haptoglobin (Hp), like Gc, was one of the first protein polymorphisms to be identified in humans. It is unique among the common genetic polymorphisms in possessing allelic forms that differ in size as well as charge. Three common alleles, Hp*1F, Hp*1S, and Hp*2, are found in all populations. Each encodes a single polypeptide chain which undergoes posttranslational cleavage yielding an alpha-chain and a beta-chain; these combine as alpha-beta dimers in the Hp 1 types and as extended multimers in the Hp 2-1 and 2 types [24].

The Hp*2 alpha-chain is nearly twice as large as the Hp*1F and *1S alpha chains, ca. 16,000 vs. 8,000. It was originally postulated that the Hp*2 allele arose through an end-to-end partial duplication. Gene sequence analysis has demonstrated that this is in fact the case [26]. The junction point for the duplication connects a point in intron 4 of the *1F gene sequence with a point in intron 2 of the *1S gene sequence, forming a FS hybrid; the appropriate splice sites were in frame, allowing the formation of a functional extended gene.

Gene sequence analysis has also revealed the basis for the Hp 2-1mod phenotype found in Blacks [27]. This phenotype is determined by a mutation in the promoter region of an Hp*2 allele; this mutation results in reduced expression of the Hp*2 allele product, possibly through altered interaction with an IL-6 response element. There is evidence that other phenotypic variation in Hp expression might be the result of mutation in the Hp promoter region.

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PSA/p30 has an amino acid substitution at its active site that precludes true kallikrein activity. The enzyme is present in an active form in semen and has been demonstrated to be involved in the liquefaction of the seminal clot by specific cleavage of a major seminal vesicular protein [14]. (In contrast, PSA/p30 leaked into blood plasma is over 90% complexed with the serine protease inhibitor alpha anti-chymotrypsin.) Although the 3-D structure of PSA/p30 has not been solved, the 3-D structure is known for a number of serine proteases; based on structural homologies, it is possible to model the physical structure of PSA/p30. This allows the use of rational drug design methods to develop specific synthetic substrates.

SALIVARY AMYLASE

Human saliva contains very high levels of amylase activity; this activity is higher by several orders of magnitude than amylase activity in other human body fluids and tissues [15,16]. Salivary amylase is also found in other primates, in rodents, and in lagomorphs but not at appreciable levels in other mammals [17]. Amylase is ubiquitously expressed in mammalian pancreatic tissue and as a result is found in blood plasma, urine, and feces. The human salivary and pancreatic amylases can be distinguished electrophoretically and have been determined to be the products of two distinct loci, AMY1 and AMY2, respectively [18]. Forensic efforts to develop enzymatic and immunological tests to distinguish the salivary and pancreatic amylases have not been highly successful.

Molecular biological studies have provided considerable insight into our understanding of amylase expression in humans. Sequence comparison at the protein and DNA levels shows that the salivary and pancreatic amylases are highly homologous; the protein sequences are 97% identical [19]. The two human amylases are more similar to each other than either is to the corresponding mouse amylases, indicating that the human AMP1 and AMY2 genes diverged after the split of the two species. The high sequence homology between the salivary and pancreatic amylases no doubt accounts for the relative failure of enzymatic and immunological tests to qualitatively distinguish the two.

The AMY1 and AMY2 genes are located in a gene complex on chromosome 1 at 1p21. The normal human genome in fact contains three AMY1 genes (AMY1A, AMY1B, and AMY1C) and two AMY2 genes (AMY2A and AMY2B); variant genotypes containing more or fewer AMY1 genes have been reported [20]. The AMY1 genes differ from the AMY2 genes in having two inserted elements, an actin pseudogene and a retroviral-like segment, located 5' to the coding sequence. A 1 Kb segment immediately 5' to the coding sequence, consisting entirely of sequence from the inserted elements, has been demonstrated in reporter gene constructs to be responsible for tissue specific expression in parotid gland; the inserts appear to have combined to form a tissue specific promoter for AMY1 expression [21]. The identification of this unique tissue specific promoter accounts for the expression of amylase in human saliva.

GROUP SPECIFIC COMPONENT

Group specific component (Gc) was one of the first genetically polymorphic proteins to be identified. At present, three common alleles are recognized, Gc*1F, Gc*1S, and Gc*2, and over 100 rare variants are known [22]; it is one of the most polymorphic of the plasma proteins. The protein possesses strong binding affinity for several biologically significant ligands including vitamin D, G-actin, complement factor 5a, fatty acids, and lymphocyte immunoglobulin receptor; its primary function is believed to be the plasma transporter for vitamin D but obviously other functions cannot be discounted [23].

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Nevertheless, the homologies in sequence and intron structure are evidence of a common evolutionary origin. Comparison of the human PAP and LAP sequences to rat secretory and lysosomal acid phosphatase sequences shows stronger homologies between enzyme types than between the enzymes from a species [7], suggesting that the PAP and LAP ancestral genes split prior to the split between primates and rodents some 50 million years ago.

The regulation of the PAP and LAP genes is markedly different. The promoter region of LAP is typical of "housekeeping" genes and the gene appears to be expressed in most tissues [5]. In contrast, high expression of PAP is known to be androgen dependent and PAP mRNA can be detected only in prostatic tissue among tissues surveyed [8]. Significantly, PAP mRNA was not detected in placental tissue, a tissue which does express ACP3; this provides evidence that PAP and ACP3 are encoded at separate genetic loci.

There have been no studies on acid phosphatase expression in vaginal tissue. Accordingly, it is not known whether PAP is expressed in this tissue or whether VAP is related to ACP3. The question of PAP's tissue specificity is thus nearly but not completely resolved. However, the tools are available to answer the question.

PROSTATIC ANTIGEN p30

The prominent semen protein of about 30,000 molecular weight has been "discovered" by at least four different groups [9]; it has been called variously gamma-semenoprotein, E1 protein, p30, and prostate specific antigen (PSA). Although there has been some argument that these proteins were not the same, sequence analyses and immunological studies have firmly established that they are in fact identical entities. Under the name PSA, the protein has become the marker of choice for early detection of prostatic cancer and for monitoring the progression of cancer in affected individuals. As p30, it has been used as a marker for semen in forensic testing.

The initial sequence analysis of PSA/p30 established its identity, theretofore unknown, as a serine protease [10]. Subsequent studies refined this identification and placed PSA/p30 in a specific family of serine proteases, the kallikreins [11]. There are two other members of the human kallikrein family, tissue kallikrein (KLK1) which is expressed in various tissues including kidney, pancreas, and salivary gland, and glandular kallikrein (KLK2) which like PSA/p30 appears to be expressed only in prostate tissue. PSA/p30 shows 60% sequence homology to KLK1 and 78% sequence homology to KLK2. The homology of PSA/p30 with KLK1 could account for the anecdotal reports of rare positive immunological reactions of anti-p30 with saliva; the two proteins could share a cross-reactive epitope recognized by some antisera.
PSA/p30, KLK1, and KLK2 have nearly identical gene structures, each with 5 exons and each spanning about 5 Kb, further affirming their common evolutionary origins. The three genes are located in a gene complex on chromosome 19 at 19q13. The gene order is KLK1 - 31 Kb - PSA/p30 - 12 Kb - KLK2.

Like PAP, both PSA/p30 and KLK2 mRNA expression is regulated by androgens; this is not surprising given the near identity of the promoter regions of the two genes [12,13]. Although KLK2 is expressed at the mRNA level, a secreted protein corresponding to KLK2 has not been identified; either the mRNA is not translated or tests with the necessary sensitivity and specificity have yet to be employed. The expression of PSA/p30 mRNA appears to be limited to the differentiated epithelial cells of prostatic tissue; this affirms previous findings on the tissue specific expression of PSA/p30 protein.

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Table 2
MOLECULAR KNOWLEDGE OF TISSUE SPECIFIC PROTEIN MARKERS

Marker	Sequence	Gene Struct.	Regulation	Homology	3-D
Hemoglobin	P, cDNA	+	+	+	+
Pros. ACP	P, cDNA	+	+	+	(+)
Pros. p30	P, cDNA	+	+	+	(+)
Sal. Amylase	cDNA	+	+	+	

PROSTATIC ACID PHOSPHATASE (PAP)

Prostatic acid phosphatase has been used for nearly 50 years as a marker for semen; it also has a venerable history as a marker in clinical testing for prostatic cancer. It was established many years ago that the activity of PAP is inhibited by tartrate and that this could be used to distinguish PAP from red cell (cytoplasmic) acid phosphatase. Subsequent biochemical studies showed that PAP belongs to a group of acid phosphatases defined by a molecular weight of 100-110,000, referred to here as the 100K acid phosphatases. The 100K acid phosphatases are dimeric proteins with subunit molecular weights of about 50-55,000; they are glycosylated and tartrate inhibition is an additional defining characteristic. [For a recent review, see ref. 1] In addition to PAP, the 100K group includes lysosomal acid phosphatase (LAP) and, of particular forensic interest, the acid phosphatase present in vaginal fluids (VAP).

Genetic studies established that there exist at least two distinct genes for 100K acid phosphatases, ACP2 located on chromosome 11 and ACP3 with an unknown chromosomal location [2]. LAP has the same electrophoretic mobility as ACP2 and was presumed to be a product of the ACP2 locus. VAP has an electrophoretic mobility similar to the ACP3 gene product and PAP migrates somewhat anodal to both. PAP, VAP, and ACP3 gene product from placenta are immunologically cross-reactive suggesting a close molecular relationship [3]. The mobility of all three can be altered by desialidation and it is clear that variable glycosylation can alter electrophoretic migration patterns. Based on this evidence, I previously suggested that PAP and VAP might be products of the ACP3 locus [3]. The major forensic implication of this suggestion is that the specificity of the acid phosphatase test for semen resides in the 1000-fold excess of acid phosphatase activity in semen relative to other body fluids and tissues; in short, it was suggested that the specificity of the test is quantitative rather than qualitative.

Recent molecular studies shed new light on the relationships among the 100K acid phosphatases and the tissue specificity of PAP. LAP and PAP have been sequenced as cDNA and the gene structure determined for both [4-6]. The gene for LAP has been determined to be on chromosome 11, affirming its probable identity with ACP2. The gene for PAP has been assigned to chromosome 3 at 3q21-ter. The secreted form of PAP has two identical subunits 354 amino acids in length. The LAP subunit is 393 residues long and consists of three parts: a 350 residue core enzyme followed at the C-terminal end by a 24 residue transmembrane segment and a 19 residue cytoplasmic segment. The sequences of PAP and the LAP core segment are about 50% homologous; critical features such as active site residues, cysteine positions, and glycosylation sites are conserved. The positioning of introns in the two genes is nearly identical; the LAP gene has an additional exon 3' to the core sequence exons which encodes the transmembrane and cytoplasmic segments. There is a significant difference in the structure of the two genes; despite the common placement of introns, the PAP gene spans about 50 Kb compared to about 10 Kb for LAP.

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A NEW LOOK AT OLD FRIENDS: THE MOLECULAR BIOLOGY OF THE PROTEIN MARKERS

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Until the advent of DNA technology, protein markers occupied center stage in the armamentarium of forensic biology. Body fluid identification is based on the detection of tissue specific protein markers, e.g., hemoglobin for blood, prostatic acid phosphatase and prostatic antigen p30 for semen, amylase for saliva, and so on. More prominently, the core group of genetic markers used for individualization and paternity testing consisted primarily of polymorphic red cell enzymes and plasma proteins; these markers now have been eclipsed for the most part by DNA markers.

