Plasma Protein Polymorphism in HIV-Seropositive Patients: Allotype Frequencies in the Gm- and Km-System

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SUMMARY

Examinations on human Immunoglobulin systems represent an important connecting point between serogenetics and immunogenetics. This paper reports on the distribution of genetic markers in the Gm- and Km-systems found in serum samples of HIV-seropositive patients. The results are discussed.

INTRODUCTION

In 1987 *Eales et al.* reported on a strong association between AIDS and the GC-system (D-binding protein). They suggested that individuals with the GC*1F subtype would take more risk to develope AIDS. Also it was suggested that the homozygous type GC*2 may render a relative protection (1).
In the meantime several reports resulted in non-confirming these observations.
However, *Eales*'s report 'has created considerable concern'.
Since genetic factors and especially genetic influences on the immune response could be important in the tendency to develop deseases, we decided to investigate immunoglobulin allotypes in HIV-seropositive patients (References see 3).

MATERIALS & METHODS

Blood samples were taken from 157 patients of the Institute of Blood Coagulation and Transfusion Medicine at the *Heinrich-Heine*-University. For this pilot study we restricted us to only one marker: HIV-seropositive. Therefore the collective included individuals HIV-seropositive only, patients with ARC (AIDS related complex), with AIDS and with *Kaposi*-sarcoma.
The typing reagents were all commercially available, all factors ($Gm^{1,2,3,10,21}$, $Km^{1,3}$) were typed by following the appropriate directions for use of each reagent.

RESULTS AND DISCUSSION

The Gm and Km phenotypes of 157 (146) HIV-seropositive patients are listed in table 1 and 2.

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The BF allele gene frequencies are different in distinct ethnic groups. The gene frequence of BF*F is significantly higher in Negroes than in Whites. Therefore and because of its physiological role in inflammatory processes and linkage to the HLA system it seemed to be meaningful to check fora hypothetical association between the BF system and HIV infection.
We examined a sample of 86 HIV-seropositive patients. The distribution for the BF system found in this collective is shown in table 1. Surprisingly we found no satisfying agreement between the observed and the expected numbers of phenotypes. Meanwhile the nonconfirming of the S type may be due to the small number of the collective. The nonagreement of the homocygous F type and the heterocygous BF*FS regarding to the expected numbers of cases is statistical significant. Comparing with a control group of obviously healthy individuals resulted in confirmation. The gene frequencies found in this group are similar to those reported by other authors on German collectives. An increasing gene frequency of BF*F in a southerly direction can be noted.

In our opinion caution is advised in interpretation of the results. Nevertheless the results are interesting because of the strong linkage between BF and the HLA system. Also frequencies in Negroes may be recalled.
One specific mark of our HIV-seropositive group - patients have been treated with 'autovaccination' - couldn't - in our opinion - influence the results. Other marks like homosexuality etc. must be itemized in further studies.
We are extending our investigations into more patients with the aim to create sufficient subgroups including different stages of disease. Nonetheless this paper reports the preliminary results because it might be impossible for only one research group to examine enough patients for definite decision on association or non-association.

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See: Huckenbeck W, Bonte W, Kuntz BME, Brüster HTH (1989) *Plasmaprotein-Polymorphismen bei HIV-seropositiven Probanden: GC- und TF*C-Subtypen, PI-, C3- und BF-System*
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Table 1 DISTRIBUTION OF BF-PHENOTYPES IN HIV-SEROPOSITIVE PATIENTS

	observed		*expected*	
Phenotype	*n*	*%*	*n*	*%*
S	53	61,63	46.15	53.66
F	11	12.79	4.65	5.41
FS	16	18.61	29.31	34.08
FVar*	2	2.32	1.40	1.63
SVar	4	4.65	4.40	5.12
Var	0	0	0.11	0.10
total**	86	100	86	100

*The following values contain variants including F1 and S1 (S 07)
**values rounded up

Table 2 DISTRIBUTION OF PHENOTYPES IN THE BF-SYSTEM CONTROL GROUP

	observed		*expected*	
Phenotype	*n*	*%*	*n*	*%*
S	100	62.50	99.23	62.02
F	7	4.38	6.60	4.13
FS	50	31.25	51.18	31.99
FVar*	1	0.62	0.61	0.38
SVar	2	1.24	2.37	1.48
Var	0	0	0.01	0.01
total	160	100**	160	100

*The following values contain variants including F1 and S1 (S 07)
**values rounded up

Gene frequences: F .1840, S .7980, Var .0180

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Smithies demonstrated a genetically determined polymorphism of the third component of Complement by using technics of agarose and starch gel electrophoresis, numerous investigations have been reported. Most of them were based on population genetics - additional on differences between Caucasians and coloured people. Hereditery defiency states of one and another of the Complement components have been documented in experimental animals and man.
Especially C3 and its conversion products (β_{1A}-globulin etc.) were examined in detail. Emphasis is placed on the fact that the Complement cascade can be triggered not only by activation of the components C1 etc. but also by activating C3 by an alternative pathway. *Whitehead* et al. and *Teisberg* et al. reported assignement of the gene for C3 to chromosome 19. The physiological function of the Complement reactions contains cell lysis, neutraliztion and lysis of virus. Therefore this study of genetic markers in HIV-seropositive individuals includes the C3 system, the Complement component with the highest serum level in man.
In a total of 86 HIV-seropositive patients we observed 3 known C3 alleles: C3*S, C3*F and C3*S^{06}. Our results of C3 phenotypes provide a satisfactory correlation to the *Hardy-Weinberg*-equilibrium. Small differences between the observed and the expected values may be due to the small number in the collective. We found the following gene frequencies (calculated by gene counting): C3*S .814, C3*F .180 and C3*Var .0060.
The results seem to be in accordance with the results obtained by other authors in Germany.
Therefore we conclude that this pilot study results in a normal distribution of C3 phenotypes in HIV-seropositive persons. Because of the small number in the collective and the restriction to only one marker (HIV-seropositive) this examination will be extended to cover the reported results.
If one sees AIDS to have originated in Africa one would expect, if an association can be assumed, a high frequency of C3*S. If one excludes the possibility of a gene drift our study does not demonstrate an association between AIDS and the distribution of the C3 system.

BF system BF - also known as C3 proactivator, C3 PA, factor B, glycine-rich glycoprotein, GBG - as one of the major participants in alternative pathway of Complement activation is coded by an locus on chromosome 6. The BF locus is closely linked to the major histocompatibility complex and it is placed between HLA-B and HLA-D. Because of this linkage the BF marker is suitable for use in the study of association between genetic susceptibility and diseases. *Bertrams* et al. described association between BF and Insulin-dependent diabetes mellitus. A possible linkage between BF and SLE (systemic lupus erythematodes) as examined by *Klemp* et al. could not be proved. *Papiha* et al. found a striking association between a special BF allele and glomerulonephritis.
However, the glycoprotein BF (mol.w. 90.000) is known as an acute phase reactant during acute inflammatory episodes.
The genetic polymorphism of the BF system was first described by *Alper* et al.. There are two common alleles BF*F and BF*S, two less common alleles BF*F_1 and BF*S_1, and rarer alleles.

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Distribution of C3 and BF Phenotypes in HIV-seropositive Patients

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SUMMARY

The paper reports on the distribution of C3 (Complement Factor 3) and Bf (Properdin Factor B) in serum samples from HIV-seropositive patients. The gene frequencies of the C3-system are similar to those in healthy persons. In the Bf-system we found unusual features of distribution. Problems of interpretation are discussed.

INTRODUCTION

In 1987 *Eales* et al. reported on a strong association between AIDS and the GC-system (D-binding protein). They suggested that individuals with the GC*1F subtype were at greater risk of HIV-infection and to develope AIDS. It was also suggested that the homozygous type GC*2 may render a relative protection. In the meantime several reports did not confirm these observations.
However, *Eales*'s report 'has created considerable concern'. Examining plasma protein polymorphism seems to be useful. In principle studies on genetic polymorphism gain further importance in connection with a possible correlation between genetic markers and various diseases and the resulting selective advantages.
In this pilot study we investigated the distribution of C3 (β_{1C}-globulin, 3rd component of human Complement) and BF (glycine-rich beta-glycoprotein, C3 proactivator, Properdin factor B) phenotypes in a sample of 86 HIV-seropositive patients.

MATERIALS & METHODS

Blood samples were taken from 86 patients treated in the Institute of Blood Coagulation and Transfusion Medicine at the *Heinrich-Heine*-University. For this pilot study we restricted us to only one marker: HIV-seropositive. Therefore the collective included individuals that were HIV-seropositive only, patients with ARC (AIDS related complex), with AIDS and with *Kaposi*- sarcoma.
We compared this group with other spot checks. The BF-control group contained 500 obviously healthy persons.

RESULTS AND DISCUSSION

C3 system Since *Wieme* and *Demaulenaere* , *Alper* and *Propp* and *Azen* and

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To our knowledge association between AIDS and the PI-system has never been discussed. We included PI typing in this pilot study because of its function as an 'acute phase' protein. In 99 serum samples of HIV-seropositive patients we found 3 persons with the heterozygous phenotype Pi*M1S, the others showed phenotypes due to the M allele only. The findings are shown in table 1. In our small collective we found insufficient correlations between the observed and expected values. Especially the homozygous PI*M2 and the heterozygous PI*M2M1 show a distribution which is not consistent with the *Hardy-Weinberg*-equilibrium. The homozygous PI*M2 phenotype is overrepresentated in this spot check. Comparison with the control group (tab.2) confirms these findings. Comparison with other studies on PI-polymorphism shows that the phenotype's distribution of the control group is similiar to those found in other German regions.

In our opinion it is absurd trying to discuss hypothetical theories of possible association. First, one must think about the risk of 'small number'. On the other hand the deviation is statistical significant. Refering to the 'GC theory' there are no comparable differences between whites and blacks. At least all associations found between the PI-system and other specific diseases are due to deficiency alleles but not to the common PI*M alleles.

Therefore we come to the conclusion that hasty attempts of explanation are not useful. However, the findings are statistical significant. Nevertheless the possibility of 'super chance' must be excluded by extended examinations on spot checks of sufficient number. One specific feature of our HIV-seropositive group - patients have been treated with 'autovaccination' couldn't - in our opinion - influence our results. Other characteristics like homosexuality etc. must be itemized in further extended studies.

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Table 1 PI-PHENOTYPES IN HIV-SEROPOSITIVE PATIENTS

	observed		*expected*	
Phenotype	*n*	*%*	*n*	*%*
M1	59	59.60	54.57	55.12
M2M1	15	15.15	25.99	26.25
M2	9	9.09	3.09	3.13
M3M2	2	2.02	2.30	2.32
M3	0	0	0.43	0.43
M3M1	11	11.11	9.66	9.76
M1-Var*	3	3.03	2.23	2.26
M2-Var	0	0	0.53	0.57
M3-Var	0	0	0.02	0.20
total**	99	100	99	100

*The following values contain variants including Pi S and Pi Z
** Values rounded up

Table 2 PI*M-PHENOTYPES IN THE CONTROL GROUP

	observed		expected	
Phenotype	n	%	n	%
M1	87	54.37	89.25	55.78
M2M1	39	24.38	35.85	22.40
M2	3	1.88	3.60	2.25
M3M2	3	1.88	4.35	2.72
M3	2	1.25	1.31	0.82
M3M1	22	13.75	21.65	13.53
M1Var*	4	2.50	2.99	1.87
M2Var	0	0	0.60	0.38
M3Var	0	0	0.36	0.27
Var	0	0	0.03	0.02
total**	160	100	160	100

*The following values contain variants including Pi S and Pi Z
**Values rounded up

Chi^2: 1.611 (Chi^{2*} 3.841; df=1; alpha=0.05)

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RESULTS AND DISCUSSION

GC-System Our examination also resulted in non-association between AIDS and GC. The distribution of GC-subtypes in our HIV-seropositive group is comparable to those found in obviously healthy individuals. We found the following gene frequencies: GC*1S .5808, GC*1F .1414 and GC*2 .2778.
Adding these results to the data of the other authors we come to the conclusion that the suspicion of association between GC and AIDS can not be upheld. But in our opinion it has shurely not been disproved that with a normal risk of infection any one allele may give a relative protection of developing AIDS. In this study we restricted us to the one marker "HIV-seropositive. Other author's data - splitting HIV-seropositive only, ARC and AIDS - were taken on small groups. Therefore we plan extended examinations on further patients - sampling has already started - which includes splitting into subgroups of sufficient volumes.

TF-system Transferrin (D-binding protein) is an iron binding glycoprotein of the ß-globulin fraction. Because of its binding capacity and transport function transferrin seems to play a part in immune reactions. The common six phenotypes are due to three codominant alleles TF*C1, TF*C2 andTF*C3. In this study we examined the distribution of TF*C subtypes of 99 HIV-seropositive individuals. We found only the common phenotypes. The observed values are similar to the expected values (calculated by gene counting). We found the following gene frequencies: TF*C1 .7374, TF*C2 .1616 and TF*C3 .1010. The results seem to be similar to those found in obviously healthy persons. The some what higher frequencies of the TF*C2 and TF*C3 alleles might be caused by the small volume of the collective. In our opinion the differences are not significant.

PI-system PI (alpha$_1$-antitrypsin) is the most important protease inhibitor of the extracellular compartment. It is coded by chromosome 14. Being a glycoprotein (mol.weight 50.000) of the 'acute phase' in inflammatory processes it has its physiological function in inhibiting neutral proteases, especially in inactivating elastase.
Since the first description of genetic variation development of specific methods led to the observation of more than 60 genetic variants. The common six PI*M phenotypes are due to the codominant alleles M1, M2 and M3. The PI*S and PI*Z alleles have lower frequencies (0.03 and 0.01 in whites).
The expression of specific alleles (S,Z I) can cause a decrease of the alpha$_1$-antitrypsin serum concentration. This may have a significant effect on health, the development of hepato and pneumopathly is dependent on these serum concentration and tissue deposition of abnormal molecules. Also association between rarer alleles and rheumatism is discussed. *Erban* et al. found significant increased alpha$_1$-antitrypsin levels in blood samples of psychiatric (schizophrenics) patients.

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Plasma Protein Polymorphism in HIV-Seropositive Patients: GC- AND TF*C-SUBTYPES AND PI-SYSTEM

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SUMMARY

The paper reports on the distribution of three plasma protein systems in serum samples from 99 HIV-seropositive patients: Gc-subtyping, Tf_C-subtyping and Pi- (alpha$_1$-antitrypsin) system. In the Gc-system - as in further studies - we found evidence for non-association of Gc-subtypes - especially 1F - with HIV-infection. Also the distribution of Tf_C-subtypes seems to be very similar to studies on healthy persons. In the Pi-system this pilot study led to the suspicion that the phenotype Pi*M2 is overrepresented. The distribution found in these patients is not comparable with the control group.

INTRODUCTION

In 1987 *Eales et al.* reported on a strong association between AIDS and the GC-system (D-binding protein). They suggested that individuals with the GC*1F subtype would be more at risk to develope AIDS. Also it was suggested that the homozygous type GC*2 may render a relative protection.
In the meantime in several reports this observation could not be confirmed.
However, *Eales*'s report 'has created considerable concern'.
Such studies on fringe groups take the risk of 'small volume'. Therefore we decided to carry out an additional examination. Furthermore we included in this study the PI- (alpha$_1$-antitrypsin) and the TF- (transferrin) system.

MATERIALS & METHODS

Blood samples were taken from 99 patients of the Institute of Blood Coagulation and Transfusion Medicine at the *Heinrich-Heine*-University. For this pilot study we restricted us to only one marker: HIV-seropositive. Therefore the collective included individuals that were HIV-seropositive only, patients with ARC (AIDS related complex), with AIDS and with *Kaposi*-sarcoma.
Because not all patients lived in the Düsseldorf area we compared this group to other German studies on distribution. Our PI-control group includes 160 obviously healthy persons.

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DISCUSSION

The study of chromosome polymorphisms is highly useful in many fields of human genetics, including paternity investigations: (Gürtler and Niebuhr, 1986; Olson et al., 1986; Balicek et al., 1978; Shiono et al. ,1985; Kamenev et al., 1986). Although not very informative, the polymorphisms of heterochromatic regions of chromosomes 1, 9 and 16 here described can be useful, at least as ancillary markers in some situations, such as:
(a) the accused man, not being excluded by the current battery of tests of the laboratory has a low paternity probability and,
(b) deceased persons are involved, but their karyotypes are available, namely resulting from previous clinical studies.

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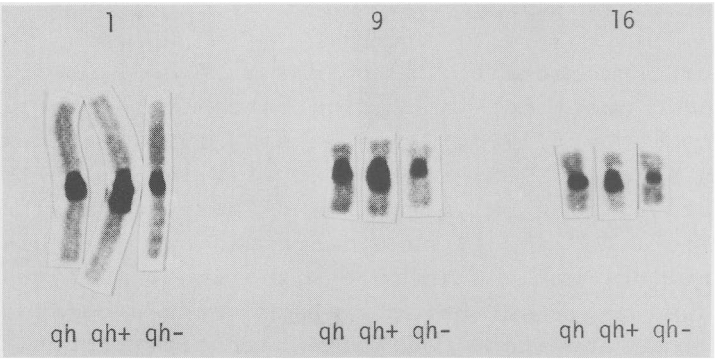


Fig 1. Polymorphism of heterochromatic regions of chromosomes 1, 9 and 16 observed in our sample.

Table 1. Frequencies of chromosome polymorphisms, *a priori* probability of exclusion (PE) and mean probability of paternity among fathers (WF).

chromosome		freq.(%)	PE(%)	WF(%)
	1qh+	3.3		
1	1qh-	4.8	7.7	54.1
	1qh	91.9		
	9qh+	4.3		
9	9qh-	2.2	6.2	53.3
	9qh	93.5		
	16qh+	3.3		
16	16qh-	0.5	3.6	52.0
	16qh	96.2		
TOTAL			16.6	59.3

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Chromosome polymorphisms in paternity investigations

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INTRODUCTION

The use of chromosome polymorphisms in paternity expertises has been rare, when compared with current classical antigen or biochemical analysis or even with HLA phenotyping. In this work we demonstrate that polymorphism of heterochromatic regions of chromosomes 1, 9 and 16, evidenced by simple routine cytogenetic techniques, may become very helpful, at least in "difficult" cases, such as those where a man is not excluded using current battery of tests but the corresponding value of W is low.

MATERIAL AND METHODS

The polymorphism of heterochromatic regions of chromosomes 1, 9 and 16 was studied in a sample (n=302) of unrelated individuals from Porto district (Portugal).

Metaphase chromosomes were obtained by a 72 hours lymphocyte cell culture (Moorehead et al.,1960). Slides were stained by the C-banding technique of Sumner (1972). At least eight, and up to fifteen metaphases were observed per individual.

RESULTS

The common polymorphisms of heterochromatic regions of chromosomes 1, 9 and 16 are depicted in Fig.1.

In Table 1 we present the corresponding frequencies in Porto district as well as the *a priori* probability of exclusion (PE) and the probability of paternity among fathers (WF).

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RESULTS AND DISCUSSION

BALB/c mice were immunized with P30 and 12 immunized mice spleen cells were fused with SP2/0 myeloma cells. From each fusion, several hundred clones were obtained. The fusion rate was mean 80%. The supernatant from each well in which cell growth could be noted was tested for specific P30 McAb by ELISA and the positive rate was mean 2.4%.

Nine mouse McAb directed against the human seminal specific protein P30 have been isolated. These McAb only recognized and bound to P30 and P30 in the seminal plasma, and did not reacted with any other human body fluid (e.g. vaginal secretion, blood, serum, saliva, urine and colostrum) and semen from goat, cat and cock.

All of 9 P30 McAb are IgG class and IgG1 subclass determined by double diffusion.

The supernatant of hybridomas and the ascites were further tested for their antibody titers by ELISA. The titers of supernatants were 16, 32, 80 and 320 fold and the titers of ascites were 1×10^4 - 6.4×10^4 fold. The titers of hybridomas which had been cultured in vitro for 3-6 months did not reduce.

The methods for the identification of semen or seminal stain using the P30 McAb have been established successfully. Seminal stain extract diluted to 32×10^4 fold could be detected. The concentration of P30 in seminal stain extract as low as 3ng/ml could be detected.

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Preparation and Application of Monoclonal Anti-P30 Antibody

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Since 1975, the technique of cell hybridisation or fusion has been applied to a large number of problems in biomedical science. In recent years the monoclonal antibody has been used in forensic science. Ryouichi Tsuda (1984) [1] identified seminal stain by sandwich ELISA using monoclonal gamma-sm antibody. Sensabaugh (1978) [2] successfully found a human semen specific protein (HSSP) from human semnial plasma. The HSSP was proved to be a 30000-dalton semen-specific glycoprotein of prostatic origin and thus named as P30. The present article describes the method of preparation of monoclonal P30 antibody and its application to the examination of semen or seminal stain.

MATERIALS AND METHODS

BALB/c mice about 8 weeks old were immunized by intraperitoneal and intradermal injections for three times, each with 100ug of purified human seminal protein P30 which were isolated and characterized in our lab. [3] For the two injections, the P30 was emulsified with an equal amount of complete Freund's adjuvant and the interval between the first and second injection was two weeks. The third injection with no adjuvant was given 4 weeks after the second injection and 3 days prior to the cell fusion.

The fusion procedure was a modification of routine method. 10^7 SP2/0 myeloma cells were mixed with 10^8 immunized spleen lymphocytes, centrifuged at 800 r.p.m. for 5 mins, suspended in serum-free RPMI 1640 medium and recentrifuged at 800 r.p.m. for 5 mins. Then, the supernatant was discarded and 0.7 ml of 50% polyethyleneglycol (PEG 1500) were added to the sedimented cells. The cell suspension was stirred carefully for 60 sec. 20 ml of serum-free medium were added to the fusion tube slowly and centrifuged at 800 r.p.m. for 5 mins. The supernatant was discarded. The cells were re-suspended in 40 ml HAT 1640 medium, distributed onto four 96-well microculture plates and incubated in 5% CO_2, 37 C, for 10-15 days. The supernatant of each well was assayed for antibody using ELISA.

The solid-phase binding assays for specific antibody were used: 1. solid phase binding antigen (P30 3ug/ml) - supernatant -enzyme labelled goat anti mouse IgG-colored product; 2. solid phase binding antibody (serum of guinea pig anti-P30 [4] 1: 3000) - antigen (P30 3ug/ml) - supernatant - enzyme labelled goat anti mouse IgG-colored product.

For cloning of hybridoma, the limiting dilution method was used.

For ascites growth, BALB/c mice were injected with 0.4ml liquid paraffin at an interval of 1 week and approximately 10^6 hybridoma cells were injected intraperitoneally. Typically, the mice were dying from the resulting tumors after 8 days. The mice were killed and the ascites fluids were obtained by washing the peritoneal cavity with saline.

Two methods for the examination of seminal stain using P30 McAb were established: 1. solid phase binding P30 McAb (ascites 1:10000) - seminal stain extract - serum of rabbit anti P30 [4] - enzyme labelled goat anti rabbit IgG-colored product; 2. solid phase binding antibody (serum of guinea pig anti P30 1:3000) - seminal stain extract - P30 McAb (supernatant)- enzyme labelled goat anti mouse IgG - colored product.

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Ultrastructural Localisation of GDA-J/F3

For ultralocalisation, dried germ cells were tested with GDA-J/F3 MoAb using IEM. GDA-J/F3 was found localised to the fibrous sheath (Fig. 2).

Reaction of GDA-J/F3 MoAb with Lining Epithelium

To exclude the reactivity of GDA-J/F3 MoAb with the lining epithelium, frozen sections from lip, tongue, buccal cavity and tonsillar area as well as from vagina and cervix were tested with the antibody using IIF and IIP tests. In both tests, GDA-J/F3 MoAb did not react with the lining epithelium of all these tissues. Instead, the antibody stained the basement membranes (Fig. 3).

Reaction of GDA-J/F3 MoAb with PBL

Leucocytes were screened with GDA-J/F3, anti-Class I HLA and anti-leucocyte MoAbs using IIF test. In contrast to the anti-Class I HLA and anti-leucocyte MoAbs which stained the PBL, GDA-J/F3 did not react with the leucocytes (data not shown).

Reaction of GDA-J/F3 and Anti-tubulin MoAbs with Fibroblasts

Using IIF test the three antibodies failed to stain the surface of the viable cells but as might be expected the microtubules of the dried fibroblasts were stained by the anti-α and β tubulin antibodies but not by the GDA-J/F3 MoAb.

DISCUSSION

Three MoAbs (GDA-J/F3 and anti-α and -β tubulin) were used to assess their suitability for sperm detection. The 3 antibodies recognise intracellular antigens inside the sperm tails. The anti-tubulin MoAbs react with the axonemal microtubules while the GDA-J/F3 with the fibrous sheath. This was based on the following:- (1) the reaction of GDA-J/F3 MoAb was limited to the principal piece, (2) non reactivity with cilia of respiratory epithelium (unpublished data) which lack the fibrous sheath and (3) immunoelectron microscopy.

GDA-J/F3 MoAb which lacks the reactivity with epithelial cells, leucocytes or fibroblasts seems to be the most specific probe for sperm; and thus the most suitable for their identification. The other 2 antibodies recognise tubulin which shows a ubiquitous tissue distribution as it forms the microtubules in all tissues (Schliwa, 1986). Currently, GDA-J/F3 MoAb is being investigated for its use in the detection of sperm in sexual assault cases.

ACKNOWLEDGEMENT

We are indebted to D Auger (Oral Pathology) for his help with the EM and The Leverhulme Trust for their financial support. We are also grateful to Denny Williams for preparing the manuscript.

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Human Fibroblast Cultures

The fibroblasts were a kind gift from T Purkis (Dept. Dermatology). For IIF screening, the fibroblasts which were cultured onto slides were tested either viable (the cells were not allowed to dry and the washing was with PBS), or after air drying.

RESULTS

Reaction of GDA-J/F3 and Anti-Tubulin MoAbs With Ejaculated Sperm

Using the IIF test, the three MoAbs stained the tails of all demembranated sperm. However, the reaction of the anti-α and -β tubulin antibodies was with the whole tail while that of GDA-J/F3 MoAb was with the principal piece only (Fig. 1).

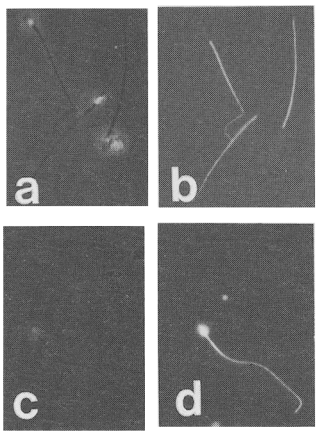


Fig. 1. IIF test of demembranated sperm with GDA-J/F3 (a,b) and anti-α or -β tubulin MoAbs (c,d). GDA-J/F3 stains the principal piece only while the other 2 antibodies react with the whole tail. (a,c: phase contrast; b,d: fluorescence microscopy).



Fig. 2. IEM of dried germ cells with GDA-J/F3 MoAb. The reaction (electron dense deposits) is seen on the fibrous sheath just beneath the cytoplasmic membrane of a spermatid (arrows).

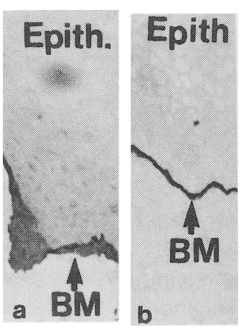


Fig. 3. IIP test of GDA-J/F3 MoAb with frozen section of (a) lip, (b) cervix. The antibody stains the basement membrane (BM), but not the lining epithelium (Epith).

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GDA-J/F3 Monoclonal Antibody: The Ideal Probe for Sperm Detection in Forensic Science

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INTRODUCTION

One essential procedure in the scientific investigation of sexual assaults is the identification of semen. The potential of using an immunological approach has been investigated by the study of three monoclonal antibodies for their suitability for the identification of sperm.

MATERIALS & METHODS

Sperm Donors

Seminal samples and peripheral blood leucocytes (PBL) were obtained from 20 medical students. The semen was handled within 1h of donation.

Preparation of Demembranated Sperm

The sperm membranes were disrupted by repeated freezing and thawing using liquid nitrogen (Jassim & Festenstein 1987a).

Monoclonal Antibodies (MoAbs)

Details of GDA-J/F3 MoAb production have been published elsewhere (Jassim & Festenstein 1987a). Mice were immunised with sperm and their precursors (spermatids and spermatocytes) obtained from seminal samples of an oligospermic donor. Anti-α and -β tubulin MoAbs were purchased from Amersham (Code N° 356, 357). The hybridoma cell lines for W6/32 anti-HLA Class I (HB95) and anti-leucocyte (HB12) MoAbs were obtained from the American Type Culture Collection (ATCC).

Indirect Immunofluorescence (IIF) and Peroxidase (IIP) Tests of Frozen Sections and Cell Suspension

These standard techniques were performed as previously described (Jassim & Festenstein 1987a).

Immunoperoxidase Eelectron Microscopy (IEM)

Sperm cell precursors obtained from an oligospermic donor were dried onto a slide. These were tested with GDA-J/F3 MoAb using immunoperoxidase test (Jassim & Festenstein 1987a). The tested cells were prepared for electron microscopy using conventional techniques (Jassim & Festenstein 1987b).

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the acrosomal part of human sperm.

To detect sperm antigens seminal stains were washed with PBS. Unspecific reactions were blocked by addition of 5% milk powder. After another wash with PBS the monoclonal antibody was added. A POX-conjugate 1:20 was added after another PBS wash; before stopping the reaction with aqua dest. 4-Cl-1-Naphtol was added.

RESULTS AND DISCUSSION

The monoclonal antibodies detecting seminal stains are being tested in current forensic casework in our laboratory. Besides we perform our experiments also on dried seminal stains produced artificially and stored for months at room temperature. Whileas the artificially produced stains show an intensive blue stain when positive, we do not always obtain a satisfactory result when analyzing cotton wool cloths from casework. Nevertheless we are working on the improvement of the method. For the future a combination of monoclonal antibodies against seminal vesicle specific antigens (Herr et al 1987) and against sperm antigens seem to be extremely promising to detect seminal stains.