Before taking full leave of the protein markers, it is instructive to look at what has been learned about them using the tools of molecular biology. Many of the protein markers have been sequenced at the protein, cDNA, and/or genomic DNA levels. This sequence information provides insight into the basis of polymorphic and tissue specific variation; it can also indicate homologies among proteins and evolutionary relationships. The 3-D structure has been solved for some of the markers, providing additional insights into structure-function relationships. Tables 1 and 2 summarize the current level of knowledge about the tissue specific markers and polymorphic markers.

The discussion that follows focuses on insights provided by molecular biology to some of these markers with an emphasis on the markers of tissue origin. Other markers are discussed in accompanying papers by Hopkinson, Mayr, and Dissing.

Table 1
MOLECULAR KNOWLEDGE OF POLYMORPHIC MARKERS

Marker	Sequence	Gene Struct.	Polymorphism	Homology	3-D
Hp	P, cDNA	+	+	+	
Gc	cDNA	+	+	+	+
Ig-Km	P	+	+	+	+
Ig-Gm	P, cDNA	+	+	+	+
PGM	cDNA	+	+	+	
AK	P		+	+	+
ADA	cDNA	+	+		
EsD	cDNA	+[a]	+	+	
ACP1	P, cDNA	+[a]	+		
GLO	cDNA				
ABO	cDNA	+[a]	+		
MN	P, cDNA	+	+	+	+[b]
Rh	cDNA	+	+		
HLA I	P, cDNA	+	+	+	+
HLA II	cDNA	+	+	+	+

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a - Detail not complete, b - Antigenic region structure modeled from NMR data

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2.2.2.4. RFLPs and microsatellites between HLA-B and HSP70
Three polymorphic genes (with 4, 3 and 7 alleles, respectively) situated between HLA-B and BAT1 can be recognised with RFLPs. Again, there is a strong gametic association between the alleles of these loci and HLA class I alleles. Furthermore, several microsatellite loci coding for a variable number of CA repeats are located between HSP70 and HLA-B.

3. CONCLUSIONS

By using molecular genetic methods, it is possible to increase the HLA polymorphism, thus reaching a better definition of the HLA haplotypes. If only the class II alleles are considered (tested e.g. by PCR-SSO), the chance of paternity exclusion reaches 90%.
Due to the very strong linkage disequilibrium between the alleles at the various loci, the informations obtained by molecular genetics are in most cases, especially if "common" haplotypes are involved, not significantly higher than the informations arising from serology. Nevertheless, the definition of HLA class I and class II alleles, of other RFLPs and of microsatellites governed by the MHC can be helpful in some paternity cases; the chance of paternity exclusion being 98% if HLA-A,B,C and DR factors are typed by serology might increase to 99%. If two putative fathers, however, cannot be excluded by the serology of HLA, it is obviously more informative to include other systems not linked with HLA (e.g. VNTR-AMPFLP or STR DNA polymorphisms) than to analyse extensively the molecular structure of the HLA haplotypes observed in such a case.

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known molecules (e.g. HLA-A3) and several supertypic patterns can be observed, but it is not possible to type for all class I specificities with RFLPs.
In HLA class II, the situation is different: using only 3 detection systems consisting of a specific DNA probe and a given restriction enzyme (DRB/TaqI, DQB/TaqI and DQB/BamHI), it is possible to detect polymorphisms which are highly correlated with the known DR, DQ and Dw specificities. Furthermore, the application of other systems reveals several splits, thus giving a better definition of the various HLA-D haplotypes. As the proportion between exons and introns is only 1 to 9, it is probable that the majority of the class II RFLPs is due to variations in the non-coding sequences. These polymorphisms, however, must show a very strong linkage disequilibrium with phenotypically expressed HLA factors detectable by serology, cellular techniques, biochemical methods and/or PCR.

2.2.1.2. Sequence data
The ultimate definition of an HLA factor must be the nucleotide sequence of the allele or the aminoacid sequence of the gene product. Up to now, the sequences of 41 HLA-A, 61 HLA-B, 17 HLA-C, 4 HLA-E, 2 DRA, 60 DRB1, 4 DRB3, 1 DRB4, 4 DRB5, 3 DRB6, 13 DQA1, 19 DQB1, 8 DPA1 and 37 DPB1 factors are known and they received an official designation by the HLA Nomenclature Committee (each allele was given a unique numerical designation placed after an asterisk following the gene name). The sequence data demonstrate that several molecules which appeared to be homogeneous can be further subdivided: for instance, HLA-A2 can be coded for by at least 12 different alleles, HLA-B27 by 7 alleles or HLA-DR4 by 12 alleles.
The knowledge of many class II sequences is the basis for the application of the polymerase chain reaction (PCR) in order to define HLA class II alleles. By constructing specific primers, it is possible to amplify selectively the polymorphic parts of the second exon of HLA class II alleles from genomic DNA; the amplified DNA is mainly analysed by hybridisation with sequence-specific oligonucleotides (PCR-SSO) although other detection methods can be used, e.g. PCR-SSP (PCR with sequence-specific primers), PCR-RFLP (digestion of the amplified DNA with restriction enzymes and subsequent electrophoresis in polyacrylamide gel) or direct sequencing of the amplified DNA. These techniques are also used in order to define HLA class I alleles.

2.2.1.3. Polymorphisms in the promoter region
The sequencing of the promoter regions of HLA class II loci revealed marked polymorphisms in these regions. For the moment, such polymorphisms are known for DRA, DRB, DQA1 and DQB1; they show a strong linkage disequilibrium with the corresponding HLA class II alleles.

2.2.2. Other polymorphisms of the HLA system
2.2.2.1. TNF
In the TNF region, there are at least 5 complex microsatellite polymorphisms (TNFa - TNFe) and 3 biallelic RFLPs. The alleles of these polymorphisms are in linkage disequilibrium with class II alleles.

2.2.2.2. TAP
Polymorphic residues have been described in TAP1 and TAP2. Four TAP1 alleles and 8 TAP2 alleles are possible, 3 and 5 of them have been observed. There is also a strong gametic association between TAP and HLA class II alleles.

2.2.2.3. HSP70
The three HSP70 loci (HSP70-1, HSP70-2 and HSP70-Hom) show only a restricted polymorphism in the coding or in the promoter regions.

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Histocombatibility Workshops increases the number of HLA-ABC phenotypes to more than 10^8.
The class II molecules defined by serology are slightly less polymorphic: 17 HLA-DR and 7 HLA-DQ alleles are officially recognised, corresponding to 154 DR and 29 DQ phenotypes. With possibly new alleles defined in the 10th and 11th International Histocombatibility Workshops, these figures increase to more than 200 and 40, respectively. Because of the extremely strong linkage disequilibrium between HLA-DR and DQ alleles, the DQ factors do not increase the HLA polymorphism; the same applies to DR51, DR52, and DR53 genes which show an extreme linkage disequilibrium with some of the DR alleles DR1 - DR18.
The number of possible HLA phenotypes which can be detected by serology taking into account the products of the loci HLA-A, B, C and DR is higher than 20 x 10^9. This figure, however, is an underestimation as the class I loci coding for antigens on T lymphocytes and some subtypic factors of well defined determinants are not included. Furthermore, the phenotypic polymorphism is increased by the fact that the DQA1 molecule of one haplotype can combine with the DQB1 product of the other haplotype ("trans-complementation").

2.1.2. Cellularly defined specificities
It is possible to measure the response of T lymphocytes of one individual against determinants defined by the HLA-D haplotypes of another one using the one-way mixed lymphocyte culture (MLC). With the help of homozygous typing cells, several specificities, the HLA-Dw factors, could be defined. Most of them are included in HLA-DR specificities and it is highly probable that the response in the MLC reflects disparities of the whole HLA class II region; with regard to the strength of the stimulation, the following hierarchy can be envisaged: DRB > DQ > DP.
Other polymorphisms can be detected by a secondary response of T lymphocytes which have been primed in a first incubation step. The main targets detectable by this technique, the primed lymphocyte typing (PLT), are the HLA-DP factors provided that the responding cells have not been primed against HLA-DR/DQ. For the moment, 6 HLA-DP specificities (corresponding to 22 possible HLA-DP phenotypes) are officially recognised.
Besides these two methods, T cells, especially cells which have been expanded in clones, can be used in cytotoxic or proliferative assays for the definition of class I or class II polymorphisms. The subtypic factors of several class I determinants (e.g. of A2, B7 od B27) which have been detected in this way correspond very well to differences demonstrable by one-dimensional isoelectrofocusing (1D-IEF). The same applies for cellularly defined class II splits which can also be detected by one-dimensional and two-dimensional gels.

2.1.3. Biochemically defined polymorphisms
The use of 1D-IEF for the detection of class I molecules during the 10th International Histocompatibility Workshop demonstrated the existence of many subtypes of HLA-A, B and C gene products. These subtypes increase the class I polymorphism by a factor of 16.
The analysis of two-dimensional gels for HLA-DRB, DQA1 and DQB1 factors showed at least 23, 9 and 9 different patterns, respectively, in 71 highly selected homozygous cell lines. These figures clearly show that class II molecules are more polymorphic than expected on the basis of serologic data.

2.2. Polymorphisms defined by molecular genetics

2.2.1. HLA class I and class II alleles
2.2.1.1. RFLPs
The restricton fragment length polymorphisms (RFLPs) detected by class I probes are not very helpful for the definition of HLA-ABC factors: some patterns correlate well with already

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In the HLA-DQ subregion, there are 5 loci: DQA1, DQB1, DQA2, DQB2 and DQB3. Both DQA1 and DQB1 chains are polymorphic; the serologically defined epitopes are situated on the B chain. The products of DQA2, DQB2 and DQB3 are not known to be expressed.
The HLA-DP subregion contains 4 loci: DPA1, DPB1, DPA2 and DPB2. The DP molecules are made up of the DPA1 and the DPB1 chain; DPA2 and DPB2 are pseudogenes.
Four other HLA class II loci, DMA, DMB, DNA and DOB, are also situated in the HLA-D region; the function of their products is not yet known.

Furthermore, besides the class I and II loci, there are several other loci situated in the MHC of man:
- at least 4 loci defining proteins of the complement system (C2, C4A, C4B and BF),
- the structural gene for the 21-hydroxylase, CYP21, and an inactive pseudogene, CYP21P,
- two loci for tumor-necrosis factors, TNFA and TNFB,
- loci for the heat-shock proteins HSP70 and several loci coding for HLA-B associated transcripts (BAT1-9); the products of these loci may belong to a cell stress response system,
- genes involved in the processing of antigens: transporter genes, TAP1 and TAP2 (TAP = transporter associated with antigen processing), and proteasome-related genes, LMP2 and LMP7 (LMP = large multifunctional protease).

The probable order of these loci on the short arm of chromosome 6 is: centromere - ... - DPB2 - DPA2 - DPB1 - DPA1 - DNA - DMA - DMB - LMP2 - TAP1 - LMP7 - TAP2 - DOB- DQB2 - DQA2 - DQB3 - DQB1 - DQA1 - (DRB1 - DRB9) - DRA - ... - CYP21 - C4B - CYP21P - C4A - BF - C2 - (HSP70, BAT2 - 9) - TNFA - TNFB - BAT1 - ... - HLA-B - HLA-C - HLA-E - HLA-A - (HLA-F, HLA-G).