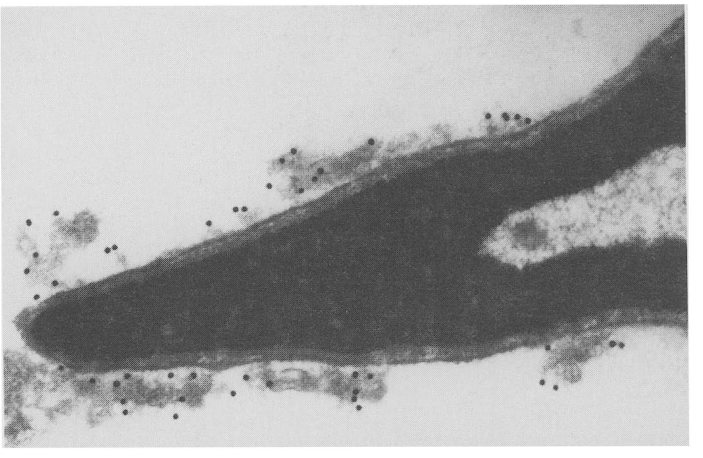


Figure 1: Pre-embedding gold staining of MOAB TÜS 1. Electron microscopy reveals the binding-site in the acrosomal part of human sperm

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MONOCLONAL ANTIBODIES DETECT SPERM ANTIGENS IN SEMINAL STAINS

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INTRODUCTION

Monoclonal antibodies that have proved to be excellent tools in different fields of medicine may as well serve to detect sperm antigens in seminal stains. The fact that we are able to produce antibodies which recognize a specific antigen determinant group is due to Köhler and Milstein (1975) who succeeded in obtaining monoclonal antibodies with cell fusion between myeloma cells and spleen cells of immunized mice.

In order to detect seminal stains searching techniques are of great importance in daily forensic casework as they allow to proceed in an economic way when a big amount of stains is to be analyzed and this analysis is carried further on with other expensive techniques like DNA-Profiling.

The acid phosphatase test as the most widespread searching technique to detect seminal stains shows lack of specifity, other searching methods having an even poorer sensitivity (Scheithauer 1988).

Our method based on monoclonal antibodies is intended to show high specifity as well as high sensitivity.

MATERIAL AND METHODS

BALB/c mice were immunized with human sperms. After repeating this procedure twice the obtained spleen cells were fused with myeloma cells. After using the limiting-dilution-technique monoclonal antibodies resulting from the hybridoma technique detected different antigens of human sperms.

After looking for cross-reactivity with a panel of human tissues and biological fluids no cross-reactivity or false positive results could be achieved with two different monoclonal antibodies showing specific reaction with acrosomal parts of spermatozoa.

By way of the pre-embedding immunogold-staining method we could show the precize localisation of the immunologic reaction between our monoclonal antibodies TÜS 1 and TÜS 19 and the sperm antigen. Immunelectronmicroscopy reveals that the antigen to antibody-reaction takes place (see Fig. 1) in the inner compartment of

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The electrophoresis for GLO-I was carried out in 1.2 mm thick mixed starch agarose gel (1:2) as described by Sehajpal et al (1986). After the electrophoresis, the gel was overlayed with Whatman filter paper no. 1 soaked with methyl glyoxal and reduced glutathione dissolved in reaction buffer and incubated at 37°C for 30 minutes. The charecteristic blue bands of GLO-I were visualised by pouring iodine solution on the surface of the gel after blotting excessive reaction mixture. The electrophoresis for G-6-PD was conducted in 1.2 mm thick mixed starch - agarose gel (1:2). The haemolysates and extracts from vaginal secretion stainswere treated with 0.05M Cleland's reagent for 20-30 minutes before loading into the gel. Using the method as described in WHO Technical Report no. 3 366 (1967) ,the gel was developed by agar overlay method. Blue bands against yellow background showed G-6-PD activity.

RESULTS AND DISCUSSION

The epithelial cells could be detected upto 4½ months whereas Doderlein's bacilli could not be detected beyond one month. Number of epithelial cells decreased with increase in the age of the stain. Rothwell and Harvey (1978) have questioned the validity of vaginal epithelial cells being identified on penile swabs. Their study indicated that false positive findings can be obtained and that this approach to determine whether a male has recently had intercourse may not be reliable one. Lactic acid was detected upto 10 months and the intensity of reaction increased with the passage of time. Mucus was not detected in dried stains but could be detected in liquid form only.

Using absorption-elution technique, 86.00% subjects were found to be secretors. The intensity of the reaction for h was more in all the groups as compared to A and B. Even in AB group, readable activity of H substance was detected. The ABO(H) substances from vaginal secretion stains showed that with the increase in the time of the stain, the number of positive results decreases. Some discrepancy in the time till which all the stains could be correctly typed was observed for different groups e.g. the samples of O group could be typed upto 40 days. The samples of B group could be typed for 46 days whereas samples of A and AB blood groups could be typed upto 42 days. The samples which showed weak reactions (3-4 cells clump) were considered as negative. After 30 days, some samples of A and O groups showed B activity. This activity increased with the passage of time. Graves et al (1978) also observed a similar phenomenon. This antigenic activity was reported to be due to some bacteria or bacterial enzyme like E.coli which might have converted the terminal sugar of blood group specific substances to change its specificity (Jenkins et al 1972).

Rh(D) antigens could not be detected in any of the vaginal secretion stains tested in this study. Similarly GLO-I and G-6-PD isozymes could not be detected in any vaginal secretion stains tested in this study. Schwed and Stock (1982) and Bryson et al (1988) also showedthe absence of GLO-I isozymes from vaginal secretions.

CONCLUSION

On the basis of above parameters, the vaginal secretion stains can be conclusively identified but cannot be individualised. For the individualisation of vaginal secretion stains,more polymorphic genetic parameters should be studied.

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CHARACTERISATION OF VAGINAL SECRETION STAINS IN FORENSIC CASES - A CRITICAL REVIEW

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INTRODUCTION

The usual contribution of a forensic scientist to the investigation of a sexual assault is to identify the offender by grouping any semen found contaminated with vaginal secretions because in these cases victim is known to us. The typing of genetic markers like cell surface antigens serum proteins and enzymes can be very valuable in the investigation of crime by establishing the the identity of the individual. However, the knowledge about the presence or absence of various genetic parameters, their level of detection, stability and the distribution of their phenotypes in population concerned will help in deciding which parameters should be studied from body fluids or even mixtures of the two body fluids.

The vaginal secretions have been identified using various tests for epithelial cells, Doderlein's bacilli, lactic acid and mucus by various workers. Graves et al (1978), Martin (1981), Martin and Cheshire (1982) and Joshi et al (1986) reported the presence of ABO(H) substances in vaginal secretion stains.

Schwerd and Stock (1982) attempted typing of PGM and AK on starch gel, EsD on cellulose acetate sheets and GLO-I on agarose gel from vaginal secretions. Sato et al (1983) typed PGM, EsD and 6-PGD from vaginal secretions by starch gel electrophoresis. Garlo (1985) reported PGM & EsD activity in post coital vaginal swabs by electrophoresis. Ablett (1983) identified SAP and VAP by isoelectric focussing. Endogenous and post coital components of vaginal swabs were studied by Bryson et al (1988).

In the Present Study, in continuation with the earlier work, the detection and persistence of epithelial cells, lactic acid, mucus, Doderlein's bacillus, ABO(H), Rh(D), G-6-PD and GLO-I from vaginal secretion stains have been reported.

MATERIALS AND METHODS

400 semen free vaginal discharge samples were collected on sterilised cotton cloth pieces, dried at room temperature and kept in serially marked envelops. Blood samples of the same subjects were also collected for typing of ABO(H) and Rh(D) blood groups and for preparing haemolysates. The information regarding name, age, time of sexual abstinence, pregnancy or non-pregnancy and date of collection of the sample was also recorded.

Epithelial cells and Doderlein's bacillus were examined microscopically after staining with Papanicolaou staining procedure and Gram's staining procedure respectively. Mucus and lactic acid were detected by chemical tests (Examination of Biological Stains, 1980). The technique of Kind (1960) was used with slight modifications for ABO(H) typing, whereas Rh(D) typing was done according to Bargagna et al (1982) technique. In case of Rh(D) typing, low ionic strength solution was used and prepared according to the method of Low and Masseter (1974).

This work is the part of the research project granted by Bureau of Police & Research Development Ministry of Home Affairs, Government of India, India.

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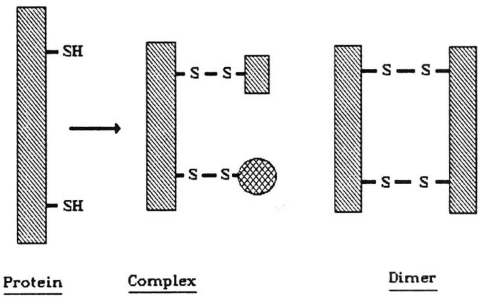


Fig. 2 Diagrammatic representation of possible complex formations.

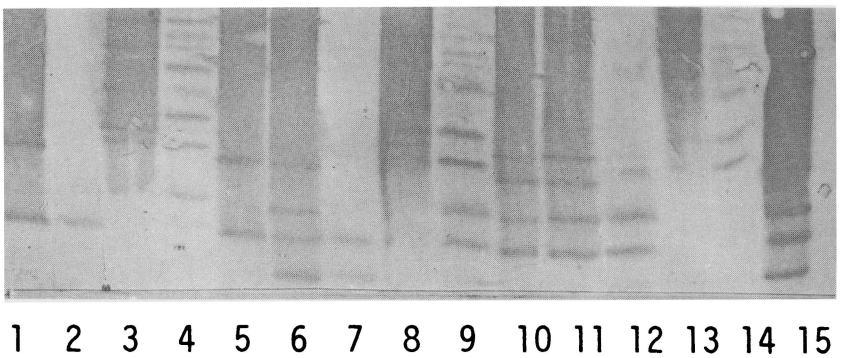


Fig. 3 PLG band patterns with and without neuraminidase pretreatment. Lanes 1, 5, 6, 10, 11 and 15 are treated plasma, 3, 8 and 13 are untreated.
Lanes 2, 7, and 12 are treated blood stains, 4, 9 and 14 are untreated. Controls PLG A: 1,2,5; PLG AB: 6,7,15;
PLG A-A3: 10,11

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In this context the third category usually produces no difficulties as the bands tend to be totally absent.

Proteins with internal disulphide bonds or cysteine residues can form several types of complex:
1.as multiple forms of the same protein eg. **Dimer**
2.combination with **fragments** of the same molecule
or 3. combination with **other** molecules (fig.2).

A good example of the third type is the binding of the Gc protein with actin released from the breakdown of platelets to form larger molecules with altered isoelectric points. In most cases this can be reversed by pretreatment with a strong reducing agent such as 6M urea to restore the original form.

Most proteins have in addition to the amino acid residues a carbohydrate and often sialic acid component. The amount varies from protein to protein and in some cases, as in Gc, between subtypes. The Gc 1 molecule for example possesses sialic residues whereas the Gc 2 protein does not (Kimura et al 1985).

The NANA residues confer a positive charge and during storage the number of residues can be reduced either through passive bond breakage or by bacterial neuraminidase activity. This can result in an irregular distribution of positive charges on the molecules and subsequent band pattern changes. By pretreating with neuraminidase all the sialic acid residues can be removed creating a uniform structure for all molecules. The solution of the problem is obviously not quite as simple because in many cases artefacts appear even after suitable pretreatment.

In the plasminogen system many bands occur cathodally even after appropriate pretreatment with neuraminidase (fig.3). With A2HS no artefacts have yet been demonstrated although without neuraminidase pretreatment bands were very often completely absent. In both cases extended treatment resulted in the disappearence of all bands. Obviously the alteration process described here will not occur in isolation and it is most probable that a complex of reactions occurs to give the resulting band pattern changes.

This problem becomes more acute when evaluating the results from mixtures of blood and other body fluids where a multiple band pattern is to be expected. Here extreme caution must be taken.

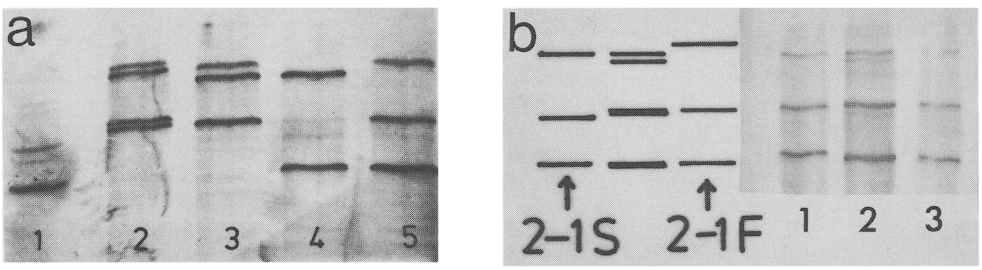


Fig. 1 a)Gc - subtypes after IEF (pH 4.5-5.4) 1)2, 2)1S-1F, 3)1S-2A7, 4)2-2A7, 5)2-1S.
b) 1)2-1S, 2)aged blood sample with extra bands, 3)2-1F.

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Evaluation of unusual band patterns in stains of biological interest

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INTRODUCTION

The polymorphic protein systems Gc, A2HS, PLG are now routinely used for individualization in paternity and stain cases. They meet the accepted standard for forensic work and have the advantage that they are generally more stable than enzymes on storage and therefore more suitable for use in stain grouping.

MATERIALS AND METHODS

Blood samples and stains from routine paternity, identification and stain cases were investigated as previously described (Rand et al 1988).

DISCUSSION

The visualisation of protein polymorphisms by immunochemophoresis depends on the presence of an intact antigenic site on the protein and not on the presence of a functionally intact enzyme molecule. The detection and interpretation of the results is not however free from complications and, in the case of stain grouping, false interpretations are possible due to insufficient care or lack of awareness of the problems involved.

The basic problem revolves around the differentiation between true variants and artefacts caused by suboptimal storage conditions. In most protein systems a variety of rare variant alleles have been found. This is particularly true for the Gc-system where at least 35 variants have been described at present. Gc 1 variants have 2 bands whereas Gc 2 variants have only 1 band (Cleve et al 1981).

This can sometimes be confusing when a discrete alteration occurs producing very few bands. A typical variant (2A7)in the Gc-system can be seen in fig.1a lanes 3 and 4 and can be distinguished from an artefact (fig.1b) seen in an old blood sample by comparison of the band concentrations . The presence of a potential third allele product would also arouse suspicion except in cases of mixtures (Rand et al 1989).

The problem can basically be divided into 3 categories:
- the **formation** of complexes
- **removal** of sialic acid or other carbohydrate residues
- and the **breakdown** of the primary structure of the protein molecule.

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The Legal Aspects

The laws of Singapore assume that a child born in wedlock is the child of the parents whose marriage is registered with the Registry of Marriages. However, due to the vagaries of mankind, the institution of marriage breaks down at a faster rate than before. In these situations, the defence of a disputed paternity almost always arise largely because of the husband's attempt to avoid paying maintenance for the physical needs of the child and his estranged wife. If it can be proven by blood group typing that he is not a father; i.e., an exclusion of his paternity is achieved, then adultery on the part of his wife is proven. In this situation he is under no obligation to provide for the maintenance. However, the situation where the putative father is not excluded from paternity is far more common and here the courts can enforce the father to continue payment of maintenance till the child reaches adulthood i.e. 21 years of age.

In Singapore, the statute laws do not acknowledge this important facility that blood group typing can achieve in the elucidation of paternities. However, case laws have demonstrated the potential use of blood group typing and the courts can direct the parties involved for blood tests to be done. However, unlike the law in England, the courts cannot draw any inference should any party refuse to submit him or herself or her child to the blood sampling and typing.

The investigations

The following blood groups are investigated : ABO, Rhesus, MNS, Kell, Duffy, Kidd and the enzyme groups : GLO, LDH, AK, 6PGD, ESD, GPI, G6PD and HLA A and B loci.

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LEGAL ASPECTS OF DISPUTED PATERNITIES IN SINGAPORE

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Disputed paternities are common in Singapore. The Department of Forensic Medicine is the only laboratory in Singapore which takes on these investigations. Requests for these investigations average about two cases per month, or about a total of 24 cases a year.

Situations of Disputed Paternity

There are two common situations of disputed paternities in Singapore. Firstly, there is the classical situation of the disputed paternity in cases of divorce where the putative father disputes paternity, alleging adultery on the part of the mother. However, in Singapore, there is yet another unique situation where the putative father claims paternity but it is the Government that disputes paternity or that the child named in birth certificate, and now seeking citizenship, is really the one that was conceived by the parents named on the birth certificate. Let me elaborate on this latter situation.

The Citizenship of Singapore is an important and expensive privilege - there are extra expenses that a non-citizen can incur more than a citizen in the areas of education, medical and health services, the right to work for a living without any restrictions. Therefore the Singapore Government tends to exclude any who cannot prove their citizenship by birth. In all of these situations, it involves illiterate or ignorant parents who, unaware of the legal implications, did not correct spelling errors to the names of their child in their birth certificates or because of ignorance of governmental regulations, their child applied for foreign passports. The line of argument by the Immigration Department in refusing citizenship to the children of these ignorant parents is that other than the errors of documentation, there is no way to prove that the person named on the birth certificate is actually that of a particular child. Another child could have been substituted by the parents and passed off as the one named in the birth certificate. This is the situation where the Government disputes that the child claiming citizenship to Singapore is really conceived by the parents.

Uncommon situations of disputed paternity encountered in the Singapore context are : a) proving that an alleged rapist is the father of the child born to a victim and b) settlement of family disputes in an equitable distribution of an estate following the death of the head of a large family.

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At this point it was now obviously clear that insufficient contact existed between the experts, as specialists in this field, and the court. In 1988 the previously recommended repeat of the anthropological investigation was undertaken. In the meantime many "new" systems, which had not previously been investigated, had become a routine component of a normal paternity case. Luckily the anthropologist referred to us the question of a possible extension of the serological findings. This request could of course be fulfilled and the extended investigation led finally to exclusions in two constellations. These are in our opinion fully conclusive as the systems have been sufficiently and extensively investigated .
The question now arises as to what conclusions can be drawn from this case sample. Firstly it must be underlined that close contact must exist between the experts and the judge and that the expert must be aware that he should function as a "fulfillment-adviser" to the judge, whether the title pleases him or not. The judge should be directed by his recommendations and runs the risk of employing out-of-date methods so long as this expert is not available.
Furthermore, when an example can be taken from the described case, the use of isoelectric focusing with the "newer" systems should be part of the standard repertoire of every serological laboratory and that an extension of the investigations using only the HLA-system does not lead to a solution in every case.
For the future it must be considered that in problem cases DNA-investigations should be carried out by every serological laboratory, whether these investigations have been legally accepted or not, and such an additional investigation should be possible by the laboratory alone or in co-operation with another laboratory.
In this context it is very important that the court is aware that such a possibility exists.

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the first man (including the results of the first expertise) was 99.58% (according to Hummel : " paternity most likely").
The court didn't accept the likelihood of paternity and ordered a third expert to make an anthropological expertise. This expertise was sent to the court in 1985. The anthropological expert arrived at the conclusion : "paternity likely" and advised a re-examination to be taken two years later.
In 1988 the third expert (anthropologist) was ordered to make the re-examination. Before starting the investigation he consulted us about the possibility to complete the serological data.
The factors Lutheran a, Colton b, Gm 3, Gm 21 and Km 3 were investigated on our behalf and we made further examinations in Gc-, PGM_1- and Tf-subtyping (additional I.E.F.) and the Pi-, PGP- and FXIIIB-systems.
We found two excluding constellations. In the child's blood we found Gc 2-1F, in the mother's blood Gc 2. Therefore the marker Gc 1F was to be expected in the real father's blood, but the putative father was Gc 2-1S. In the FXIIIB-system the marker 3 was to be expected from the real father but the putative father possessed only FXIIIB 1.
So after a period of 8 years the 4th expertise excluded the man from paternity in this case.

DISCUSSION

There are a variety of reasons for publishing casuistic relevant paternity cases (1). Although the reported case is not a sensational example the facts of the case show some interesting aspects which should be seriously considered. Firstly it must be clearly emphasized that no reproach should be made against the serological results of the three expertises. The data were correct.
The first expertise was made at a time when the systems which were later additionally investigated were not in the routine use in any serological laboratory. Here an additional investigation incorporating the HLA-system was correctly recommended. This investigation was completed in 1982 and lead to a plausibilty of paternity of W = 99,85 % for one of the putative fathers and to an exclusion of both the others. The statement contained no further recommendations for the court and it was therefore inevitable that the necessary contact between the expert and the court was interrupted. It is known that there are still civil courts which are satisfied with a court decision of "paternity probable" (Hummel's predicate) but in this case the court did not accept this. Because no recommendations for an extension of the serological investigation were given - although new systems were available at this time but not yet recognized (2, 4, 6) - the court decided to take the old-fashioned way and ordered an anthropological examination of the people involved. The third expert obviously did his best, especially within the scope of his limited examination possibilities. His assessment of "paternity likely" gave, after all, only the third highest grade of plausibility of paternity for an anthropological examination. Here a repeat examination of the persons involved was recommended in two years time.

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A Remarkable Case of Affiliation: Exclusion from Paternity after 8 Years

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SUMMARY

We report a case in which a man was excluded from paternity after a period of 8 years. Two exclusion factors were ascertained in the 4th expertise: in Gc-subtyping and F XIIIB.
Elimination was not possible in the HLA-system.
This case verifies the importance of investigating the "newer" blood group systems in such problematic affairs.

CASUISTIC

While a revised version of the guidelines for paternity testing is under discussion (3,5) - the current regulations were published in 1977 - we report about an affiliation case which verifies the importance of extending basic blood group expertise in problematic cases to the "newer" systems and not torely only on the HLA-system:
The child was born in 1980. In 1981 the first expert was ordered by the court to examine the three-man-affair.
Although 22 systems were investigated, none of the three possible fathers could be excluded from paternity.
The statistical values for likelihood of paternity (W% for log y/x+10) were low : 57,8 % for the first, 4,5 % for the second and 37,7 % for the third man.
According to Hummel these values can be translated to the verbal predicates :
1st man: "indifferent on the positive side"
2nd man: "paternity very unlikely"
3rd man: "indifferent on the negative side".
The expert advised the court to order a second expert to extend the investigations using the HLA-system.
In 1982 the second expertise (including the HLA-system) was sent to the court. The child received the HLA-markers A 25 - Bw 44 - Cw 4 or A 25 - Bw 35 - Cw 4 from its mother. Therefore the child must have inherited the markers A 3 - Bw 35 or A 3 - Bw 44 from its father. These markers were only present by the first man. The second and the third men could be excluded from paternity. The statistical value (W%) for the paternity of

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CONCLUSIONS

Paternity cases where a single exclusion is detected can now be dealt with using a uniform biostatistical approach weighting all the evidence available, no matter what class of exclusion is observed. The difficulties arising from the expression, in the same case report, of quantitative and qualitative expert's opinions are avoided, allowing a better understanding of the facts at stake by a layman.

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APPENDIX

For a n (>2) allele codominant genetic system, μ can be estimated from the frequency of mother-child 1st. order exclusions (f):

$$f = (n-2)\ \mu\ [\sum_{i=1}^{n} p_i^2(1-p_i) + \sum_{i>j}\sum 2p_ip_j(1-p_i-p_j)]$$

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APPLICATIONS AND DISCUSSION

Reports on the occurrence of 1st. rule exclusions in situations where kinship is not doubtful have accumulated recently and data are now available in the literature for estimation of mutation rates in some genetic systems (Martin, 1981; Wetterling, 1988; Werret et al., 1988).

If we look upon PGM1, for instance, 4 exclusions by the first rule out of 12,682 mother-child pairs were found (Wetterling, 1988). The same author concludes that "at the present, it is preferable not to base any paternity exclusion on the PGM1 system alone".

We agree in the sense that no exclusion should be made in a qualitative way, specially if based on an isolated incompatibility, disregarding the rest of the genetic evidence. However, we think it is very difficult to sustain before experts and laymen that the same system can be reliably used when "including" but it is suspicious when "excluding".

In fact, using the algorithm above, a unified biostatistical approach to such cases is possible, allowing a quantified opinion on paternity using all the information available.

Indeed, the available data lead to a conservative estimate of $\mu = 4.4 \times 10^{-4}$ (see Appendix). Using this estimate, gene frequencies from the same source and the above algorithm, we get L values ranging from 10^{-2} to 10^{-3}.

Since the test battery used now in many laboratories is sufficiently powerful to produce an average L among true fathers exceeding 3×10^{2}, we believe that no longer a single 1st. rule exclusion in PGM1 is a major difficulty for paternity expertises.

Another illustration of the use of the algorithm comes out from a paternity case we analysed where, after the routine battery, the child evidenced a previously undescribed gene product, absent from both mother and accused man in ESD system (phenotypes: 1, 1 and 1-V, respectively). However, using the other systems, paternity index was 9.4×10^{3}. Extended investigations, including HLA, were performed and L raised to 2.1×10^{7}, no other exclusion being found.

In order to join all the genetic evidence in a single figure we would need estimates for both mutation rate and gene frequency, both non-existing in the present case. Anyway, even assuming a very low mutation rate (10^{-6}) and an absurdly high gene frequency (10^{-2}), the global paternity index would be still 41.

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the genetic results under the opposite hypothesis. For the calculation of these probabilities, estimates of population parameters are needed: gene frequencies (including silent genes) and mutation rates. Mutation will be understood here as any violation of the formal genetic model established for the system under analysis that cannot be explained by a silent gene. It includes, therefore, along with "classical" mutations, intragenic recombinations and unequal crossovers.

DERIVATION AND PROPERTIES OF THE ALGORITHM

Let us assume that we observe a trio where the child demonstrates a gene, absent from both mother and putative father (phenotypes: 1-X, 1 and 1, respectively). It is obvious that such a situation can only be reconciled with the hypothesis of paternity, assuming a mutation event during gametogenesis, either maternal or paternal. Thus, the probability of occurrence of this observation under the hypothesis of paternity is:

$$p^2.\mu.p^2(1-\mu) + p^2(1-\mu).p^2.\mu = 2\,p^4\mu(1-\mu) ,$$

where p stands for gene 1 frequency and μ for mutation rate. Correspondingly, the probability of the same observation under the hypothesis of non-paternity, is:

$$[p^2.\mu.p + p^2(1-\mu).q].p^2 = p^4[\mu p+q(1-\mu)] ,$$

where q is gene X frequency. Thus,

$$L = 2\mu(1-\mu)/[\mu p+q(1-\mu)].$$

From the expression it is easy to infer that L is minimum when μ is low and q very high; in other words, and in accordance with common sense, the reliability of the exclusion is high when mutation rate is low and the gene present only in the child is common in the population. On the other hand, the maximum value of L=1 (assuming no practical interest in a theoretical situation where μ could be higher than q) is reached when $q=\mu$ and $p=1-q$. That is to say: the reliability of the exclusion is minimal when the gene present in the child is so rare that it is equally probable to explain its occurrence by mutation or by normal gametogenesis from an unknown true father. More bluntly: if the described situation occurs in a monomorphic system and the gene is detected for the first time, the reliability of the "exclusion" is zero.

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Paternity probability calculation in "exclusion" cases

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INTRODUCTION

Recent advances of paternity investigation have shown two apparently contradictory developments: (a) the growing information power of the tests, corresponding to very high paternity probabilities among true fathers, and (b) the loss of absoluteness of the "exclusion" concept, since the number of false exclusions is expected to grow with the increasing number of genetic systems employed.

This has been widely recognized, and operative solutions have been advanced for "difficult" cases where single 2nd. rule exclusions are observed (Gürtler, 1977; Polesky and Souhrada, 1988).

The discovery of false 1st. rule exclusions at a relatively common rate (from 10^{-4} up to more than 10^{-3}) in some genetic systems (Wetterling, 1988; Zang and Blin, 1988; Werret et al., 1988) demonstrates that even this kind of exclusion, when isolated, can no longer be treated in a qualitative fashion, but, on the contrary, must be quantified along with the rest of the genetic evidence gathered in the case under dispute.

Since we have already discussed in a previous work (Amorim and Rocha, 1988) the disadvantages of a non-uniform treatment of the genetic results when an exclusion is present, we shall limit ourselves here to the derivation of an algorithm able to deal in a biostatistically uniform manner with results of 1st. order exclusions.

DEFINITIONS

We will call paternity index (L) to the ratio between the probability of occurrence of the genetic results under the hypothesis of paternity and the probability of occurrence of

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commonly shared HLA A and B antigens between the AF and M are the most common antigens at these loci. Sharing of a single such antigen resulted in only a slight decrease in the HLA PI. However, if the AF and M share 1 A and 1 B, or other combinations of two or more antigens, then the HLA PI was significantly decreased. Analysis of the 1 A and 1 B AF/M antigen sharing group (n = 64) reveals that the low HLA PI values are usually associated with 1 of 3 types of situations: 1) the child has a blank and the AF and M share the one antigen detected at that locus, 2) M and C are HLA identical, or 3) the AF's "other" haplotype (unshared with mother) is common and is the obligatory haplotype.

The HLA PI is also reduced when the child has either an A locus or B locus blank. Analysis of the antigens detected at the locus with a blank reveals, as expected, that they are usually the more common antigens. Therefore, the child is usually homozygous for a relatively common antigen. The Y value in the denominator of the PI is increased relative to cases in which the paternal antigen is less frequent. This results in a lower quotient - the PI value. However, when the AF has a B locus blank, the HLA PI value tends to increase due to his apparent homozygosity for the B locus antigen. The resulting X value of the numerator is increased relative to the Y value. The Y value of the B locus genes tends to be low due to the B locus gene frequencies. Common A locus antigens tend to have higher frequencies. Therefore, an A locus blank in the AF does not have a strong influence on the PI.

SUMMARY

HLA antigen sharing between a true father and mother can result in a significant reduction of the HLA PI if the father and mother share more than one HLA antigen. HLA blanks in the child, and a B locus blank in the AF, can also produce a significant change in the HLA PI when compared to cases with no blanks. These variances can be attributed to the gene frequencies of the HLA system. Relatively low HLA PI values result when the AF and C share a common antigen but they may also be caused by other factors.

CONCLUSIONS

In non-exclusion cases where the AF is the true father:

Low HLA PI values may be associated with:

1) Sharing of relatively common obligatory antigens between AF and C.
2) Sharing of two or more antigens between the AF and M.
3) A or B locus blanks in the child.

High HLA PI values may be associated with:

1) Sharing of a relatively rare obligatory antigen between AF and C
2) B locus blank in the AF
3) No antigen sharing between the AF and M

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Table 1. HLA A,B antigen sharing between white true fathers and mothers in paternity cases

Antigens Shared	No	%	Median HLA PI	p
None	219	42	31	
One A	148	29	28	ns
One B	67	13	24	ns
One A & B	64	12	15	0.003
Other Combinations	18	4	12	0.002
Total Cases	516	100	26	

Table 2. HLA blanks at the A & B loci in 516 white true father paternity cases

	Blanks Present			No Blanks		
A locus blanks	n	%	Median HLA PI	n	Median HLA PI	p
Alleged father	89	17	22	427	27	ns
Mother	88	17	31	428	26	ns
Child	84	16	19	432	28	0.006
B locus blanks						
Alleged father	62	12	40	454	25	0.060
Mother	57	11	29	459	26	ns
Child	50	10	20	466	27	ns
A or B locus blanks in AF,M,C	265	51	28	251	25	ns

DISCUSSION

Intuitively, one would expect the HLA PI values to be reduced whenever there is antigen sharing between the AF and M since there would be less diversity and therefore less discrete information available. The data in this study support this expectation. However, the differences in the distributions of the HLA PI values in the study groups are not marked unless the AF and M share two or more HLA antigens (Table 1). The most

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The Effect of Blanks and Antigen Sharing in the HLA System on the Paternity Index

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INTRODUCTION

The HLA system is the single most polymorphic, well defined, genetic system in use today as a component of the testing profile in cases of disputed parentage. Over 40,000 phenotypes can be detected utilizing HLA-A and HLA-B locus markers. In spite of this, antigen sharing between the alleged father (AF) and mother (M) is relatively common. Missing antigens, blanks, are also commonly observed, especially in blacks, but also in whites as well. This study was undertaken to determine the effect of HLA-A and/or B antigen sharing between the AF and M, and blanks at these loci, on the HLA Paternity Index (PI) in white non-exclusion cases where the AF was assumed to be the true father.