A strong linkage disequilibrium can be seen between various HLA alleles which is due to the preferential gametic association of some of the alleles, that means that some haplotypes are found more frequently than expected and other ones more rarely. The cause of the linkage disequilibrium is not known, the easiest explanation for its existence is the assumption that selective forces habe been acting in favour of or against some allele combinations. Because of the strong linkage disequilibrium found in the HLA system, especially in the HLA-D region between DQB1 and DRA, not all haplotypes which can be constructed by combining the alleles of the various loci are found in the population. A recombinational hot-spot is localised between HLA-DQA1 and DPA1; there is no strong positive gametic association between HLA-DP and HLA-DQ/DR alleles. The amount of the linkage disequilibrium between HLA-DQ/DR and HLA-B or between the class I loci is considerably smaller than the gametic association of the HLA-DR and DQ alleles.

2. HLA POLYMORPHISMS

2.1. Polymorphisms defined by conventional methods

2.1.1. Serologically defined factors

The class I loci HLA-A, B and C show a remarkable multiple allelism: taking into account only the specificities accepted by the HLA Nomenclature Committee 23 HLA-A, 46 HLA-B and 9 HLA-C factors are recognised. From these figures, 277 HLA-A, 1082 HLA-B and 46 HLA-C phenotypes can be computed; the number of possible HLA-ABC phenotypes amounts to 13.8×10^6. The inclusion of new factors described during the 10th and 11th International

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THE MOLECULAR GENETICS OF HLA

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1. THE HLA SYSTEM

The HLA system (for review, see Tsuji et al, 1992) which represents the major histocompatibility complex (MHC) of man, encompasses approximately one thousandth of the human genome (3.8 centi-Morgans corresponding to 3800 kilobases, kb) and is localised on the short arm of chromosome 6 (band 6p21).
In the HLA region, three major classes of genes can be found:
1) the HLA class I and class II genes,
2) immune function related genes (C2, C4A, C4B, BF, TNFA and TNFB, transporter and proteasome genes, HSP70, ...) and
3) other genes apparently not related to immune functions (e.g. CYP21, valyl-tRNA synthetase, ...).
This article will consider only the HLA class I and II genes, as well as immune function related genes with the exception of the complement genes C2, C4A, C4B and BF.

The HLA class I (HLA-ABC) loci code for glycosilated polypeptides with a molecular weight of 44000 daltons (44 kDa). The HLA complex contains around 30 such loci; most of them, however, are pseudogenes which are not expressed. The loci HLA-A, HLA-B and HLA-C are class I loci as well as other loci defining alloantigens and possibly differentiation antigens on T lymphocytes. HLA-E, HLA-F and HLA-G are further class I loci with expressed products which might be polymorphic. The size of each class I locus represents around 10 kb, the whole class I region which is situated on the telomeric end of the MHC corresponds to 1800 kb. ß2-microglobulin (ß2m), a monomorphic product of chromosome 15 (band 15q21 - 15q22) with 12 kDa, is non-covalently bound to the gene products of the class I loci. The allodeterminants of class I molecules are carried on the two distal domains of the heavy chain; for the formation of these sites, the presence of ß2m is necessary as the union of both molecules deeply influences the quaternary structure of the heterodimer.

The HLA class II (HLA-D) loci code for A and B chains (glycosilated polypeptides with 33 and 29 kDa, respectively). Both chains are non-covalently associated; the alloantigenic epitopes are expressed on the distal domain of the extracellular region. Each of the class II loci comprised approximately 10 kb; the size of the whole region is 1000 kb. The HLA class II region can be subdivided into at least 3 subregions: HLA-DR, HLA-DQ and HLA-DP (205 kb, 100 kb and 90 kb, respectively); within these subregions, several loci define A and B chains.
The HLA-DR alloantigens are situated on molecules consisting of the gene products of HLA-DRA and DRB1 (DR1 - DR18), DRB3 (DR52), DRB4 (DR53) and DRB5 (DR51). The product of DRA shows a rather limited variability, DRB2 as well as DRB6 - DRB9 are pseudogenes. The number of DRB genes on a haplotype (haplotype is the designation for the combination of the alleles of each locus situated on one chromosome and inherited en bloc) is variable and depends on the main DR specificity defined by DRB1.

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The elucidation of the molecular basis of the PGM1 polymorphism provides immediate and simple methods for genotyping [23]. For example the mutation at nt 723 abolishes a restriction endonuclease recognition site for Bgl II AGATCT in allele 2, when it becomes AGATCC in allele 1; but it creates a new recognition site for the enzyme Alw I (AGATCN4 to GGATCN4 in the reverse strand). Similarly at nt 1320 the DNA sequence CATG in allele - is a recognition site for Nla III but this becomes TATG in allele + and the site is lost. Restriction enzyme digestion of PCR generated products of exons 4 and 8 will therefore easily and specifically detect the PGM1 1/2 and +/- types. The system has already been tested out on forensic blood stains and works very well [26]. The mutations can also be resolved rapidly and easily by SSCP analysis on the Phast system with simple silver staining.

Conclusions

The classical genetic polymorphisms of human red cells continue to occupy an important place in the forensic field. They are well known and understood by the forensic scientists in the laboratories and in the courts by the police, lawyers, judges and to some extent by the general public. Also they are supported by a wealth of documentary evidence from family and population studies in a vast range of different human populations from all over the world [48]. The screening methods for phenotype analysis are rapid, cheap and effective and in many cases, especially for those red cell markers which are serologically or immunologically defined, can be automated at relatively low cost.

Most of the important blood group loci and red cell isozyme systems can now be analysed directly by molecular DNA methods and the genotypes correlated precisely with the phenotypes. The molecular methods will enhance these systems enormously by providing higher levels of heterozygosity and greater accuracy in the assessment of genotypes for the common and for the rare mutations.

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Phosphoglucomutase (PGM1) isozyme polymorphism

The PGM polymorphism was first identified 30 years ago by conventional starch gel electrophoresis as a 2-allele, 3-phenotype system with a world-wide occurrence [20]. In due course, as mentioned earlier, isoelectric focussing revealed further complexity and the PGM1* 1 and the PGM1* 2 alleles were subdivided into PGM1* 1+ and PGM1* 1- and PGM1* 2+ and PGM1* 2- respectively. Shortly afterwards it was predicted that intragenic recombination might lay at the heart of this polymorphism [21]. For example, if 1+ was the founder allele and 1- and 2+ arose from it by 2 independent point mutations, the 2- allele could arise by intragenic recombination in a 2+1- heterozygote. Recent successful attempts to clone PGM1 have allowed this hypothesis to be tested and also provide new methods for PGM1 typing which maybe valuable in the forensic field [22,23].

The human PGM1 cDNA was cloned by a more roundabout route than the ABO and Rh genes described above. The first step was to raise an anti rabbit PGM1 antibody and then use this as a reagent to screen a cDNA expression library to isolate a rabbit PGM1 cDNA. The latter was then used as a probe to isolate the human PGM1 cDNA.

As expected from what was known about the rabbit PGM1 polypeptide sequence, the human PGM1 cDNA encodes an enzyme of about 60 kDa molecule size. Structural studies have revealed that the PGM1 protein is a monomer arranged in four major domains with the active site buried at the confluence of the domains. The coding sequence is divided into 11 exons but the intron/exon junctions do not coincide with the domain boundaries [24].

We have now analysed the whole of the coding region of the human PGM1 gene by DNA sequencing in individuals of known PGM1 isozymes phenotype [23]. Only 2 mutations were found, both C - T transitions, at nt 732 in exon 4 and 1320 in exon 8. These mutations correlate exactly with the protein phenotype: in exon 4, at nt 732 individuals showing the PGM1 isozyme carry the Arg codon CGT whereas individuals showing the PGM1 2 isozyme carry the Cys codon TGT. Similarly in exon 8, at nt 1320 individuals with the PGM1 + isozyme carry the Tyr codon TAT whereas individuals with the PGM1 - isozyme carry the His codon CAT. The charge changes predicted by this amino acid substitution are entirely consistent with the isoelectric profiles of the four PGM1 isozymes. Thus there is very strong evidence to support the view that only 2 point mutations are involved in the generation of the four common alleles and that one allele must have arisen by homologous intragenic recombination between these mutation sites. Convincing further evidence to support this hypothesis has come from the molecular analysis of the PGM1* 3^+, 3^-, 7^+ and 7^- alleles [25].

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names but here they are referred to as CDE, with the antithetical allelic antigens cde and they are encoded by a gene complex on chromosome 1p. At first it was not clear how many genes comprised the Rh gene cluster and one very famous theory was the trinitarian structure due initially to R. A. Fisher. Biochemical evidence eventually pointed to the occurrence of 2 types of Rh polypeptide chains and Patricia Tippett took this evidence into account together with serological aspects of the system and proposed a 2 locus model (the 'D' locus and the 'Cc Ee' locus) in 1986.

The attempts to clone the Rh gene has produced similarly complex results which have until very recently been difficult to interpret. For example, 2 groups [14,15] independently isolated an identical Rh cDNA clone using oligonucleotide probes based on N-terminal amino acid sequence from purified Rh polypeptides and it was not clear whether the cDNA was derived from the D or the C/E locus. It now emerges that there are several different types of Rh cDNA but they fall into 2 main classes, which show a very high level of sequence identity and the new data are in support of the 2-locus model of the Rh blood group polymorphism [16,17,18].

The D locus is present in most individuals, it is expressed in red cells and encodes a 417 residue hydrophobic polypeptide which contains about 12 regularly spaced transmembrane domains and is firmly embedded in the red cell membrane. The polypeptide corresponds to the major D antigen responsible for the well known Rh positive blood group. Rh negative (dd) individuals are homozygous for a deletion of the D gene sequence [19].

The C/E locus encodes the E/e antigens and the C/c antigens but these polypeptides differ in length due to alternative splicing of the C/E transcripts [18]. Thus the E/e antigens are full length transcripts of 417 residues, the same length as the D polypeptide. The difference between the E and e antigens depends on a point mutation in exon 5 which substitutes Ala for Pro at residue 226. The C/c transcripts are shorter due to the splicing out of exons 4, 5 and 6 or exons 4, 5 and 8 and lead to the synthesis of polypeptides 266 or 267 residues in length respectively. These differ from each other and from the E/e polypeptides in their patterns of integration into the red cell membrane. The splicing leads to the loss of exon 5 and the E/e specificity and the exposure of N-terminal residues which are cryptic in the E and D polypeptides but confer C/c antigenicity. The differences between C and c appear to reside in four amino acid substitutions in exons 1 and 2.

These data summarise very recent findings but clearly there are now opportunities to characterise precisely the molecular events underlying the many low frequency Rh antigens and to use PCR based methods for Rh genotyping in forensic case work.

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specific probes [8]; this is a fairly lengthy procedure. The second is a much more rapid multiplex PCR procedure [11] which takes advantage of the critical single base deletion in the O gene at nt 261 and the nucleotide substitutions at nt 526 and nt 703 which usually distinguish B from A and O. Using four different sets of primers in a single reaction, 41 individuals of known ABO type were unambiguously genotyped and no exceptional cases were encountered. The third method [12] uses denaturing gradient gel electrophoresis (DGGE) to separate a 250 nt fragment of genomic sequence which spans the site of the single nucleotide deletion (nt 261) associated with the O allele. The procedure is also very rapid but requires specialised equipment and GC clamps on the oligonucleotide primers for the PCR. However, DGGE distinguishes hitherto unrecognised polymorphisms associated with the O and B alleles. In testing, 95 European individuals, 4 different O alleles, 2 B alleles and 1 A allele were identified by DGGE and the level of recognisable heterozygosity and hence the information content of the locus as a genetic marker was raised from 3/95 (3%) to 66/95 (70%). The mutations underlying this heterogeneity have not yet been defined precisely but it seems most likely that the single base deletion characteristic of O is the major feature which separates O from A and B on the denaturing gradient gels. Combinations of point mutations in intron sequence flanking this deletion presumably lead to the four O and three B subtypes.