METHODS

All paternity cases involving 1 child in whites processed from Aug 1981 to Apr 1989 were reviewed independently by the authors. The case material was limited to non-exclusion trios in which the Combined PI (CPI), using an average of 10 genetic systems, exceeded 18. Therefore, it was assumed that the study cases involved true fathers since the minimum probability of paternity was 98% using 0.7 as a realistic prior probability (based upon the observed exclusion rate of 30% from experience in casework over this time). The combined power of exclusion of this test panel was 99% or higher. Each case was classified into a subgroup according to HLA-A and/or B antigen sharing between the AF and M. In addition, the cases were also classified independently into subgroups based upon the presence or absence of HLA-A and/or B blanks in each of the three parties of the case. Frequency distributions of the HLA PI values were constructed for the entire study group (n = 516) and for the various subgroups of the study. Comparisons between the groups were made using the Mann-Whitney U Test for significance.

RESULTS

There were 537 non-exclusion cases in the total study material resulting in a median HLA PI of 25. 21 cases had a CPI below 19. These cases were excluded from the study group which consisted of the remaining 516 cases with CPI values all exceeding 18 (true father cases). The median HLA PI value of this group and the subgroups are displayed in Tables 1 and 2.

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cannot be allocated to any one of the three racial groups; rather, the person belongs simultaneously to all the three - and this in accordance with the established blood pertions 70% Indio blood, 20% Spanish blood and 10% Negro blood. If one applies seroanalysis in this way (and only now can one strictly speak of analysis), one is no longer working with hypotheses and probability values in terms of decision theory, but in the field of descriptive measurement. Although the sum of the values obtained is 1 (or 100%), the values themselves are no longer probabilities of accuracy or error.

> In connection with the biostatistical evaluation of blood group findings to calculate e.g. a figure for the probability of fatherhood, a seroanalytical allocation tells us which ethnic allele frequencies must be used for the person in question. Seroanalytical mix-values tell us in which proportions frequencies from diverse populations must be mixed to do justice to the person in question.

This differentiation between two seroanalytical procedures may cause some uneasiness about finding a common theoretical foundation for the seroanalytical method. But there is a relativily simple way to combine them. If in a case of allocation the probability of the alternative hypothesis is negligible, and one allocates a person categorically to a specific population, this simply means that one deprives the alternative hypothesis of all value by arbitrarily reducing its probability to zero. This must regarded pragmatically, given a high probability in favour of the null hypothesis, one decides - for reasons of economy - to disregard the alternative hypothesis. By analogy, in the case of an exclusion from paternity one treats the hypothesis "non-paternity" as 1 and the hypothesis "paternity" as zero, notwithstanding the fact that in reality 1 (= 100%) and zero in natural science and statistics will never be reached.

Thus, in terms of approach, seroanalysis is not a technique of deciding for or against. Rather, the method always provides percentage portions for frequencies. However, those frequencies which lie below certain limit values are subsequently disregarded; such low frequencies are either unrealistic or at least devoid of practical relevance.

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In the case described, Spaniards, Indios and Negroes were treated as "uniform" in terms of blood. With reference to their respective superordinate major races - Caucasian, Amerindian and Negroid - these populations can be treated as separate entities. The major races are not completely homogeneous but are so removed from one another that in relation to one another they are "genetically uniform".

Populations combining different portions of major races may also be regarded as "uniform", provided that the mix has persisted for a very long time - at least many hundreds of years - and has not been disturbed by immigration during the period. Hence, it is possible in seroanalysis to treat Chinese, Japanese, Koreans, Vietnamese, South Sea Islanders, Australian Aborigines and other peoples of the Far East and Southeast Asia as "uniform" in terms of their blood, although they are all mixed in varying degrees of Pacific and Mongoloid races. - In other mixed populations the particular mix has not yet persisted long enough, or has been disturbed by fresh immigration, so it is impossible for one to speak of genuine panmixia. This holds, for instance, for North American Negroes and Latin American populations. Whereas the so treated "uniform" peoples of East and Southeast Asia have each become a "mixed people", the former are still a "people mix": mixed-bloods of every conceivable mixture live alongside pure representatives of the major races.

The greater the differences in serotype frequencies between populations, the greater the degree of certainty with which a person can be allocated to a specific, uniformly treated population. Among Caucasians there is, for example, a not inconsiderable difference between the frequencies of Central Europeans like Germans and those of Indians, Iranians, Afghans, North Africans or Lapps. The accuracy with which a person can be allocated to the mentioned fringe Caucasian populations is correspondingly high. There is far less certainty of accurately allocating a person to the Italian, Turkish, Greek or any other typical European population.

So far, the seroanalytical allocation we have been dealing with is a typical decision technique in statistics that gives the expectation of error for a categorical decision in favour of either the null or the counter hypothesis. For a W value of, e.g., 99% a specific allocation is "highly probable"; for W = 99,73% and above a person's membership of a specific ethnic group is "practically proven". Specific allocations are impossible for W values below 99% (down to 1%). But they can still be useful indicators, provided that they are based not on a uniform, panmixed population, but on a "people mix". Then percentages can be related to the blood portions, above all if the blood portions refer to different major races. For example, if, using Indio, Spanish and Negro frequencies, one denotes the frequency of an urban Mexican's phenotype as $f(X_i)$, $f(X_s)$, $f(X_n)$. The respective probabilities in favour of the three races are given by the three formulas:

$$W_i = \frac{f(X_i)}{f(X_i)+f(X_s)+f(X_n)},$$

$$W_s = \frac{f(X_s)}{f(X_i)+f(X_s)+f(X_n)},$$

$$W_n = \frac{f(X_n)}{f(X_i)+f(X_s)+f(X_n)}.$$

If the calculated values for a certain person are, say, W_i = 70% (Indio percent), W_s = 20% (Spanish percent), W_n = 10% (Negro percent), the person

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Seroanalysis and Expert Opinion in Practice

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In 1980 an opinion on paternity in our Institute was faced with the question of whether a Puerto-Rican with a Spanish name - of whom there was no photograph - was a Spanish, Indio or Negro descent, or a mixture of these. The child's German mother had named the Puerto-Rican as the child's father. The pigmentation of the child's eyes, hair and skin was darker than it is common among, say, German children. Notwithstanding this it was impossible to draw any definite conclusion about the father's race from the child's appearance. This case encouraged the development of an appropriate technique - now known as seroanalysis - to solve such cases and was the first to which it was applied.

In the case concerned I started with two pairs of hypotheses:

I a) null hypothesis: the man is of Spanish descent (= X),
   b) counter hypothesis: the man is of Indio descent (= Y_1):

II a) null hypothesis: the man is of Spanish descent (= X),
   b) counter hypothesis: the man is of Negro descent (= Y_2).

This takes into account the presence on Puerto Rico of pure Spaniards, pure Indios (though seldom) and pure Negroes.

If one multiplies the phenotype frequencies of a person in different genetic systems in Spaniards, Indios and Negroes one obtains the corresponding serotype frequencies among Spaniards, Indios and Negroes. Taking a neutral prior probability, which means assuming that the man is equally likely to be Spanish, Indio or Negro, the Bayes function gives the probability that in blood terms the man is a Spaniard: $W_{Sp}(1) = f(X)/[f(X)+f(Y_1)]$.

The probability that in blood terms he is Indio is given by $W_{Ind} = 1-W_{Sp}(1)$.

Taking as alternative hypothesis Negro descent, the probability for Spanish descent is $W_{Sp}(2) = f(X)/[f(X)+f(Y_2)]$,

and for Negro descent $W_{Neg} = 1-W_{Sp}(2)$.

If in 100 cases with comparable circumstances one were to assume in each case Indio (and not Spanish) descent, the expectation of error would be $W_{Sp}(1)$, and if one always assumes Negro descent the expectation of error would be $W_{Sp}(2)$. The evaluation of the case in question gave values above 99,99% for $W_{Sp}(1)$ and $W_{Sp}(2)$.

Thus, it was "practically proven" that the man in question was of pure Spanish descent. Therefore, Bayes' Theorem enables one to allocate a person to a specific, uniformly treated racial circle, and this with a defined degree of probability.

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CONCLUSION

Anyone who uses scientific expertise to quote a probability of paternity in court has the duty to "tell the whole truth and nothing but the truth." This clearly includes all the assumptions made in calculating the probability, and an evaluation of the validity of these assumptions. Anything less than such a full interpretation is surely both scientific fraud and fraud on the court.

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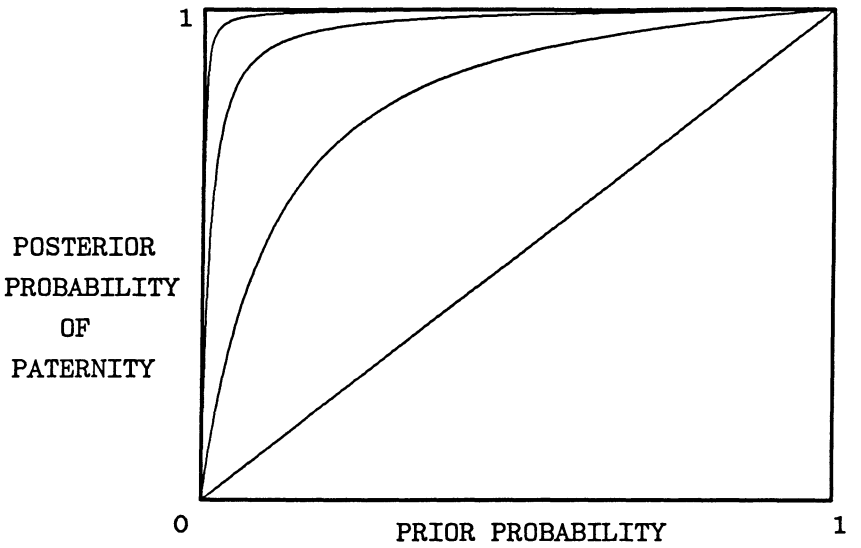


Fig. 1. Plot of the posterior probability of paternity (ordinate) against the prior probability (abscissa) for four values of the paternity index: 1, 10, 100 and 1000, giving rise to a diagonal line and three successively higher lines, respectively.

calculate a reasonable prior probability of paternity. This has been suggested by several authors (see especially Baur et al. 1981). Using such knowledge is analogous to using our knowledge of the physical structure of a die to assume that the prior probability of a "six" is 1/6. The law, however, assumes that this information is not relevant and so a prior probability *based on accusation alone* cannot be used. It is therefore up to the courts to decide, on the basis of the *independent* evidence presented, what the appropriate prior probability is. The expert witness who does the blood testing must somehow summarize the evidence from the testing data alone. This could be done by quoting the paternity index (together with all the assumptions made to calculate it) or, and I personally believe this is preferable, by quoting the probability of paternity (again, together with the assumptions made) as a function of the prior probability. This can very easily be done by plotting the probability of paternity against the prior probability over its whole range from 0 to 1. Figure 1 illustrates, for four different values of the paternity index, what this function looks like. In particular, I think it is essential to point out in court that this curve goes through the point (0, 0) whatever the paternity index is. Otherwise a judge who is convinced that the alleged father had no access to the mother may be confused by a high probability of paternity.

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"calculations," and to what extent their validity has been verified.

RED HERRINGS

Li and Chakravarti (1985, 1986, 1988) have given three reasons why they believe the paternity index, used as I have indicated, is not valid. First, they point out that it is higher among fathers than non-fathers 'for algebraic reasons.' In other words, it is not paternity *per se* that causes the index to be high. This is analogous to saying that it is not the color of one's skin *per se* that causes one to go to college. As we have seen, this is irrelevant. Second, they say that the paternity index "is not a comparison of two non-overlapping alternate hypotheses, as claimed." But the hypotheses "the alleged man is the father" and "some other man is the father" are clearly two distinct, non-overlapping alternate hypotheses. Third, they point out that when using the paternity index it is possible in certain situations to conclude that "the alleged father non-excluded by three tests is *more* likely to be the true father than if he is non-excluded by two *additional* tests." Because they find this possibility unreasonable, Li and Chakravarti recommend that the paternity index be calculated on the basis of observing merely that there has not been an exclusion (Baur et al. 1986; Elston 1986). If we know the phenotypes of the mother, child and alleged father, we certainly know whether or not there has been an exclusion, whereas the converse is not true. Therefore, Li and Chakravarti are not making use of all the information available, which violates the principles so clearly stated by Fisher. I have shown elsewhere (Elston 1986) in what sense their method of calculating the probability of paternity has "validity", but less "utility." Using the principles enunciated by Fisher, however, their probability is not even valid.

THE PRIOR PROBABILITY

Finally, I want to discuss what should be done about the prior probability, without which no probability of paternity can be calculated. At this point a clear distinction should be made between the scientific approach, which is to make a rational inference on the basis of *all* the information available, and the legal approach, which considers certain kinds of information irrelevant - even though it may not be rational to do so. The legal system (in America, at least) operates on the premise that an accused person is innocent until proved otherwise, and that the mere act of being accused should not be construed as suggesting guilt. But the fact that a man has had his blood tested for paternity singles him out from the rest of the population as being someone who comes from the class ("sub-aggregrate" in Fisher's language) of all men who are, or have been, tested for paternity. Provided this class is stable over time, a scientist should use past information about this class (which should probably be laboratory-specific) in order to time, a scientist should use past information about this class (which should probably be laboratory-specific) in order to

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This shows that all the information contained in the test data that is relevant to the probability of paternity is given by the fraction .35/.12, known in this case as the paternity index, i.e.,

$$\frac{P(M,C,A \mid \text{alleged father is the father})}{P(M,C,A \mid \text{some other man is the father})} . \quad (3)$$

The paternity index is often equated to

$$\frac{P(M,C,A \mid \text{alleged father is the father})}{P(M,C,A \mid \text{"a random man" is the father})} = \frac{P(C|M,A)}{P(C|M)} , \quad (4)$$

where this last equality assumes random mating. Changing "some other man" to "a random man" is legitimate so long as we understand that by "a random man" we mean a man randomly chosen from a population of all men who could possibly be the father, each weighted by the probability that he is in fact the father (these probabilities being based on information that is known independently of the test data). In other words we have to assume a probability distribution for the phenotypes of all possible fathers (this may or may not be the same as the distribution of phenotypes in a natural living population).

Provided we define the paternity index such that "the random man" appropriately reflects the distribution of phenotypes among all such men, it is appropriate. To my knowledge, the difficulty of ensuring this is rarely stressed by "expert witnesses" who quote probabilities of paternity. Nor do they estimate either the numerator or the denominator of the paternity index as an observed limiting frequency ratio in an actual population. Rather they make assumptions, which again they rarely explain to the courts. The two major assumptions usually made are:

(1) the observed phenotypes are caused by a set of Mendelian systems whose population distributions are mutually independent
(2) all allele frequencies of the systems tested are known without error for the population from which the father comes.