ABO genotyping is of course already extremely valuable in linkage analysis and gene mapping on human 9q34. Previously uninformative families, O by O matings for example can now be scored by the DGGE technique and the AO and BO heterozygotes can easily be resolved from AA and BB homozygotes respectively. Applications in the forensic field are easily envisaged in paternity work and criminal investigation.

As an aside it is also interesting to note that the gene for the H transferase, which acts on the precursor of the ABO substrate, has also been cloned and sequenced [13]. The gene, located on chromosome 19, encodes an α (1, 2) fucosyltransferase. Its isolation opens the door to the investigation of variations in the ABO system which are due to the action of this gene - in particular the Bombay and para Bombay phenotypes. Preliminary studies have already revealed that in some of these rare individuals, the H gene is disabled due to point mutations leading to small deletions and frame shifts, premature stop codons or substitutions affecting the active site.

Rh Blood group system

From the very start it was clear that Rh is a complex blood group system showing enormous diversity in human populations. Literally dozens of different phenotypes can be defined by serological means and the terminology is necessarily extremely complex. At the simplest level 3 sets of antigens have been defined. These have been given various

The markers are arranged in roughly chronological order of discovery. In most cases progress has been made in cloning the gene(s) responsible for the polymorphic phenotypes and this is indicated by the references alongside. The lists are not comprehensive and similarly this review cannot be all embracing. In order to illustrate the general points for the purpose of this Symposium, this review focuses on three red cell systems - the ABO and Rh blood groups and the phosphoglucomutase (PGM1) isozyme polymorphism.

ABO Blood group system

The molecular basis of this polymorphism has been known for many years to be mediated via carbohydrate determinants and variant glycosyltransferases encoded by the ABO locus on the long arm of human chromosome 9. Classical biochemistry led to the discovery that the A enzyme was an N-acetyl-D-galactosaminyl transferase and the B encoded enzyme was a D-galactosyl transferase. All the biochemical and serological evidence indicated that the O gene was an amorph.

The molecular cloning of the ABO locus came about following the purification of the A-transferase from human lung tissue and the determination of part of the amino acid sequence. This provided the basis for the design of an oligonucleotide probe for cDNA library screening and the derivation of the complete sequence of the A-transferase mRNA [6]. Comparison of cDNA clones derived from human adenocarcinoma cell lines of known ABO phenotype showed that the DNA sequence of the B gene differs from A at 7 nucleotides which result in 4 amino acid substitutions in the catalytic domain and these account for the different sugar specificities of the A and B transferases [7,8,9]. The O gene was found to be identical to the A coding sequence except for a single base deletion at nt 261 which leads to a frame-shift, then a premature stop codon and the synthesis of a functionally inactive O transferase of only 117 amino acid residues which lacks most of the catalytic domain. Thus the O gene is indeed an amorph as predicted from the phenotypic data. The minor subtype of A, the A_2 transferase differs from A1 by a single base substitution at nt 467 giving rise to Leu for Pro in the catalytic domain at codon 156 and by a single base deletion at the C terminus which produces a frameshift and 21 extra amino acid residues [9]. Several other well known but uncommon classical types such as cis AB, A^x, $B^{(A)}$ have also recently been characterised [10].

The cloning of ABO and the identification of DNA sequence differences between the A, B and O alleles provides a means for direct genotyping. Three methods have been described all involving PCR amplification of genomic DNA. The first method involves diagnostic restriction enzyme digestion of the PCR products derived from the sites of the substitutions which distinguish A, B and O, followed by Southern blot analysis with

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to the discovery of the phosphoglucomutase (PGM1) sub-types and the transformation of a classical 2-allele, 3-phenotype system into 4 alleles and 10 phenotypes.

This then, very briefly, is the background to the classical markers which are at the heart of our Symposium. We shall review the transformation wrought by the introduction of DNA technology which allows us to analyse these markers directly at the level of the gene. We shall discuss the merits (and demerits) of the molecular approaches which give direct information about genotype and we shall review the benefits which continue to acrue from the further analysis of these long established classical polymorphisms. In many cases predictions were made several years ago from detailed phenotypic analysis about the nature of the mutations which might be responsible for several of these polymorphic systems. In some cases the predictions are being fullfilled but in others we find, to paraphrase the cliche, that the truth of the DNA genotype is stranger than the prediction of the protein phenotype.

Table 1 lists the classical red cell polymorphisms which have been extensively studied in forensic medicine and used as tools in this arena.

Table 1: "Classical" red cell polymorphisms

1. Red cell antigens		Reference
1.1	ABO, H, Secretor & Lewis	[6-10,13,27, 40]
1.2	MNSs	[28-38, 40]
1.3	Rh	[14-19, 40]
1.4	Kell	[39, 40]
1.5	Kidd	[40]
1.6	Duffy	
2. Red cell isozymes		
2.1	Acid Phosphatase	[Dissing, this Symposium]
2.2	Phosphoglucomutase	[22-24]
2.3	Phosphogluconate dehydrogenase	[41]
2.4	Adenylate kinase	[42]
2.5	Adenosine deaminase	[43]
2.6	Glutamate pyruvate transaminase	
2.7	Esterase D	[44]
2.8	Glyoxylase	[45]
2.9	Peptidase D	[46]
2.10	Cytidine deaminase	[47]

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Molecular analysis of classical red cell markers

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Introduction

The application of blood groups to criminology was predicted by Landsteiner in his first major paper on the ABO polymorphism in 1902 [1]. This proved to be a far sighted observation. Since then more than 20 independent blood group systems similar to ABO have been identified by serological means [2]. The major driving force behind these discoveries was the importance of blood group antigens in blood tranfusion and in the pathogenesis of haemolytic disease of the newborn. However many of the systems were sufficiently variable in major world populations to be used as tools in forensic and anthropological investigation.

About fifty years ago the technological approaches to the analysis of red cell markers changed with the introduction of electrophoretic techniques. First, the many different forms of haemoglobin and their variants were revealed by the relatively crude techniques of zone electrophoresis using paper, cellulose acetate and agar gels as the support matrix. More sophisticated methods, such as starch gel and polyacrylamide gel electrophoresis were then introduced in the fifties and sixties and the range of genetic variation revealed by these procedures rapidly expanded to other abundant proteins in blood such as the haptoglobin and transferrin systems [refer to Sensabaugh this Symposium] and also to much less abundant proteins, enzymes, which were demonstrated as isozymes using powerful new specific detection methods [3].

The serological methods, originally devised to investigate the classical erythrocyte antigens, led on to the discovery of many other polymorphisms such as the platelet antigen systems and the highly variable histocompatibility (HLA) systems [refer to Mayr this Symposium], which have found extensive use in forensic work. The electrophoretic methods have been equally diverse and rewarding. The introduction of refinements such as isoelectric focussing were particularly important both in improving the resolution of known polymorphic markers and in identifying sub-types in classical systems. Forensic application of these classical markers was often an important driving force behind the introduction of these techniques; for example, I well remember the excitement generated in my own field by the work of forensic laboratories in the UK [4] and in Germany [5] leading

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2. Molecular biology of classical markers

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In our experience, it seems that even in judicial cases, when the informed consent is assumed, there is a lack of information. Therefore, we suggest that, in Brazil, in order to minimize the problem, an active participation of the experts, close to the courts and parties, is necessary to clarify the possible results expected in genetic association studies, in order to have an actual informed consent.

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he is allowed to behave open-heart without the fear of betrayal. In Forensic Medicine, the expert also faces another confidence compromise: the solicitant authority expects all obtained related information to reach, in the context of the process, a final decision. Although the general clinical confidence relationship does exist, the latter, with the authority, imposes an apparently contradictory procedure: it is necessary to investigate all possibilities, to analyse all results and to expose them, to discuss the relevant ones and, therefore, to conclude.

In this way, the expert is obliged to present an answer, as accurately and objectively as possible and, to use all available means to clarify the question. This includes, not only the performance of all available tests in individuals directly involved (and if necessary in all other related, as long as they agree to participate and/or give their informed consent) but also, to execute the proper "visum et repertum". It is part of the expert's compromise to present all observed results, whether or not directly requested, without concern on pleasing or not whoever, regardless of other implications they could bring. Nonetheless, the expert is linked to silence in relation to all facts and circumstances (he was occasionally aware of) not directly involved with the expertise itself. Consequently, regarding disclosure of absence of legally assumed kinship, all obtained results should be presented, but, in order to preserve the right to privacy, the expert should limit the discussion only to relevant information to reach the final conclusion.

In our opinion, in Brazil, the main reason for these situations is the lack of proper orientation of the parties due to poor knowledge and misinterpretation of scientific data: not only are there false expectancies about the interpretation of non-excluding results but also ignorance about the extension of their discriminating excluding power. The first of them usually leads to a deception because it will not be reported that X is actually Y's father and creates opportunities to extensive and confused inquiries. Although the certainty of genetic association is expected, the parallel high discriminating power of the used systems (that reaches almost certainty) is ignored.

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ETHICAL CONSIDERATIONS ABOUT UNEXPECTED FACTS DISCLOSED DURING GENETIC ASSOCIATION STUDIES

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Family studies formulated for populational genetic purposes usually disclose the absence of genetic association among some individuals supposed to constitute one family. Although an informed consent was given aiming to a research, the communication of these findings to the individuals involved is meaningless; the importance of such data is restricted to the interpretation of the research in itself. On the other hand, in Forensic Medicine, disclosure of unsuspected absence of genetic association can interfere with the conclusions of the expertise.

We faced three of these experiences: a) maternity (or paternity) investigation, when the assumed and legal parent is excluded in relation to the offspring; b) paternity investigation, when a deceased alleged father is not the biological father (BF) of one or more of his legal offsprings; c) suspicion of swapped babies, when only the man is not the BF.

In Forensic Medicine, the expert's ethical problem is when an information omitted on the absence of kinship (kept by one of the involved individuals and about which there are no questions in the process) hinders the straight conclusion of the expertise. The expert's question is the fluctuation between someone's right to privacy, that can be invaded by the disclosure of a hidden fact, which was to remain a private secret on one hand, and his duty to present all results obtained ("visum et repertum") and to discuss the ones relevant to the conclusion of the expertise regardless of their other possible developments, on the other.

The relationship between a physician and his patient is based on the patient's confidence to his doctor. This includes not only technical-scientific aspects but full assurance that

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error when - for the given decision limit - the case is decided one way or the other.

If judges were to orient themselves by Bayes' expectation of error, the level of their demands upon the evidence would always be the same, independent of, say, progress in medico-scientific opinions. For example, the demands placed upon serostatistical evidence of real paternity would remain at eg W = 95 %. In reality, over the years judges have demanded increasingly higher probabilities for their rulings. Therefore, they obviously orient themselves by the actual level of W values produced in similar cases. By choosing the Neyman-Pearson model instead of Bayes' principle, they demonstrate their awareness of the contemporary potential of evidence and, thereby, their flexibility and acceptance of progress.

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light of the evidence presented in the actual case. He will set the assessment limit in a way that optimizes the benefit he seeks to provide through his judgment. To better picture the degree of the benefit associated with the decision limit, he will privately orient himself by similar cases in his own body of legal experience and cases in the literature as well as by his own experience of life in general. If the value for the present case (either pro or contra the "null hypothesis") lies above this decision limit he will be able to pronounce judgment: the action will be either upheld or dismissed. If the value lies below this decision limit, it will be necessary to seek further evidence.

The statistician will have noticed that the underlying principle of this process is not Bayes'but another. There is a "posterior probability" in Bayes'sense, ie, an integration of all evidence, including a prior proportion. This probability value is not assessed as such. It is incorporated into the distribution of values of other cases within the judge's body of experience, in which actions upheld and actions dimissed have their own respective distributions. This procedure corresponds to the Neyman-Pearson decision model.

A significant factor is the quota of error to be expected eg for non-fathers falsely ruled to be fathers, ie, the tail probability for non-fathers at the point applicable to the specific case. For the most cases of disputed paternity it is very low, practically zero. A ruling is thus possible.

Why does the judge use Neyman-Person instead of Bayes? The answer is simple. No judge will have such breadth of experience as to have dealt with 100 cases with the same circumstances. In reality, in no two cases are the circumstances exactly the same. Thus, it is impossible for the judge to decide according to Bayes, to declare that on the given evidence one may expect for instance that 99 of 100 cases with the same circumstances will be decided correctly and one falsely. Rather, the judge must picture the present case as one of a series of comparable (even if not identical) cases so as to decide for himself whether it better fits the distribution of upheld or the distribution of the dismissed cases. In other words, he must use the Neyman-Pearson distributions to orient himself about the statistically

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disposal. The experts charged with collecting material evidence as well as the persons responsible for examinations, interrogations and investigations all provide pieces of evidence that alone are not, as a rule, decisive for the verdict. In most cases, no single individual providing these auxiliary services will have heard or seen all the evidence.

All the evidence is gathered together for the first time in the judge's summing-up. He weighs up the pros and cons of each individual piece of evidence, assessing each piece in relation to the rest as well as to a specific person, either the defendant or the plaintiff. This process produces a "probability of incrimination". Not all individual facts refer directly to people involved in the case. For instance, if traces of paint or tools have been identified, it is the duty of the judge to relate these findings to a suspect; his conclusion may be either positive or negative. In other instances the evidence may refer directly to the defendant, eg, a high probability of paternity or an "identical" blood test. But even in these apparently relatively uncomplicated instances the judge must associate the findings with a person; for this alone enables him to pass judgment.

Thus, the purpose of gathering evidence and relating the individual pieces to each other is to characterize and individualize the case as far as possible. This enables us to establish its type, and allocate it to as small a group of analogous cases as possible. If this particular group is also part of the judge's body of experience, the resulting judgment will fit the facts. The more strongly the case is focussed and the more differentiated the judge's body of experience, the sooner judgement will be passed and the more just it will be.

After the weighted evidence as a whole has been considered the judge has two bodies of arguments at his disposal: one in support of the allegation and the other against the allegation. He must now decide whether the probability of the allegation (a purely conceptual value) exceeds a - similarly conceptual - assessment limit or not. The judge himself determines the assessment limit. He uses principles of "utility", in that he tries to achieve a balance between the interests of the respective parties - or of the accused and the general public, all in the

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type I and type II errors. Bayes' and Neyman-Pearson's methods have their respective fields of application. In terms of the court setting one may describe Bayes'as that of the expert and Neyman-Pearson's as that of the judge.

Of the two, the Neyman-Pearson model is much the easier to comprehend. Misunderstandings and a lack of appreciation of Bayes' principle have led to unjustified criticism and a strong anti-Bayes' body of opinion, this for a long time. The misunderstandings arise from the process of transferring the weighted ratios of mass statistics to the individual case. As Ball and v.Mises put it: there are no statistics for individuals and no individuals for statistics. That is true. But one can treat an individual - or a single event - in relation to a reference class, ie, a collective, and allocate a statistical probability to this person or event as a member of a certain reference class. Two reference classes correlate with the hypothesis pair, eg. father - non-father, instigator - non-instigator, perpetrator - non-perpetrator. To define the two reference classes one employs as many pertinent factors as possible, eg, in paternity cases a large number of hereditary characteristics in order to distinguish fathers from non-fathers. The greater the class-typing information, the greater the certainty of allocating a specific individual or case to one or other reference class. Of course, it is always true that any single event is unique and cannot, therefore, be repeated. Consequently, a single event cannot, strictly speaking, belong to a certain reference class.

In addition to the objection that every probability statement is relatively indeterminate with respect to the individual case, there is the objection that the empirical values for very narrowly defined reference classes are not fully reliable in terms of mass statistics. Lothar Sachs(7) puts the objections in perspective elegantly and pragmatically: reliable statements are imprecise, precise statements are unreliable. Any expert who knows his biostatistics is aware of this problem. He takes adequate account of it by applying statistics to the individual case only if this case can be allocated to a sufficiently narrow and, at the same time, sufficiently large reference class.

Only the judge has all the evidence pertaining to a case at his

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each hypothesis. In accordance with his "postulate", one uses a neutral one, ie. the same prior probability for each of the hypotheses (unless specific numerical values are available).
A balance may help to illustrate the situation. One arm represents the null hypothesis, the other one the counter hypothesis. Initially, the pans, which will hold the informations presented in the case, are in equilibrium, as shown by the vertical position of the needle. One assumes "a priori" that both hypotheses have an equal chance of being true, ie, have the same initial probability. Using data he has obtained himself or has at his disposal the expert works out the weights that each hypothesis should have and puts them in the appropriate pans. The judge adds further (usually non-numerical) arguments from the rest of the evidence. He then studies the final position of the needle and tries to draw a convincing conclusion.
Let us term the null hypothesis X and the counter hypothesis Y. The expert obtains a weight in favour of X = G(X) and a different one in favour of Y = G(Y).
The ratio between them, L = G(X)/G(Y), is termed the likelihood ratio. By standardizing the values for G(X) and G(Y), one obtains the probabilities W_X and W_Y for both hypotheses:

$$W_X = G(X)/[G(X)+G(Y)] ;$$
$$W_Y = G(Y)/[G(X)+G(Y)] = 1-W_X .$$

Bayes was the first to correctly apply this formula to the individual case. De Laplace(4), Boole(3), Ball(1) and v.Mises(5) subsequently treated the problem analytically. Ever since, Bayes' Theorem (as it is generally known) not only has great legal significance, but also plays an equally important role in medical diagnostics, industrial production, archaeological research, etc.
Bayes, an Englishman, was a Presbyterian minister. His preoccupation with mathematics was a private interest. The treatise on which his fame rests was found in a drawer of his bedside table after his death in 1761. The mathematician Price recognized the importance of the work and shortly afterwards presented it in a lecture at the Royal Society of London.
One and a half centuries after Bayes, in the mid 1930s, another method of decision procedure took its place besides Bayes' Theorem: the Neyman-Pearson model(6). It works with limits and

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THE EXPERT's EVIDENCE AND THE JUDGE's EVALUATION OF ALL INFORMATION IN COURT DECISIONS

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A great part of legal judgments rests on deterministic evidence. Such evidence is always sufficient in law. But evidence may also be of a circumstantial, ie, a "probabilistic" nature. This type of evidence is not as "absolute" as the former. In consequence, an important role is reserved to the judge's subjective conviction, ie: he reaches his conclusion through the sum of the circumstances.

In reaching a decision the judge holds the sum of evidence to be either "sufficient in law" or dismisses it as insufficient. An Appeal Court may reach a different conclusion - which, given the nature of probabilistic evidence - should not surprise. This does not imply arbitrariness in matters of judgment. It is a reflection of the fact that each individual judge makes his own independent evaluation, which, in most cases, is not - indeed, cannot be - a purely formal matter; the assessment of evidence is 'ipso facto' creative.

Litigation makes sense only if allegations appear to be based on facts. In this context, allegation should have some initial probability, ie, some evidence for the initial suspicion. Without facts of this nature it would be impossible to institute any legal proceedings. Hence, all legal proceedings start with a foreknowledge. In most cases these is fairly limited. The judge therefore requires additional informations to reach a legal decision. The additional evidence is "added" to the initial probability. If the latter is large, less additional evidence is needed than if it were small. When experts provide facts to help the judge to reach a decision these are often in the form of numerical values that support either the null or the counter hypothesis. As mentioned above, the experts must be satisfied that the two alternatives are realistic, in other words, that they have prior probabilities. For this reason, in his formula Bayes (2) allotted a "prior probability" to the frequencies of

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Experts in parentage testing agree that the posterior probability of paternity cannot be derived without a prior probability. The Essen-Möller formula, W=1/(1+Y/X) does not contain a specific value for the prior and thus it assumes a neutral prior of 0.5.[6]

Hummel, Conradt and Kundinger have given a modification of the Essen-Möller formula to include a realistic prior probability and suggested that a realistic prior, the non-exclusion frequency, be used.[6]

Elston has stated that the expert scientist has an obligation to consider the validity of all assumptions used in probability estimates and that anything less constitutes scientific fraud and fraud to the court.[7] He has suggested that the report could give the probability of paternity over a range of priors. This, in fact, has also been recommended by Ellman and Kaye[8] and is in keeping with the summary recommendations of the Parentage Testing Committee of the American Association of Blood Banks following the Airlie Conference.

Most laboratories in the U.S. continue to use the neutral prior of 0.5 in their case work. However, if a realistic prior is to be used, each laboratory should be aware of possible subgroup differences of the priors within their experience.

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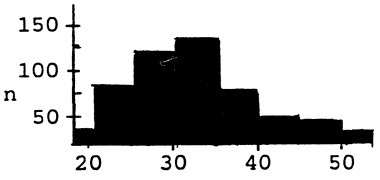
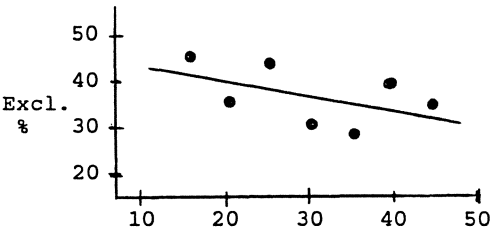


Average Age of AF & M



Average Age of AF & M

The exclusion rates tended to be somewhat higher when the alleged father (AF) and mother (M) were young with a gradual decrease in exclusion frequency in older couples. No significant differences in exclusion rates were found among subgroups, based upon racial origin, of the total study population of trios. Subgroups based upon surnames of the trio differed from each other (p=0.043). The most significant differences were between DSS vs. SSS (p=0.01) and SDS vs. SSS (0.04). These probably represent divorce actions. When AF or M paid for the test, the exclusion rate was higher than when they shared in the cost (p=0.018). If the court ordered the test to be done, the exclusion rate was lower than when it was not a court ordered case (p=0.002). Midway through the study period, the charge for the test was greatly increased. The exclusion rates before and after the charge increase did not differ significantly.