(They also assume, for systems in which there is dominance, that the genotypic frequencies in the populations from which the father and the mother come are in Hardy-Weinberg proportions). I wonder what is the nature of the evidence that these are reasonable assumptions in the case of many DNA markers. Cellmark Diagnostics has stated in its literature "The odds that two unrelated people possess the same DNA FINGERPRINT have been calculated to be on average, 30 billion to 1. Given that the Earth's population is about 5 billion people (only 2.5 billion males), it is impossible to be more sure of a paternity determination than this with any other available test." It is obvious that the ratio of 30 billion to 1 could not have been observed in any human population and must have been "calculated" on the basis of certain assumptions. The courts should surely be made aware of all assumptions used in making any such

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one to go to college. Note also that we started with a "prior" probability of .45 that the man is white (from our knowledge that he resides in New Orleans) and then, on the basis of the further observation that he is attending college, we modified this prior probability to arrive at a "posterior" probability of .7.

The probability of paternity is calculated in an exactly analogous manner. Whereas in the above example, we started with the knowledge that either the man is white or he is black, in this case we start with the knowledge that either the alleged father is the father of the child or somebody else is the father. If we know the prior probabilities of these two possibilities, they can be converted, using the blood testing results, into what is known as the probability of paternity. Can we assume that if we know absolutely nothing about the prior probabilities, they are equal? This is the flaw that is in Bayes' original essay - the assumption that lack of knowledge somehow implies that the prior probabilities are equal - which Bayes knew was an illogical assumption. Morris (1982) has stated that the assumption of equal prior probabilities is "neutral" - whatever that means. When a die is thrown, it will certainly turn up either "six" or "not six"; is it logical, or even "neutral," to assume that these two events have equal prior probabilities?

It should be fairly obvious that the additional information observed when blood testing is performed comprises the phenotypes (i.e., the results of antigen-antibody reactions or electrophoretic bands) of the mother (M), child (C) and alleged father (A). Let us suppose that the probability of obtaining the particular results found is .35 if the alleged father is the father, but only .12 if some other man is the father. We can then use Bayes' theorem, just as before, and arrive at a probability of paternity of 0.7. Thus, to arrive at a correct probability of paternity, we have to start with a correct prior probability of paternity and we have to modify this with correct probabilities of what we observed under the two possible "hypotheses" - the "alleged father is the father" and "some other man is the father". At this point I want to discuss these last two probabilities in more detail, leaving the prior probability until later.

THE PATERNITY INDEX

It is clear that I can equivalently write the above formulation (1) as follows, dividing through the top and bottom of the fraction by .12:

$$\frac{(.45)\left(\frac{.35}{.12}\right)}{(.45)\left(\frac{.35}{.12}\right) + .55} = .7 \qquad (2)$$

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I take this to be axiomatic, not because the majority of those calculating probabilities of paternity would agree with Fisher, but because I have thought long and hard about the problem and have come to the conclusion that Fisher best expounds my point of view in this regard. When we talk about the probability of paternity we are not talking about a future event that will or will not take place as a result of a random process, but rather the probability of an event that has already occurred or not occurred, so that its probability must depend crucially on the amount of information we have that is relevant to that event. For any of the information to be "irrelevant" we must be able to show that it does not affect the resulting probability.

BAYES' THEOREM

The probability of paternity is calculated using "Bayes' theorem" often without regard to the reason why Bayes himself, knowing that it contained a flaw, did not publish his essay on probability. The important thing to remember about Bayes' theorem is that it only allows one to change probabilities, not to create them. Let us first consider an example that has nothing to do with paternity. Suppose a colleague telephones me to say that he is speaking with a man who is either black or white, and he wants me to determine the probability that the man is white. Without further information, there is no way that I can determine such a probability. If, however, he tells me that the man is American, I can immediately use the fact that about 85% of such American men are white and quote him the probability of .85. If, on the other hand, he tells me that the man is a resident of New Orleans, I can use the fact that 45% of the males in New Orleans are white and quote a probability of .45. These probabilities are, in fact, limiting frequency ratios in the sense that if in each case men were repeatedly sampled at random from either America or New Orleans, the frequency ratio of whites would tend towards .85 or .45, respectively.

Suppose my colleague then tells me that the man he is speaking with, in addition to being a resident of New Orleans, attends college. Using the principles enunciated by Fisher, I must use this information if it is relevant. Now, it is well established that in New Orleans at the present time, whereas about 35% of white males go to college, only about 12% of black males do so. Thus the probability that a New Orleanian male is white and attends college is $(.45)(.35)$, while the probability that he is black and attends college is $(.55)(.12)$. Given that one of these two events actually occurred, we must divide each by their sum to arrive at the new probabilities that he is white or black. Thus the probability that the man is black now becomes

$$\frac{(.45)(.35)}{(.45)(.35) + (.55)(.12)} = .7 \ . \qquad (1)$$

Note carefully that this is a limiting frequency ratio and has nothing to do with whether the color of one's skin *per se* causes

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The Probability of Paternity

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INTRODUCTION

Much has been written on how to calculate the probability of paternity when testing fails to exclude the alleged father from being the true father of a child, and it is clear from what has been written that the concept of "probability" is often not well understood. I have therefore decided to focus my talk specifically on this problem. I shall review the principles and assumptions underlying the "calculation" of the probability of paternity, which are the same whether DNA or non-DNA markers are used, and give you my opinion of how, without scientific fraud, this information should be transmitted in court.

PROBABILITY

There are many ways of defining probability, but the frequency definition, i.e., defining probability to be a limiting frequency ratio on repeated sampling from the same population, is the only definition that we can be sure has any relevance to the real world. I shall take as axiomatic what Fisher (1959) wrote on this topic:

"It has been often recognized that any probability statement, being a rigorous statement involving uncertainty, has less factual content than a assertion of certain fact would have, and at the same time has more factual content than a statement of complete ignorance. The *knowledge* required for such a statement refers to a well defined aggregate, or population of possibilities within which the limiting frequency ratio must be exactly known. The necessary *ignorance* is specified by our inability to discriminate any of the different subaggregates having different limiting frequency ratios, such as must always exist ...
"... although in the deduction of statements of certainty it is legitimate to draw inferences from some of the axioms available while ignoring others, or, in other words, to base a valid argument on a chosen subset only of the available axioms, no such liberty can be taken with statements of uncertainty, where it is essential to take the whole of the data into account, though some part of it may be shown on examination to be irrelevant, and not to affect the result."

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3 General Biostatistics

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Table 1

Relationship between PLG- and kallikrein phenotypes as examined by IEF

PLG		Kallikrein	
n	Phenotype	n	Phenotype
		111	a
119	A		
		8	a*
		86	ab
92	AB		
		6	a*b
18	B	18	b
3	AA3	3	aa3
1	BA3	1	ba3
1	BM2	1	bm2

Discussion

PLG and kallikrein are definitely not independent systems. But, on the other hand, there is not a complete correspondence of the band patterns of PLG and kallikrein. Additional bands in kallikrein were observed in particular with a.

The relationship between the two systems is unclear with the presently available results. A genetic interpretation of these results would suggest chromosomal linkage of the genes for PLG and kallikrein at the long arm of chromosom 6 with the PLG gene locus at 6q26-27 (13). Alternatively, a physiological interpretation could be entertained by the assumption of complex formation or other interactions.

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Fig 5 shows the destribution of PLG and kallikrein phenotypes in 16 "families".

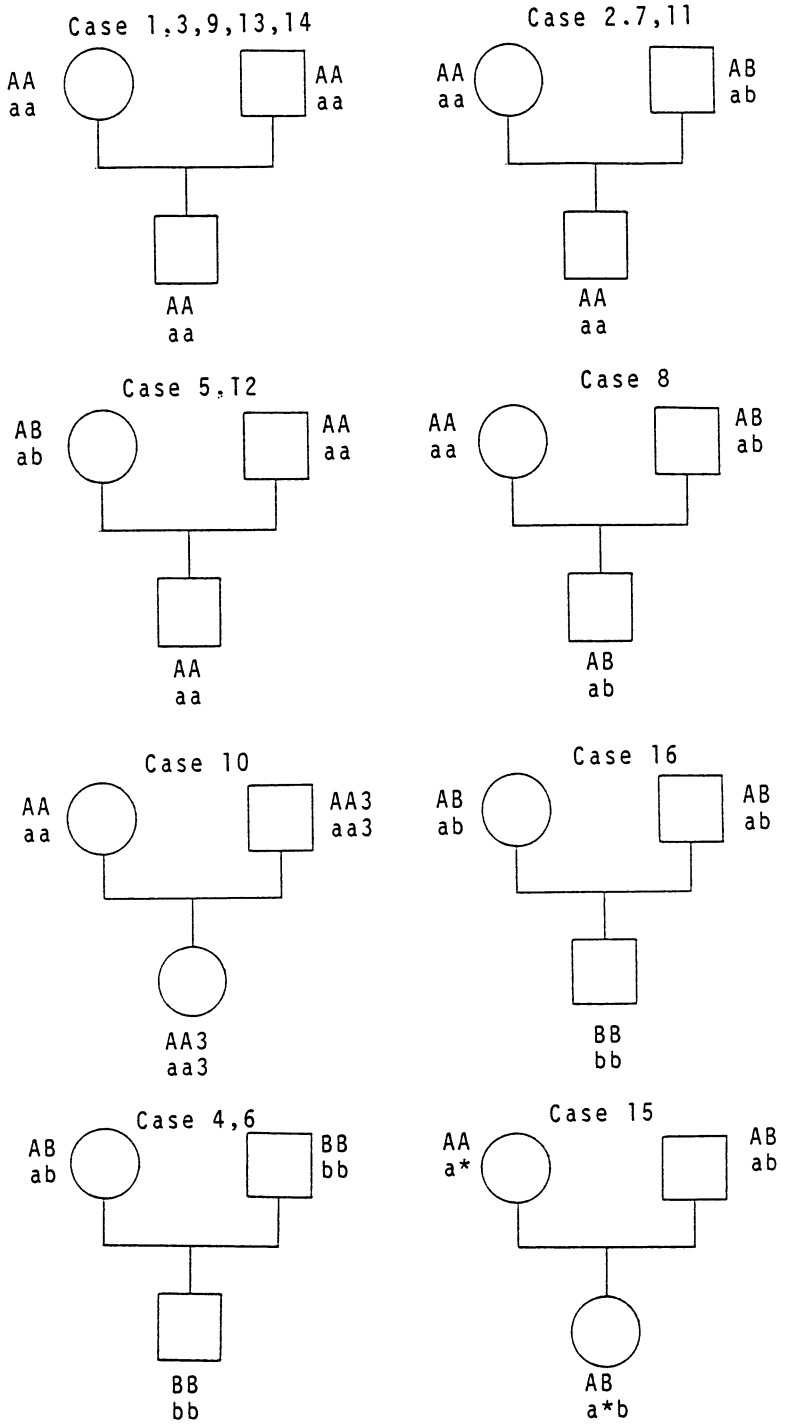


Fig. 5: Pedigrees of non excluded (> 99.9 %) paternity cases

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Antibody specificity was tested with highly purified kallikrein and PLG. We did not observe immunological cross reactions (Fig 3).

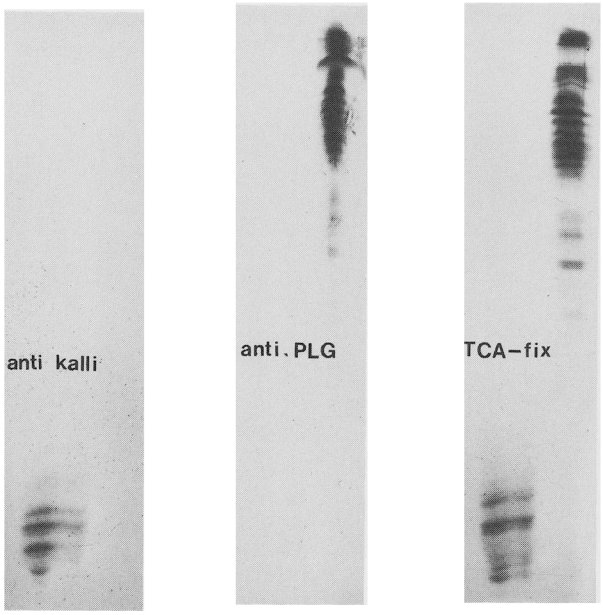


Fig. 3: Antibody specifity tested with purified PLG and kallikrein

The relationship between PLG- and kallikrein phenotypes as observed by IEF and immunological detection with blotting technique is shown in table 1. In some sera kallikrein band patterns are found which show additional bands not observed in PLG - a*, a*b (Fig.4).

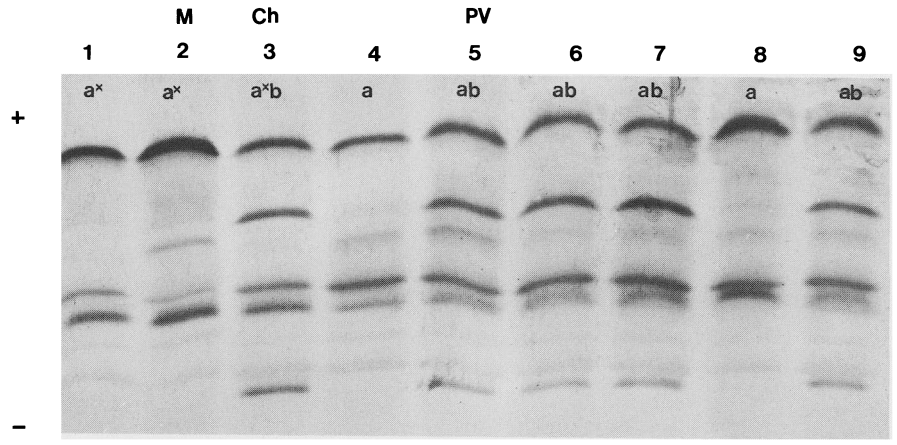


Fig. 4: Different kallikrein a phenotypes of neuraminidase treated sera. Paternity case 15 (Fig. 5) Lane 2, 3 and 5

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Results

Fig 1 shows kallikrein phenotypes of untreated serum samples demonstrated with isoelectric focusing and immunoblotting. The band pattern is observed in the pH-range from 5.9 - 7.3. In the cathodal side of the band pattern the different phenotypes can be read off.

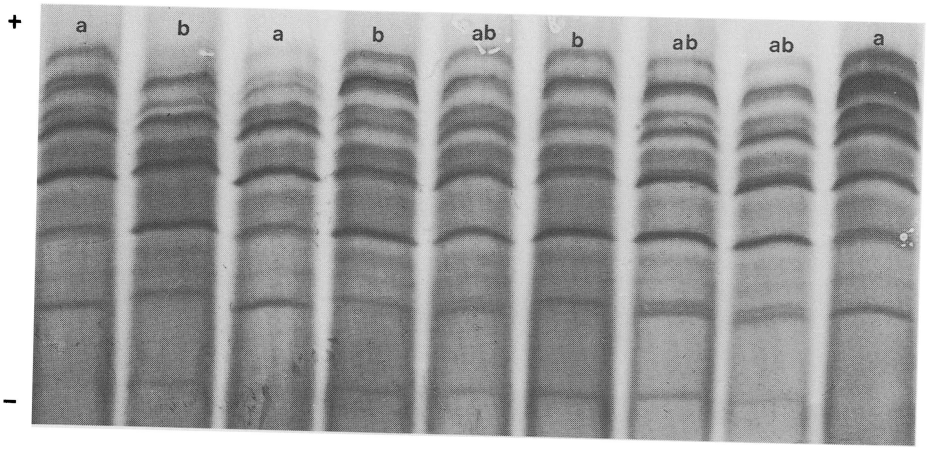


Fig. 1: Kallikrein phenotypes demonstrated by IEF and immunoblotting of neuraminidase untreated serum samples

Fig 2 demonstrates kallikrein after neuraminidase treatment. The band pattern is focused between the marker bands 6.4 - 8.3. Three common kallikrein phenotypes a, ab, b are observed. The neuraminidase treated as well as the untreated material shows a polymorphism correlated to plasminogen. The correlation does not depend as a cross reaction of the antibodies.