Discussion

The overall exclusion rate observed in our Laboratory during the study period of 1986-1993 was 35.6% which is higher than the reported national average of 27-30% in the United States.[1] This high exclusion rate is probably related to the mix of trios in our case work population, rather than our combined average power of exclusion (PE), since most all laboratories performing parentage tests in the U.S. have average PE values which exceed 95%.[2] Examination of the subgroups in our study population reveals that in 23% of the trios, the AF, M and C all have the same surname. This is due to the fact that our laboratory does not participate in state or regional contract work for welfare cases so that we have a high proportion of private pay and divorce cases.

High exclusion rates were reported from divorce cases in 1976 by Dykes and Polesky in the U.S.[3] Valentin, Henningsen, Nijenhuis, Mayr, Martin, and Salmon all reported high exclusion rates in divorce cases in Europe at the Airlie Conference in 1982.[4]

Lower exclusion rates in court order cases have been reported by Lincoln in England and Nijenhuis in the Netherlands.[4] Our testing results in both of these categories (same surnames and court ordered) also follow these patterns of observations.

Professor Hummel surveyed the exclusion experience in different countries and geographic areas in Europe and found that exclusion rates varied in 1974-1978 according to country, with the highest rate of exclusions in Austria and the lowest rates in Denmark.[5] He suggested that these differences are probably not related to differing PE values, legal or political systems, gene frequencies, sample size or illegitimacy frequencies. He concluded that in one-man-one-child cases in German speaking areas of Europe that high exclusion rates are probably due to concealment of promiscuity by the mother or uncertainty about the identity of the true father rather than the intentional naming of false fathers.

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THE ASSOCIATION OF INDIVIDUAL SOCIODEMOGRAPHIC FACTORS WITH PATERNITY TEST RESULTS

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Introduction

The paternity testing laboratory has available to it information other than the laboratory results regarding the tested individuals. This accessory data relates to sociodemographic factors which are requested prior to the testing. Analysis of these factors in our institution was undertaken to determine if there was an association between one or more of these factors and the outcome of the laboratory analysis as determined in our laboratory.

Method

The study group consisted of 500 consecutive cases from our files in which the testing was performed between the years 1986-1993. During this time, each individual was tested for genetic markers in multiple genetic systems. The systems utilized included traditional laboratory antigens, the HLA system and RFLP-DNA systems. The combined power of exclusion was, on the average, 99% or higher. The overall observed exclusion rate in the total study group of 500 cases was 35.6%.

Subgroups of the total study cases were identified based upon sociodemographic factors in order to determine whether or not the exclusion rates of the subgroups would differ from each other or from that of the overall exclusion rate.

Results

				Total Observations	Exclusions No.	(%)	p value
Race of trio							.27
White				362	124	34	
Black				116	48	41	
Other				22	6	27	
Surname of trio							.043
AF	M	C					
*D	S	S		278	90	32	
S	S	S		111	51	46	
S	D	S		52	15	29	
D	D	D		32	10	31	
S	S	D		15	8	53	
Payer							.035
AF				186	77	41	
M				33	14	42	
AF/M				21	3	14	
Court or unknown				258	84	33	
Court ordered							.0002
No				373	151	40	
Yes				126	27	21	
Charge for tests							.572
Lower				214	73	34	
Higher				286	105	37	

*D = different, S = same

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blood sample for DNA purposes. Would you really be happy about your genetic data floating about the system for the rest of your life? Would you be secure in the knowledge that no mistake could possibly happen with that information?

Some, like Professor Williamson of St Mary's Hospital Medical School in London, who support a national database with all adults, argue that DNA samples and data should be the property of, say, the judiciary rather than the police. But I doubt if that would be either practicable or a meaningful check against abuse.

Professor Jeffreys has recently suggested that a national data-base would not be politically acceptable, but that there might be a case for keeping the profiles of previous offenders. I agree, with some hesitation, although it means that suspects must be told that if they are convicted their data can be used for other purposes. That is an exception to the basic principle (as with fingerprinting), but an acceptable one. Anything more would be "a step too far".

3.5 Finally, we must all recognise that however many laws you pass, the process, particularly the scientific process, is not static. There must therefore be a link to another process, the democratic process, to ensure that the rights and responsibilities in this area are not left unmonitored. We have no Minister of Justice in the UK (although we will have one one day). But we do have Parliamentary Committees of the House of Commons. It should be the function of one such committee to oversee the developments in this are - to monitor, to investigate, to listen to the views expressed, and to be a public and democratically accountable forum for protecting rights.

CONCLUSION

As a lawyer, I believe that I have a duty beyond the mere interests of my client.

As scientists, you will have your own creed. In the Judith Ward case, the appeal court said that, in future, it should be spelled out in the clearest terms to scientists that they must act in the course of justice.

Perhaps Gustave Lebon (in the nineteenth century) got closest to it, when he said: " Science has promised us truth - an understanding of such matters as our minds can grasp; it has never promised us either peace or happiness".

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Practice covering the use of firearms or the use of plastic bullets by the police in Northern Ireland. The Code should cover the whole procedure from receipt of samples to giving evidence in court. There must be full and frank disclosure of scientific methods, equipment used, notes taken and tests carried out, even though the results may cast a shadow of doubt on the guilt of the suspect. These are minimum standard requirements, and failure to comply with them should result in the evidence being excluded.

I can tell you, these things are not done in the UK. In a recent murder case in which, crucially, the blood-grouping on the defendant's shoe was alleged to match the blood group of the deceased, a Home Office scientist went into the witness box. He had, he said, 20 years experience as a forensic scientist. He had carried out certain tests, he had come to certain conclusions, and that was his evidence. But it was just not true. The original file, produced late in the day at court, showed that he had not carried out any of the tests, although he had supervised one. The tests had been carried out by scientists of three years' experience and the opinion was theirs.

3.3 Thirdly, there must be a pool of experts available to work for the defence, a library of information and expertise, ready and willing to help in technical fields. A defendant and his lawyers must have access to justice. The Royal Commission has gone some way towards this in its proposals for a new Forensic Science Advisory Council and that public sector laboratories should also help the defence. I prefer, however, well-qualified expertise to come from sources independent of public bodies.

3.4 Fourthly, data protection. I am deeply worried about the idea of the database to solve crime. My concerns centre on the risk of misuse and abuse of DNA profiles kept on a comprehensive database. As Professor Howard Slater of the University of Wales put it:

> "In the last few years in many celebrated cases, convictions have been set aside becuase of shoddy and fraudulent forensic evidence and false or fabricated police evidence. It seems to me, whatever the basic civil liberties issues may be, the public simply will not trust the police and closely-related agencies to apply a complex, highly-sensitive technique such as DNA fingerprinting with the necessary rigour and honesty."

Just think for a moment. If you were the victim of a terrible mistake, such as mis-identification - common enough - and you were arrested, charged, but fortunately acquitted. And you had given a

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Meanwhile, the UK governmnent, worried about serious miscarriages of justice, set up a Royal Commission, to investigate, among other things, the use of science in the forensic process. This summer the Commission recommended an extension to existing police powers. The police should have wider powers to take samples, and everyone convicted of a serious offence should have DNA taken for analysis, even though it had no bearing on the crime committed. The records would be kept for all time and for any purpose. Only last week the government announced that these proposals would soon become law and that about 500,000 samples a year would be taken (we have plenty of serious crime), with the aim of creating a national database of convicted criminals' DNA profiles - described by the Data Protection Registrar, the official custodian of public data storage, as "a step too far".

Herein lies the conflict. On the one hand there is a strong public feeling that the steady rise in crime should be halted, at almost any cost. On the other hand, there is a growing concern that the methods adopted for this purpose involve serious infringements of civil liberties. Obviously a balance must be struck, between the desire to catch criminals and the rights of the individual not to be falsely accused, let alone falsely convicted. But where to strike the balance?

That takes me to the third and final part of my talk.

PART III THE PROTECTION OF HUMAN RIGHTS.

3.1 It seems to me, first and foremost, that there must be rigorous control over the whole process. It is extraordinary that the use of DNA profiling in the forensic process has developed piecemeal, sometimes illegally, without any overview of the principles and consequences.

There must be legislation controlling the taking of samples, the necessary consents, the retention and destruction of samples and of radiographs and of information taken from them. Wherever the boundaries are to lie, they must be clearly stated. It is a first principle of human rights that the citizen must know what those rights are.

3.2 Secondly, the scientists should operate under a published, statutory-based Code of Practice, just as there is a Code of

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all. But if it is possible, we must protect against it as best we can.

So, what about the specific process of DNA profiling?

Since 1984 the police, in the UK, have been able to take body samples from a person suspected of serious crime in the following circumstances. An intimate sample (blood, semen, urine, saliva or pubic hair) may be taken, but only with consent. A non-intimate sample (non-pubic hair, nail clippings, footprint) may be taken without consent.

If convicted the sample may be kept, and presumably, although this is not stated, may be used for other purposes (as with a fingerprint). If the suspect is cleared of the offence, the sample must be destroyed (as with a fingerprint). In other words the starting principle is that a sample is taken for a specific purpose. Once that purpose is fulfilled, the suspect's privacy (of personal body information) is protected, by the destruction of the sample. That is the principle, at least. If convicted, the principle is amended and the right to privacy is forfeited. That seems to me to be a reasonable, if not wholly consistent, way of working.

But there is a problem here which has caused much confusion. The British Act of Parliament (the Police and Criminal Evidence Act 1984, sections 61-65) is silent on whether the information taken from the sample should also be destroyed. This grey area has been exploited by the police who decided, secretly, to keep thousands of files containing DNA profiles on both convicted and suspected (but acquitted) sex offenders. Roy Williams, a 25 year old convicted burglar, gave a blood sample to the police in 1982, before the advent of DNA profiling. Six years later he was asked to give a further sample, this time for DNA analysis. His blood type had been found to match a bloodstain in a murder case. Although he was cleared from the murder inquiry the police refused to erase his DNA profile which had been stored on computer. He was told that it was police policy to keep such details.

This was almost certainly done in breach of the Data Protection Act which states that information gathered for one purpose cannot be used for another. At the very least it was contrary to the spirit of the Act which controlled samples. Indeed the police in London took legal advice and were advised as such. And, much to the horror of senior detectives, some 4,000 DNA files were then deleted from computer records, or so we are told.

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PART II THE PROBLEM OF GENETIC PRIVACY.

There is, of course, no such thing as absolute genetic privacy. If I examine my own genes, I am also looking at information about my parents and my children. I might want information on an inherited disease such as Huntington's chorea. So might an insurance company in trying to assess my life expectancy. And the state, of course, wants to compile an ever-increasing file of information on all of us, not least in the detection of crime. Senior police officers in the UK would like to have all adult males on file.

One of the most interesting debates at the moment is raised by the general question - How far should the state be allowed to go in collecting and keeping genetic information for the purposes of detecting crime?

I have to say that I am bound to approach this issue from the cynical end of the spectrum. As a hard-bitten lawyer, having led a working life of crime (at second-hand, of course), I am only too conscious that where there is life, there is corruption - sometime, somewhere. (Italy has been particularly active recently in proving this point.)

Regrettably, police officers as a class cannot be excluded from this maxim. From time to time they fabricate evidence; they plant evidence; they give perjured evidence; they produce false confessions. I once met a man who used to be in the Flying Squad in London (a special police unit dealing with violent crime). He was described as the "script writer"; he sat in a room on the top floor of the police station, with a bottle of whisky, writing out the confessions. He retired from the police force and became a lawyer. Not all police officers, of course, are corrupt. But it only takes one or two to bring about a miscarriage of justice.

It is therefore necessary to produce a system which does its best to protect the innocent from that element of corruption. It is very difficult to make use of a fingerprint corruptly; planting a print is very difficult. Playing about with blood samples is less difficult. It is easy enough for exhibits to become contaminated or deliberately mislabelled, long before the scientists get their hands on them.

Experience shows that scientists may make mistakes, that they may fall into the trap of partisanship, and at worst they may conceal and deceive. All of this is possible; we are all human beings after

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lies and statistics". That is particularly relevant here. Statistical confusion has led to the exclusion of evidence in two cases at the Old Bailey. David Balding, the British statistician whose work has been cited in court, has suggested that if police scientists corrected their statistical methodology to take his criticisms into account, the odds of someone else having the same genetic match would be reduced typically from 10 million to one, to one million to one. The layman might say, with some justification, where the hell has the 9 million gone? In a celebrated New York murder case, the probabilities of a chance match were put at 1 in 500 with one statistical method and 1 in 739 billion with another. Such variations do not exactly inspire confidence.

Matching profiles has caused problems too. The approach to matching has been far from uniform: whether to use the naked eye or fragment sizing or any other technique. Laboratory error, as in the Castro case, can never be ruled out, particularly with degraded material. Profiling procedures are not standard, are not covered by published guidelines, and are therefore likely to be open to criticism in court. All of which weakens the value of the evidence presented.

No doubt as scientists, you have the answers to all these difficulties. No doubt as scientists, you blame the lawyers. As Victor McKusick, professor of medical genetics at Baltimore, put it: "It's the lawyers who keep the pot boiling".

But as a lawyer involved with the liberty of the subject, I am not so sure. Science rarely stands still. Even now (so I am told) the view, which so far has been widely propagated, that DNA profiles are produced solely from non-coding DNA, is being questioned. I look forward to the discovery that DNA has the structure of a triple helix.

This all goes to show - what exactly? Not that DNA profiling should be thrown out, but that a technique such as DNA profiling should not be introduced into the criminal process without adequate safeguards and controls. I was amazed when it was first "discovered" and used in the UK. It just appeared, suddenly, almost without warning, in the courts. It was tried and tested, of course, but quietly and in private. The experts all worked for the prosecution and the courts accepted the evidence without knowing what it was all about. For all the judges knew, Alec Jeffreys was a member of the Magic Circle.

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Things are moving so fast in this field, it seems to me as a layman, that this speech will probably be given in five years time by a pig; by that, I don't mean a police officer - that really would be an advance, and one beyond the scope of science, I suspect.

But the serious and important subject which I wish to address today is the human rights implications of DNA profiling in the forensic context. Let me raise some of the problems and, somewhat more tentatively, suggest some of the answers. I shall deal with it in three parts: PART I A new scientific technique without controls. PART II The problem of genetic privacy. And PART III The protection of human rights.

PART I A NEW TECHNIQUE WITHOUT CONTROLS.

Science has been used in the forensic process to variable effect. While fingerprint comparison has stood the test of time well, tests for explosives, for example, have fared less well.

It was the dubious scientific evidence in the IRA bombing cases which propped up the false confessions and led to the worst wrongful convictions in modern British history. To discover that swab tests giving a positive reading for explosives could have been caused by everyday items such as playing cards was bad enough. But a series of errors, omissions and conspiracies by forensic scientists gradually emerged from these cases.

In one case, that of Judith Ward, who was convicted of an IRA coach bombing in which 12 died, the appeal court held that government scientists "took the law into their own hands" and "succumbed to the dangers of partisanship". And these are scientists who inevitably have high credibility in the eyes of judges and juries.

One old idea, which never quite made it to court (in the UK, at least), was the polygraph. The British government did start using it, only a few years ago, for vetting security officers. But phase 2 of the project was postponed indefinitely when 40% of MI5, the secret service, failed the test. Thomas Hobbes was no doubt right when he wrote in the seventeenth century that science was "The skill of proceeding upon general and infallible rules". Some rules have proved to be more fallible than others.

As you will know, the use of DNA profiles in court has not been without controversy. It is well-known that "there are lies, damned

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D N A - A HUMAN RIGHTS PROFILE

PETER THORNTON QC

INTRODUCTION

I am a lawyer and not a scientist. I used to think that a single locus probe was an investigation into the lifestyle of adults without partners, until I discovered DNA profiling. As a lawyer, I have a healthy mistrust of science in the forensic process. On the whole I subscribe to the sentiments of the old proverb: "Much science, much sorrow."

Let me give you an example. In a recent double murder case - the bodies of two women were found in a car in North London. The prosecution wanted to prove that the women had been killed in the flat which they shared with a man, the defendant. The link had to be made with blood found on the car, which was considered to be post-mortem blood, with blood in the flat. Over a period of months extensive blood testing, DNA testing and PCR testing took place, but no link was ever established between the two sets of blood. Just three days before the trial the government scientist made a final witness statement, saying, for the first time, that the blood was the same, post-mortem blood, because it looked the same.

As scientists, you will no doubt say, she was just using that celebrated scientific instrument, the naked eye. As a lawyer, I merely questioned why she had not made a note of this important finding nine months before. Not exactly "Much science, much sorrow"; more a case of "Little science, late in the day".

But I am here to talk about a specific aspect of DNA. I am not going to talk about prolonging life with "ageing genes", although it reminds me of the 45 year old lawyer who died in a car crash. When he arrived at the gates of heaven, he complained bitterly to St Peter: "What did I do to deserve death at 45?" And St Peter replied: "45? Actually, according to your billing hours - you're 96." Nor am I here to talk about identifying sexual orientation through genes, a subject which led to one creative newspaper headline in London: "Abortion hope after 'gay genes' findings".

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1. Genetic typing and human rights

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laboratory protocols; b) centralized databanks; c) well-defined reference populations; d) common sets of DNA markers. It is time to join efforts. I know from my personal experience in Italy that the forensic scientists are very open-minded in this respect: I hope this Congress will be remembered as the one where the problem of identifying the suspect is paired also to the problem of identifying ourselves and our origins.

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very indirectly. In the past, less advanced societies, however, vertical cultural transmission (from parents to children) was common, and horizontal transmission (between unrelated people) rare, while it is very frequent in modern society [26]. Cultural traits transmitted vertically and therefore in a way more similar to that of genes must evolve like them, and one can expect to see coevolution of genetic and cultural traits thus transmitted, including languages.

6. Prospects for the future

I would like to close this note by linking the soft *scenario* I just presented to the hard bench work of the forensic haematologist. A considerable part of his job is today that of comparing DNA profiles. More precisely major efforts are spent to calculate with as much precision as possible two likelihoods: 1) that of two matching DNA profiles coming from the same person; 2) that of two matching DNA profiles coming from two different individuals randomly drawn from a structured extant population. I would suggest that the former likelihood depends on the technology, i.e. the future: mini- and microsatellites, PCR-based typing, direct sequencing,etc., shall increase the degree of resolution by which matching of bands will be assessed or excluded with a more and more decreasing error. The latter likelihood is depending on how the population is genetically structured, i.e. on its past, its history, that history we try to interpret from their current genetic geographic images. The forensic scientist lies at the border: he looks at the future challenged by a moment of much innovative activity in DNA technology; but he must look back at the past as well in considering how populations evolved to the present structure. A way of not loosing our head by this kind of squint is to share the challenge with scholars of other disciplines, and clearly I am thinking mainly of geneticists and statisticians. The study of human variation is one of the principal areas of interest for geneticists and the introduction of techniques that allow the direct study of DNA is going to add new dimensions to the analysis of human diversity. World-based and European projects have been proposed with the aims of planning systematic studies of genetic differences in suitably chosen samples of our species and saving, for future analyses, the DNA of a significant proportion of individuals representing current human variability. Many of the problems they are going to face are common to those of the forensic community, mainly the establishment of: a) standardized

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5. Major expansions and gene-culture evolution .

The genetic map of the world seems to carry the traces of many human expansions. Each of them was most probably the result of some technological innovation which generated population growth and helped spreading people from the nuclear area of origin to new regions. Some of these may have been unoccupied or only more sparsely settled before, and especially in the latter case genetic clines must have formed.

Some major expansions occurred in Paleolithic and their traces are the cause of the major differences we still see among aboriginal populations of continents and parts of them. After Paleolithic, the major expansions were determined by the development of agriculture, which arose at very similar times in widely different regions. The earliest were in the Middle East , in China (several centers, one in the north and at least two in the south) and Mexico-northern Andes. Spread from the Middle East is very clearly visible towards Europe, towards Central and South Asia and to some extent towards North Africa. Arab and Bedouin migrations to North Africa in the seventh century AD may have made it more difficult to follow the initial spread. Similarly, genetic data do not yet do much to clarify the genetic effects of the Chinese agricultural expansions; but we know that north and south China have different ecology, neolithic history, genetic and cultural maps [25]. They grew at least initially different crops (millet in the north, rice in the south). Genetic data are not yet detailed enough to show much more than a sharp difference between north and south.

In summary, major expansions prior to the modern era provided a series of "punctuations" [3] due to cultural innovations which we consider major determinants of our genetic change. The spread of a population entails the spread of its culture: therefore it is simply natural that the first settlers carried their language with them: this was certainly true for the spread of Europeans after the great geographic discoveries. However, during long periods such as that of the spread of Neolithic farming to Europe which lasted nearly 4,000 years (from 9,500 to 5,500 years ago), the initially present languages would transform and differentiate sharply between regions and their differentiation is difficult to trace back, as, by definition, no written record of prehistoric events exist and prehistoric languages are inferred

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a thus strong indication of an expansion west and north starting from Japan; the period is difficult to establish, but the date of population peak of 4,000 years ago may be a reasonable date for the expansion.

The genetic map of Africa is reasonably well-known, apart from the Sahara which is presently inhabited only in restricted zones. The synthetic map of the first PC values shows the distinction between the Caucasoid populations inhabiting the northern part of the Sahara, and black subsaharan populations. The Sahara was not a desert until a few thousand years ago, and was the seat of agricultural development, especially cattle breeding which may have been in part local. With the drying up of the Sahara, 3-4,000 years ago cattle had to migrate south and farmers (mostly dark-skinned) started domesticating plants like sorghum, millet and others in the Sahel region, immediately south of the Sahara. Much of these new agricultural developments were in West Africa, from where a well-known expansion began heading toward the east and south around 3,000 years ago or earlier. Its latest and most important part is usually called the Bantu expansion, because people at the boundary between Nigeria and Cameroon spoke Bantu languages and spread them over all of Central and most of South Africa. This radiation from West Africa is well visible on the synthetic map of the second PC values.

The synthetic maps of the Americas are more complicated, mostly because of extreme drift [22]. The synthetic map of the first PC values shows the approximate locations of three major groups of American natives: *Eskimos* in the extreme north, *Na-Dene* below them, and *American Indians* all over the rest. This classification was suggested by the linguistic work of Greenberg, odontological work of Turner, and preliminary genetic work of Zegura [23]. The synthetic map of the second PC values shows a heavy hybridization with European settlers in the east coast of North America, and the basic homogeneity of the Andean region. The analysis of the whole continent, however, fails to single out regions of spread of agriculture which, as well-known, started on the Mexican plateau and the northern Andes. Agriculture spread only lately from Mexico to North America, perhaps because it was difficult to cross the Sonoran desert in the north of Mexico, and the regions bordering Mexico further north were also very arid. It was only some 5,000 years after the beginnings of agriculture in Mexico that the first agricultural communities developed in the southern United States, and agriculture started spreading across the prairies [24].