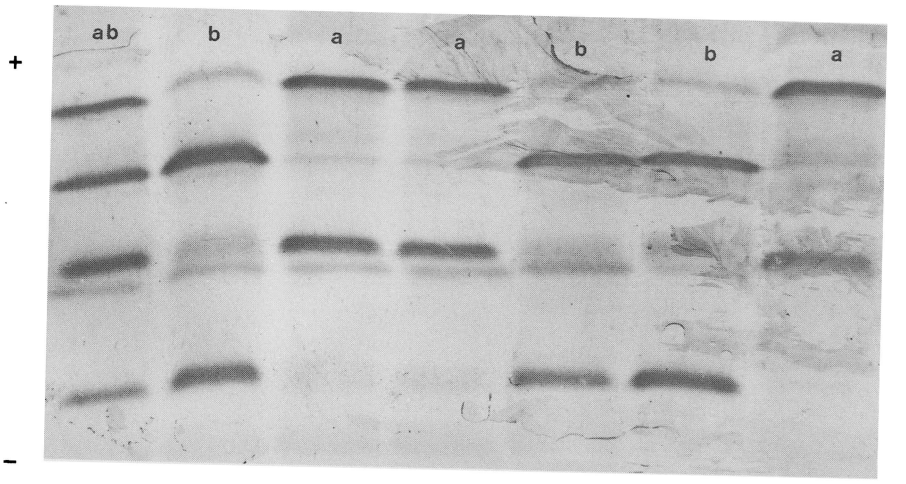


Fig. 2: Kallikrein phenotypes after neuraminidase treatment

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KALLIKREIN PHENOTYPING BY ISOELECTRIC FOCUSING

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Introduction

The influence of kallikrein on the coagulation and the fibrinolytic system depends on the reciprocal activation with factor XII. The inactive prekallikrein exists as a single polypeptide chain of 619 amino acids with a molecular weight of 85.000. Activation by factor XIIa is achieved by the cleavage of an internal Arg-Ile bond. Plasma kallikrein is then composed of a heavy chain (371 amino acids) and light chain (248 amino acids) held together by disulfide bonds (1, 4, 5, 6). The aminoacid sequence of human plasma prekallikrein shows 58 % identity with factor XI (2, 3). The heavy chain is composed of repeated sequences homologous to factor XI. The light chain contains the catalytic part of the enzym and is homologous to the trypsin family of the serine proteases (1). In plasma prekallikrein circulates in a complex with high MW kininogen (5). The normal plasma concentration is 50µg/ml. Prekallikrein deficiency causes abnormalities in the coagulation, fibrinolytic, complement and kinin systems (7). Several different kallikrein genes are found. The nucleotid sequences show homologies (10). Several DNA polymorphisms in the kallikrein genes are observed (11). The human renal kallikrein and prostate specific antigen (PSA) genes were localized to chromosome 19q13 (8, 9).

Material and method

Neuraminidase treated and untreated serum and plasma samples from unrelated, healthy blood donors and from paternity cases were examined. For pI-determination marker proteins from 4.7 - 10.6 were used. Kallikrein antibodies were purchased from Behring (FRG) or donated by Dr. Müller-Esterl, University of Munich. Antibody specificities were tested with highly purified kallikrein and plasminogen isolated by Dr. Müller-Esterl (12).

Kallikrein was demonstrated by isoelectric focusing in 0.8 % agarose gels with the pH-range from 5 - 8. Immunolocical detection was done by immunofixation or immunoblotting.

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Table 3. PEPC segregation analysis in family material from NW Portugal

Mating type	nr. families	offspring 1	6-1	6	1-V	total
1 x 1	40	127				127
1 x 6-1	33	49	67	5		121
1 x 6	10	2	30			32
6-1x 6-1	7	6	14	6		26
1 x 1-V	1	2			1	3
total	91	186	111	11	1	309

Concerning population genetics, the observed phenotype distribution agrees well with Hardy-Weinberg expectations based upon a codominant model in the German sample. The fit is poor in the Portuguese material, with a deficiency of heterozygotes, probably due to the relatively common silent gene detected in family analysis.

Therefore it seems that PEPC can be reliably used in paternity expertises and, given the gene frequencies found in both populations, it proves to be a rather informative marker.

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Table 1. PEPC phenotype distribution in SW Germany and NW Portugal

Population	N	phenotype 1	6-1	6	2-1	others	X^2
SW Germany	442	235 (235.5)	175 (174.2)	32 (32.3)	0	0	0.01
PEPC*1=.730 ; PEPC*6=.270							
NW Portugal	320	212 (206.3)	89 (100.2)	18 (12.2)	1 (0.3)	0 (0.0)	3.91
PEPC*1=.803 ; PEPC*6=.195 ; PEPC*2=.002							

Table 2. PEPC segregation analysis in family material from SW Germany

Mating type	nr. families	offspring 1	6-1	6	total	X^2
1 x 1	66	196			196	
1 x 6-1	92	130 (130.00)	130 (130.00)		260	0.0
1 x 6	11		29		29	
6-1x 6-1	35	35 (25.75)	40 (51.50)	28 (25.75)	103	6.0
6-1x 6	13		19 (19.50)	20 (19.50)	39	0.1
6 x 6	4			12	12	
total	221	361	218	60	639	

Segregation analysis (Tables 2 and 3) supports an autosomal genetic model with codominant alleles. However, in Portuguese material, evidence for a silent gene (2 families, PEPC*0=0.006) was found.

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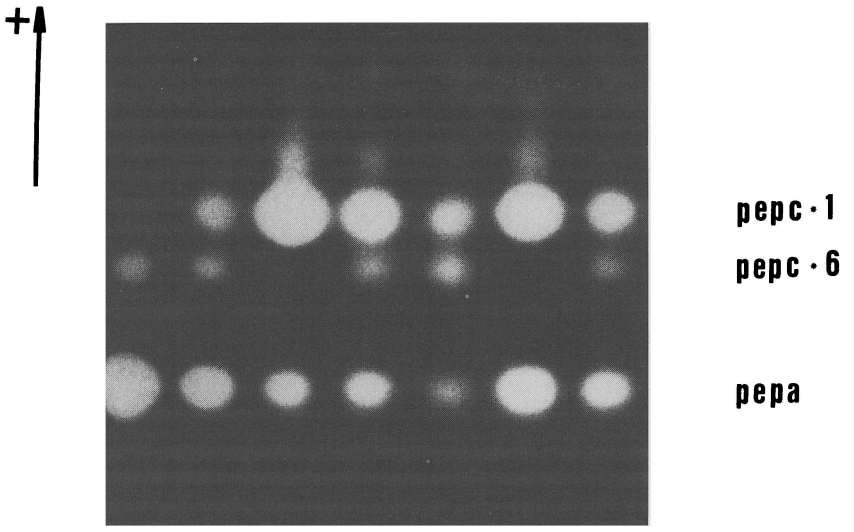


Fig.1. Common electrophoretic phenotypes of PEPC

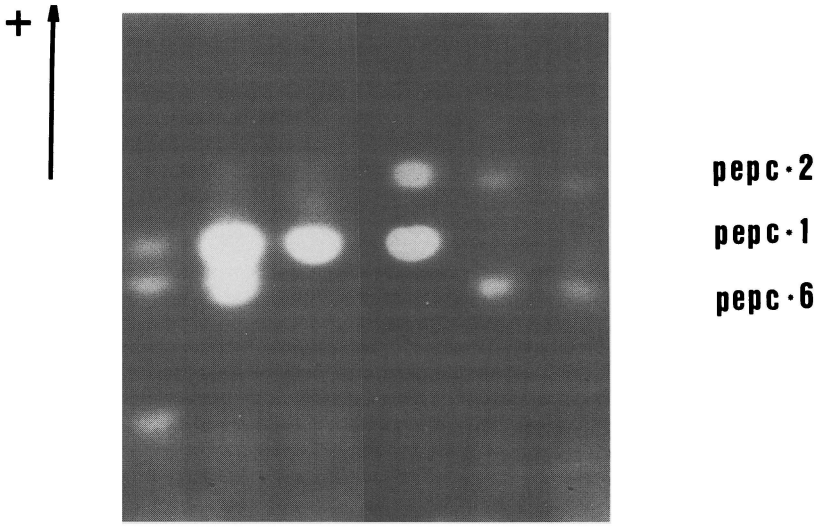


Fig.2. Rare electrophoretic phenotypes of PEPC

DISCUSSION

Electrophoretic patterns detected are consistent with a monomeric structure for PEPC (Figs 1 and 2). Apart from the common gene products PEPC*1 and PEPC*6, rare variants were found (Fig.2).

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Genetic polymorphism of human peptidase C (PEPC)

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INTRODUCTION

Peptidase C (PEPC) was recently demonstrated to be polymorphic in man (Kömpf et al. 1989).

In this work we present data on the formal and population genetics of PEPC from SW Germany and NW Portugal and we discuss the use of this marker in paternity expertises.

MATERIAL AND METHODS

Blood samples were collected by venipuncture. Phenotyping was performed either in leucocytes or in red cell lysates, used fresh or after storage at -20°C up to 7 years. Sample treatment, electrophoretic separation and staining were performed as described previously (Kömpf et al. 1989).

RESULTS

The common phenotypes (1, 6-1 and 6) of PEPC are shown in Fig.1. In Fig.2 the rare phenotypes 2-1 and 6-2 are depicted.

Population genetics data from SW Germany and NW Portugal are presented in Table 1.

The results of the segregation analysis in nuclear families from SW Germany and NW Portugal are shown in Tables 2 and 3.

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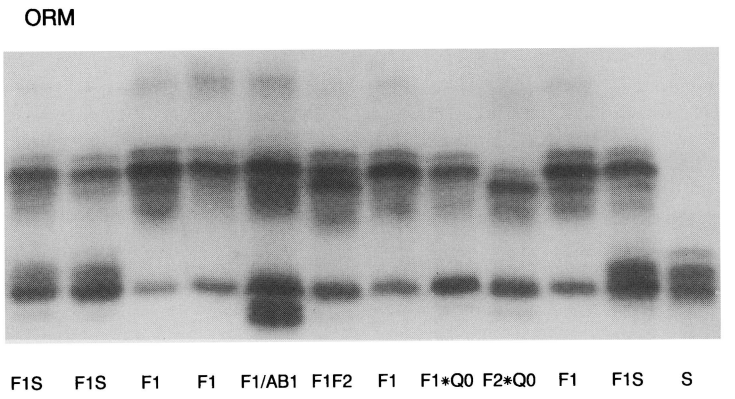
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Vox. Sang. (in press)

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Conclusions:

The reduced level of ORM1 in the child and in the alleged father in comparison to controls indicate the presence of an inheritable null allele. **ORM1*Q0** should be taken into consideration when using this effective system in paternity testing.



ORM1 frequencies in blood donors of Baden-Württemberg/West Germany

	n = 1007	n = 484
Phenotype	Frequency (%)	Weidinger et al. South Germany (1987) Frequency (%)
ORM1 F1	35.65	38.22
ORM1 F1F2	2.98	4.55
ORM1 F2	0.3	0
ORM1 F1S	42.40	41.74
ORM1 F2S	2.68	2.48
ORM1 S	15.99	12.81
ORM1 F1S1	0	0.20

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A Silent Allele in the Orosomucoid (ORM) System

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Orosomucoid 1 (ORM1) - or alpha-1-acid glycoprotein assigned as a genetic polymorphism to human chromosome 9 - with its common three codominant autosomal alleles ORM1*F1, ORM1*F2 and ORM1*S is a very useful system in paternity testing. In 1986 the existence of a second structual locus ORM2 was discovered and has been confirmed in the meantime. This study seems to present evidence for a silent allele ORM1*Q0.

In a paternity case 31 different genetic marker systems were tested [10 red cell membrane systems, 12 plasma-protein systems and 9 red cell enzyme systems: ABO, MNSs, P, Rh, Kell, Duffy, Kidd, Xg, Lu, Co, Hp, Gc*, Gm, Km, C'3, Bf, Pi*, Tf*, FXIII B, PLG, A2HS, ORM1, Gt, acP, PGM1*, AK, ADA, ALT, EsD*, 6-PGD and GLO, * = includes subtyping].

One single apparent exclusion was found in the ORM1 system showing an opposite homozygousity:

child ORM1(F1), mother ORM1(F1), putative father ORM1(F2).

Methods:

Routine ORM phenotyping was carried out using PAGIF with PHARMALYTES at pH 4.2 - 5.4; sera were treated with neuraminidase. After IEF human acid alpha-1-glyco-protein antiserum from ATAB was used for immunofixation. There was a clear difference in the intensity between the pattern of mother and child, which was confirmed by quantitative investigations with NOR-PARTIGEN plates (Behring). The ORM concentration in the mother was normal; whereas, the concentrations in the sera of the child and the alleged father were only approximately 50 % of normal. No additional exclusion was observed by HLA-testing. The biostatistical evaluation showed a likelihood of paternityin the remaining systems including HLA (without ORM) of

W = 99.9998 %.

PI 31 946 **PI*s** = 10 542 **A** = >99.99 %.

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DISCUSSION

SIEF combined with immunoblotting achieved a considerable technical improvment of ORM1 subtyping. The distribution of ORM1 subtypes found by this technique for an extended population sample from North Germany was in good agreement with the Hardy-Weinberg equilibrium. 11 different variants were observed, 9 apparently caused by gene duplication. Family data were available to elucidate inheritance of the homozygous ORM1 F2 subtype hypothetical until recently. The segregation of ORM1 subtypes in 115 Northern German families with 126 children was in accordance with an assumed autosomal codominant mode of inheritance. The single exclusion chance for non-fathers (SECNF) being 0.2137, subtyped ORM1 can be considered as an informative marker for paternity testing.

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Table 2. Family data

parents				children							
ORM1			n	n	F1	F2	S	F1F2	F1S	F2S	F1-F2.S
F1	x	F1	12	16	16	-	-	-	-	-	-
S	x	S	2	2	-	-	2	-	-	-	-
F1S	x	F1S	19	20	3	-	2	-	15	-	-
F1	x	S	8	8	-	-	-	-	8	-	-
F1	x	F1S	43	46	22	-	-	-	24	-	-
F1	x	F2S	5	6	-	-	-	4	2	-	-
S	x	F1S	15	16	-	-	6	-	10	-	-
F1F2	x	F1S	6	6	2	-	-	1	2	1	-
F1F2	x	F2S	1	1	-	-	-	-	1	-	-
F1S	x	F2S	3	3	-	-	-	2	-	1	-
F1S	x	F1.F4-F2.S	1	2	-	-	-	-	-	-	2
total			115	126	43	-	10	7	62	2	2

other matings not observed

The allele frequencies of subtyped ORM1 in various European and non-European populations correlated to our own data are given in Table 3.