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Before leaving Europe for Asia, I should like to show you another relevant facet of the biology of Europe: what about anthropological traits?. More than a year ago, when the title of my talk was announced, I received a kind letter from a member of our Society expressing the hope that I would not restrict "genes" to "blood genes". In fact, the author of the letter - Dr. Ròsing from the University of Ulm - is coauthor with Dr. Harding and Prof. Sokal of an interesting analysis of craniometric data collected by Dr. Ròsing himself and Prof. Schwidetzky [20]. They consider 10 cranial measurements taken from fossils of three different periods: 1) before 3,000 B.C.; 2) 3,000-2,000 B.C.; 3) 2,000-1,000 B.C. The contour maps of their geografic variation in Europe show an East-West gradient very similar to that of our analysis of the third principal component of genes. The interpretation of this finding is not easy, even less so because of the low number of sites, around 50, on which the analysis is based. We can reasonably exclude, as the authors correctly do, an influence of the Neolithic spread of farmers on the cranial morphology, but two possible explanatons remain open: a) a demic diffusion of genes (Indo-European?) from the Eurasian steppes as suggested by our genetic data or b) an unidentified influence by environmental factors which are known to affect craniometric and in general morphological traits more directly than genetic types.

The synthetic map of the first PC values of Asia shows a strong west to east gradient which has an apparent origin in the Middle East. This is likely to represent the eastern expansion of agriculture from the Middle East, which certainly reached as far as India in the eastern direction, and the steppes in the northern direction. The synthetic map of the second Asian PC values is an almost perfect gradient north to south. In the map of the third PC values there is an apparent center of expansion in the middle of Japan . In the last glacial period there was an almost complete continuity between Japan and continental Asia, both north and south, and the sea of Japan was practically an internal sea. Thanks to an archaeological survey of almost 28,000 sites [21], the curve of population growth in Japan has been reconstructed since the year 10,000. Around 4,000 B.P. the Japanese population reached its maximum of about 260,000 with a density of 0.89 per Km^2 and considerable variation from region to region . The period is called "Neolithic", although there was no agriculture until about 1,700 years ago. Foraging and fishing produced high population densities in Japan without agriculture. There is

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in the central Russian steppes. A connection was suggested between the kurgan culture and migrations of Indo-European speakers by Gimbutas [14].

Recently, Renfrew suggested that Indo-European languages came to Europe from Anatolia with the spread of farmers [15]. His rationale was that farmers did spread as discussed above, and they must have carried to Europe their original language. This must have undergone considerable evolution in the process of spread, but replaced almost all Pre-Indo-European languages spoken before in Europe, with one clear exception: Basque. Hypotheses on the place of origin of a language are notoriously difficult to support or disprove. Different authors suggested many different homelands for Indo-European [16] emphasizing the uncertainty associated with this decision. The problem is compounded by the possibility that there may have been several origins, scattered in time and space. The Gimbutas hypothesis of an origin in the kurgan region and spread during the Bronze Age (between 4,000 and 2,500 BC), which seems to have greatest support from archaeological and other considerations discussed in [17], is really not alternative to the Anatolian origin. Both hypotheses may be correct, the kurgan origin being much later, and secondary to the spread of agriculture from its origin to the steppes, and the establishment there of the new economy of pastoral nomadism.

The success of pastoral nomads was not limited to the expansion of the kurgan culture to Europe, but extended to a great number of other, some quite recent radiations (e.g. Turks in the last millennium). Unquestionably, however, the most spectacular of these secondary developments of agriculture was pastoral nomadism in the steppes of southeastern Europe and central Asia. The steppes extend from Romania to Manchuria in an almost uninterrupted way. The domestication of the horse took place in the western steppes, about 5,000 years ago or earlier, and was soon followed by military applications [18,19]. The way of life of pastoral nomads, their migrations in large numbers, demanded the development of considerable mobility, logistic and military skills. War chariots and later cavalry were important innovations which gave considerable military and political power to nomadic sheferds of central Asia. Nomads generated a hierarchical society in which they formed the highest castes, and the original farmers were organized in various lower castes distinguished by geography and profession. This social system was introduced by pastoral nomads from the Russian steppes in the Indian continent, where it survives today except where the Hindu religion never acquired control, or lost it to the Moslems.

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in the Middle East, beginning around 10,000 years ago . Cereals and animals domesticated in the Middle East spread slowly in all directions shortly after 10,000 years ago. The expansion to Europe is best known and took place at the average rate of 1 km. per year [11]. The question was asked, if the spread from the area of origin was cultural (i.e., it was farming which diffused) or demic (farmers radiated) [12].

We originally developed synthetic maps [9] as a device to further test this hypothesis. If farmers truly spread from the Middle East in early Neolithic to all of Europe, it might be possible to detect still today traces of this migration in the genetic map of Europe. In particular, a comparison was made between the geographic map of the spread of agriculture to Europe, based on archeological dates of first arrival of farming in various regions of Europe [11] and the genetic maps. The correspondence between the archaeological map and the synthetic map corresponding to the first PC was remarkably clear [9]. On this evidence it was concluded that the diffusion of farming from the Middle East to Europe was a spread of farmers and not simply that of the technology. The correlation of the first PC values with the dates of first arrival of Neolithic farming (given in [11]) is 90%. Maps of Europe obtained with PCs of lower rank are also quite interesting. The second map of Europe shows a north to south gradient, which may reflect a climatic effect, but may also be at least in part of ethnic origin. In the north of Europe live Lapps, who have genetic similarity with other north-Eurasian populations from Siberia, and speak a language from the Uralic family, found today mostly east and west of the Urals. Lapps have undergone very substantial admixture with Scandinavians [13] and in spite of their probable west Siberian origin are now classified as Europeans, although they diverge considerably from all of them, being the extreme European outliers. Southern Scandinavians expanded into the area occupied in the north by people of Uralic origin like Lapps, establishing a gradient. Thus the north-south gradient may in part be due to selective effects of climate, and in part to contributions from East Asian populations related to the Uralic ones.

The third PC of Europe strongly suggests another possible expansion. It clearly defines a nuclear area between the Dniepr and Volga rivers, north-east of the Black Sea and north-west of the Caucasus. This is the same region where archaeological data place the beginnings of the "kurgan" culture, believed to have been developed, starting around 6,000 years ago, by pastoral nomads of the Eurasian steppes. Kurgan is the Russian name for a burial mound, very common

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One cannot exclude that navigation may have helped Africans to reach S.E.Asia (and finally Australia) along the southern coasts of Asia. Great advantage must have been provided by a fast and precise system of verbal communication while expanding to new territories and environmental niches, involving quite different climatic conditions not to mention flora and fauna.

3. Synthetic genetic maps of the world

Population movements after Paleolithic did not have the time to completely erase the differences generated in Paleolithic, but added new genetic patterns still recognizable today. No single gene would allow to clearly trace changes operated by later migrations on the original differences, but combining the information from as many genes as possible allows such reconstructions. We introduced the methodology of "synthetic" genetic maps, which are geographic maps of lines of equal values of principal component values (PCs) calculated from many gene frequencies [9]. This map generates a "genetic landscape" which is independent of all the others, and explains a fraction of the total genetic variation. We have shown by simulation that different PCs can separate expansions which started at different times and places [10]. The genetic variation explained by a PC is a function of the magnitude of genetic differences between the expanding population that migrates to a new area, and the aboriginal populations.

The synthetic map of the world [1] is in excellent agreement with the hypothesis of an expansion of modern humans from Africa: an extreme PC value is in Africa, the other in Australia. The synthetic map cannot choose between these two poles as origin of the expansion, but the archaeological records shows Africa was occupied by modern humans (and their ancestors) long before Australia. It also shows that much of human variation is in the west-east direction and therefore that adaptation to different climates cannot be the major determinant of variation observed at the genetic level.

4. Neolithic and post-neolithic expansions

The Neolithic transition is the passage from foraging (hunting and gathering), i.e. food collection, to food production by agriculture and animal breeding. Its earliest development was

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densities were especially low, and the effect of genetic drift was therefore quite extreme, while later numerical increases progressively reduced the importance of drift and genetic differentiation. We should like to elaborate on this idea and to show how these expansions "punctuated" the evolution of human populations [3].

There were probably at least two major expansions of the genus *Homo*. Starting between one and two million years ago, *Homo erectus* spread from Africa to all of the Old World. Anatomically modern *Homo sapiens sapiens* is believed by a number of paleoanthropologists to have started spreading from Africa between 130,000 and 60,000 years ago and to have continued moving until the present. The finding of old and reasonably well-dated modern humans in West Asia may somewhat change the origin of this second *Out of Africa* expansion, but archaic *Homo sapiens* closest to modern humans seems to come from Africa [4]. Other paleoanthropologists [5], however, prefer a hypothesis of local development of differences based on local continuity of East Asian skull morphology.

The stimuli to these major radiations from Africa or less probably from East Asia must have been either cultural or biological developments, or both, connected directly or indirectly with the efficiency of food collection and/or with drives or incentives to expand. The first radiation from Africa is that of humans with a larger brain and a relatively sophisticated set of stone tools. It seems obvious that an improved tool arsenal gave considerable advantage in the yield of food collection, and the chances and opportunities of spread to new habitats. A major coevolution of culture and perhaps brain biology has probably anticipated the origin of modern humans and their radiation. The human brain had reached its present size already before the second expansion, with the appearance of *Homo sapiens*. It is also likely that during this period modern human language went through the last stages of its evolution to its present degree of sophistication, and was the major support of the second expansion. While this is speculative, there is hard evidence that in the period between 100,000 and 60,000 years ago, prior to the expansion or accompanying its beginning, there was a substantial modification of the tool arsenal [6]. In addition, local cultures showed increased differentiation of stone tools, as if an incipient linguistic differentiation favored partial segregation and divergence of rapidly evolving local technologies [7]. Use of rafts, boats etc. must have been common because around 55 or 60,000 years ago several sea tracts had to be passed for reaching Australia [8].

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HISTORY AND GEOGRAPHY OF HUMAN GENES

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1. Introduction

The collaboration between the Forensic Community and the scholars of human population genetics, among whom I place myself, has always been fruitful and of reciprocal benefit in Italy as much as in Europe and in North-America, in the latter with a more dialectical attitude as shown by recent rather hot debates. DNA analysis is today offering new possibilities of collaboration. Case work and search for reference populations complement in the daily activity of the forensic scholars. Substantial DNA databases have already been established for a number of population groups, but the development of new standards and new reference databases is likely in the immediate future following the implementation of PCR-based DNA typing systems, and a strong argument can be made for population geneticists to share protocols and markers with the forensic community in order to type well-defined reference populations and from them to contribute to the analysis of human genetic diversity. My talk, however, does not address to the future, but rather to the past: I have had the chance to analyze, over the past ten years, many genetic data from human populations and I am going to give a short review of our recent analyses. Our main interest lies in their interpretation in terms of prehistory and history of our species: a more comprehensive treatment will appear in a forthcoming book written in collaboration with Cavalli-Sforza and Menozzi [1].

2. Expansions in Paleolithic

A large fraction of the earth was settled by modern humans already a long time ago. At the end of Paleolithic, about 10,000 years before present, the number of inhabitants of the Old World was somewhere between one and ten million inhabitants [2] . We believe major genetic differences had already evolved in Paleolithic and that the expansions which took place at *that* time left a genetic trace which has not been completely erased by later events. Population

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Opening lecture

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