Table 3. Allele frequencies of ORM1 subtypes in various populations

population		allele frequencies				references
	n	ORM1*F1	ORM1*F2	ORM1*S	others	
Danes	215	0.5810	0.0330	0.3860	0.0000	THYMANN and EIBERG (1986)
French	112	0.5625	0.0491	0.3884	0.0000	YUASA et al. (1986)
Germans						
Hanover	1934	0.5776	0.0258	0.3966	*)	this study (1989)
Münster	167	0.5625	0.0298	0.3929	0.0149	UMETSU et al. (1989)
Munich	272	0.6103	0.0404	0.3475	0.0018	WEIDINGER et al. (1987)
Swiss	329	0.5927	0.0015	0.4043	0.0015	EAP et al. (1988)
Filipinos	115	0.7904	0.0000	0.1687	0.0409	UMETSU et al. (1988b)
Japanese	200	0.6800	0.0000	0.1525	0.1675	YUASA et al. (1988)
Taiwanese	200	0.7255	0.0000	0.1805	0.0941	UMETSU et al. (1988a)

*) not taken into account

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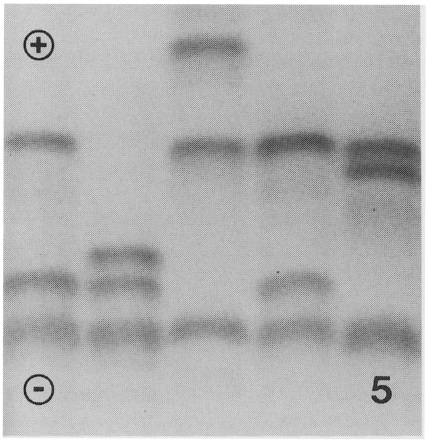


Fig. 2. Two ORM1 variants by SIEF and immunoblotting. From left to right, 1: F1S, 2: F5S, 3: F1F6, 4: F1S, 5: F1F2; F5 and F6 being provisional designations

Nine further variants apparently caused by gene duplication found in this study are not shown.

The distribution of ORM1 subtypes observed in 1934 unrelated individuals from Hanover and Lower Saxony is listed in Table 1.

Table 1. Population data (with exclusion of variant phenotypes)

phenotypes	observed		expected	allele frequencies	
ORM1	n	o/o	n		
F1	645	33.35	645.18		
F1F2	55	2.84	57.63		
F2	1	0.05	1.35		
F1S	889	45.97	886.16	ORM1*F1	0.5776
F2S	43	2.22	39.65	*F2	0.0258
S	301	15.56	304.22	*S	0.3966
total	1934	99.99	1934.19		1.0000

chi-square: 0.5392; p: 0.95 > p > 0.9; df: 3; SECNF 0.2137

Table 2 presents the results of family studies with 115 matings and 126 children.

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Immunoblotting

Blotting: passive blot (0.45 µm NC, Schleicher & Schüll); 20'. Blocking: 2% liquid gelatin (NORLAND) in washing buffer; 1 h. 1st antibody: rabbit anti-human ORM (Dakopatts); 1/500 diluted in blocking solution; 1 h. Washing 1: 5x5' in washing buffer (0.01 M Tris/HCl, 0.9% NaCl, 1% Triton X-100; pH 7.4). 2nd antibody: swine anti-rabbit IgG, AP-conjugated (Dakopatts); 1/1000 diluted in blocking solution; 45'. Washing 2: 2x5' in washing buffer. Color development: BCIP: 15 mg/1 ml dimethyl-formamid (DMF). NBT: 30 mg/1 ml 70% DMF solution. 1 ml BCIP and 1 ml NBT diluted in 100 ml carbonate buffer (0.1 M NaHC03, 1.0 mM MgC12; pH 9.8); 30'. Stopping: distilled water.

RESULTS

An original blot of common ORM1 phenotypes including the rare F2 homozygote is shown in figure 1.

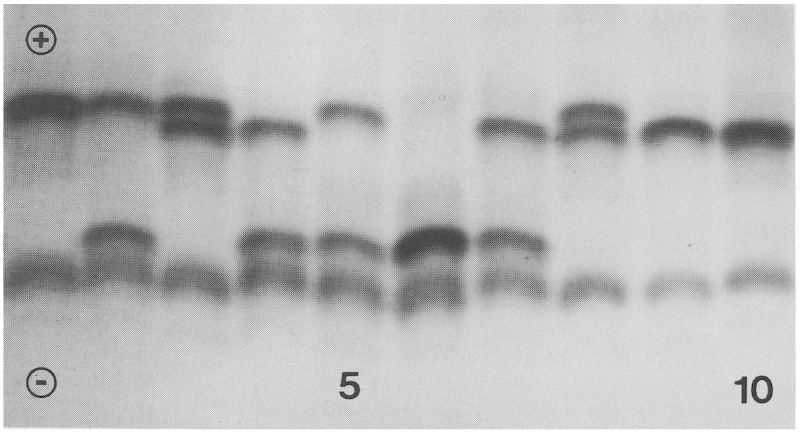


Fig. 1. ORM1 subtypes by SIEF and immunoblotting. From left to right, 1: F1, 2: F1S, 3: F1F2, 4: F2S, 5: F1S, 6: S, 7: F2S, 8: F1F2, 9: F2 (hypothetical until recently), 10: F2

The homozygous F2 carriers are siblings (sister lane 9, brother lane 10), the father's type (lane 8) is F1F2. The type of the deceased mother was deduced from those of her living siblings (lane 4 - 7) to be F1F2 or F2S.

Figure 2 represents another blot including 2 variant ORM1 phenotypes.

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ORM1 Subtypes in Hanover and Lower Saxony, FRG, by PAG SIEF and Immunoblotting (N=1934); 11 Variants

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INTRODUCTION

ORM1 subtypes have first been described by Thymann and Eiberg (1986). Since then, their existence has been confirmed by several working groups, e.g. Yuasa et al. (1986), Weidinger et al. (1987), Eap et al. (1988), Umetsu et al. (1989), and Luckenbach et al. (1989). The technique of IEF with different modifications (HIEF, IPG, SIEF) and following immunoprinting or immunoblotting was applied by all these investigators.

The aim of our study using the advantages of SIEF and immunoblotting was an improvement of protein separation and visualization. Furthermore, the exploration of population and family data for a Northern German population sample was intended.

MATERIALS AND METHODS

Sera of 1934 unrelated healthy individuals from Hanover and Lower Saxony (plus 29 variant carriers) and of 115 families with 126 children were processed.

Sample preparation

45 µl serum, 5 µl CPN (Sigma, type V; 10 U/0.4 ml 1 M KH_2PO_4 buffer, pH 7.0), incubated 24 h at RT.

Separator isoelectric focusing (SIEF)

Gel: 230x105x0.5 mm; T=4.8%, C=3.0%. Separator: 0.0175 M ACES. Ampholytes: Pharmalytes; 3.8% pH 4.2-4.9, 2.6% pH 4.5-5.4. Additive: 13% glycerol. Electrolytes: anolyte 1 M H_3PO_4, catholyte 0.2 M NaOH. Running conditions: prerun 30'; 5 W, 2000 V, mA unlimited; run 150'; 20 W, 2000 V, 16 mA; cooling 8 °C. Sample application: 3 µl CPN-treated serum on 4x4 mm Whatman 1; 2 cm from cathode for 30'. Apparature: LKB; Multiphor II, Macrodrive 5, Multitemp II.

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minor bands in places of bands from other phenotypes is a disadvantage of immunoblotting. In the begin of this series, differentiation between the phenotypes F_1 and F_2 was difficult in some cases. Better resolution through immunoblotting has improved this differentiation). Extended focusing times sharpend bands, but the minor bands also became more intense.
In contrast to other studies in southern Germany (Weidinger, 1987) our allel frequency for F_2 is a little lower. This might have technical reasons as mentioned above. Further studies with our improved method might solve this question.

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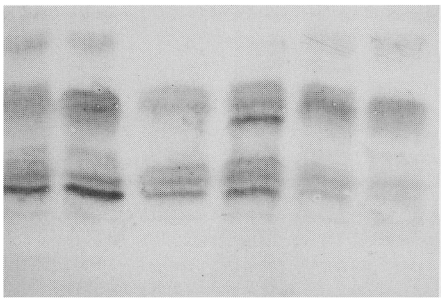


Table 1

phenotype	observed n	observed (%)	expected n	expected (%)
F_1	188	40.17	196	41.77
F_1 S	220	47.01	205	43.78
S	46	9.83	53	11.47
F_1 F_2	9	1.92	9	1.87
F_2 S	5	1.07	5	0.98
	468	100.00	468	99.87

$Chi^2 = 2,3486$; $p < 0,05$

Based on these results we calculated the allele frequencies: ORM1*F_1 = 0.6463, ORM1*S = 0.3387 and ORM1*F_2 = 0.0145. The theoretical chance for the exclusion of non-fathers was calculated to be 18.07%.

Discussion

The separation of Orosomucoid (ORM) in agarose is comparable to conventional polyacrylamid gels. Introducing immunoblot for detection has sharpend the bands in comparison to immunofixation. Also the costs for antibodies were reduced. Detection of weak

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Isoelectric focusing of Orosomucoid in agarose gels

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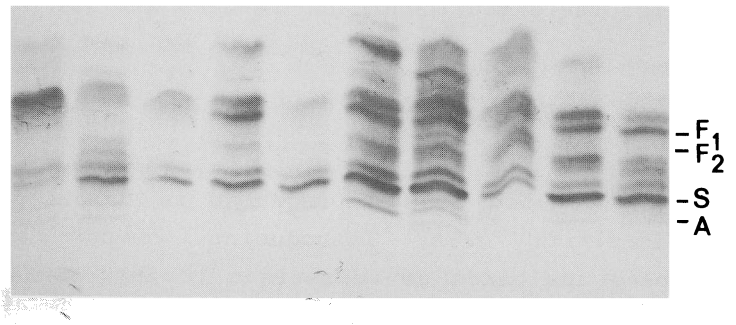
A genetically determined polymorphism of the plasmaprotein Orosomucoid has been originally demonstrated by Schmid et al., 1965. Isoelectric focusing of this protein in agarose was first published by Thymann and Eiberg in 1985. Meanwhile many investigators seemed to prefer polyacrylamide gels (Weidinger 1987, Wimmer et al., 1987) because of better separation. Agarose is in comparison a nontoxic technique. In our study we experimented with the immunoblot for detection of the samples.

Material and methods

We examined 468 samples of healthy, unrelated blood donors in southern Bavaria. The samples were treated with neuraminidase (CPN, Boehringer, Mannheim) 0.04U/50µl serum for 18h at 37°C. Gels were composed of 0.8% agarose (Pharmacia),10% sorbitol, 18.6ml aqua dest, 0.7ml ampholyte, pH 4.2-4.9 (Pharmalyte), 0.7ml ampholyte, ph 4.5-5.4 (Pharmalyte). As electrode solutions we used for the anode 0.25m acidic acid, for the cathode 0.25m NaOH. 8µl of sample (diluted 1:4) were applied cathodally. Separation was performed at 8°C, 30min prefocusing (setting 8W), 30min salt run (0.8W), 90min or more (8W).
Immunoblotting was done for 45min with a 0.45µm NC-membrane. For blocking and washing (3 x 10min) 5% skim milk powder in 0.1m PBS, pH 7.3, was used. The first antibody, a 1: 500 dilution of acid-α_1-Glykoprotein (ORM) from goat (AHS) was applied for 14h at room temperature, followed by 3 x 10min washing.
As second antibody a 1: 500 dilution of anti-goat from rabbit (AHS) was used for 2h at room temperature. For visualisation 30mg 4-chlor-1-naphtol in 10 ml ethanol and 20µl H_2O_2 in 50ml 0.1m PBS pH 7.3, were used.

Results

The separation of the different phenotypes is shown in 2 gels. Table 1 gives the the statistical analysis of our data.



F S F FF F FF FF FF FF FS
1 1 12 1 12 12 12 12 2

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RESULTS

The revealed genic frequencies are shown in Table 1. F-XIII B phenotype pattern obtained by PAGIEF and semi-dry electroblotting is shown in Fig. 1.

Table 1. Distribution of F-XIII B phenotypes in Ancona

Phenotype	Observed	%	Expected	Gene frequency
1 - 1	172	57.91	171.23	F-XIII B*1= .7593
2 - 2	2	0.67	1.56	
3 - 3	8	2.70	8.41	F-XIII B*2= .0724
1 - 3	76	25.59	75.91	
2 - 3	8	2.69	7.24	F-XIII B*3= .1683
1 - 2	31	10.44	32.65	
Total	297	100.00	297.00	

$\Sigma\chi 2 =$ 0.311 df = 3 P > 0.95

Rare allotypes were not seen. The observed phenotypes do not present any meaningful deviation from the Hardy-Weinberg equilibrium.

The Italian population sample shows some significant differences from other European countries. In particular Italians have a higher frequency of F-XIII B 1 and a lower frequency of F-XIII B 2 while F-XIII B 3 seems almost identical to other Caucasian populations.

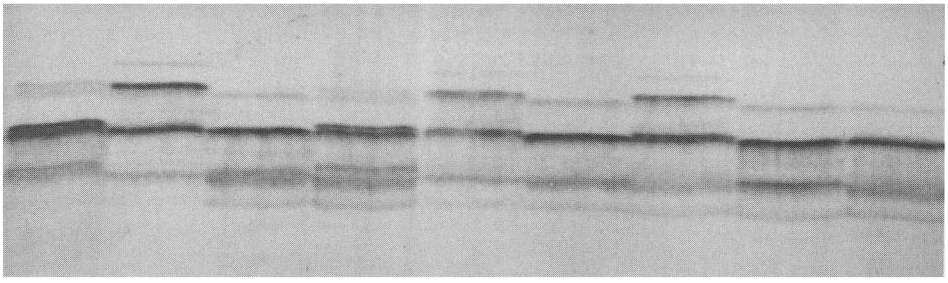


Fig. 1. F-XIII B phenotypes after PAGIEF in the pH range 3.5-9.5 and semi-dry electroblotting with enzyme immunoassay. Cathode is at the top. From left to right: 1-2, 1-3, 1-1, 1-2, 3-3, 1-1, 1-3, 1-1, 1-1.

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Study of Genetic Polymorphism of Coagulation Factor XIII B in a Italian Population Sample using PAGIEF and Semi-dry Electroblotting

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INTRODUCTION

Coagulation factor XIII (fibrin-stabilizing factor) shows a quadrimeric structure (2A and 2B subunits) in plasma. No enzymatic activity has been described for the B subunit, which seems to act only as a plasma carrier.

Genetic polymorphism of both subunits were investigated first in Australians by Board (1979, 1980) with electrophoresis in agarose gel and immunofixation. Later the B subunit polymorphism was also investigated in other population samples, while new methods were progressively introduced (Mauff et al. 1983; Olaisen et al. 1983; Pytlik et al. 1984; Leifheit et al. 1985; Kuhnl and Spielmann 1985; Hjalmarsson 1985; Dykes et al. 1985; Nakamura and Abe 1985; Nakamura et al. 1986; Leifheit at al. 1987). These investigations have provided further information about the factor XIII B system that was suggested as being determined by three common alleles in a single autosomal locus: F-XIII B 1, F-XIII B 2 and F-XIII B 3. Some rare variants have also been described.

This study is the first conducted on a Italian sample and uses some technical modifications to current methods in order to display the F-XIII B bands more rapidly and clearly.

MATERIALS AND METHODS

297 EDTA plasma samples were obtained from healthy blood donors living in Ancona. Neuraminidase treatment and isoelectric focusing were performed as described by Nakamura and Abe (1985) with slight modifications (for example, a 10 U/ml concentration of neuraminidase was used). Proteins were transferred to nitrocellulose sheets (O,45 µm LKB) by semi-dry electroblotting (nova blot LKB). The transfer was carried out, as suggested in the LKB technical note, for 45 min. at 0.8 mA/cm2 without refrigeration.
The enzyme immunoassay was performed as follows:
-blocking 120 min. in BSA-TBS 3% + Tween 20 0.05%.
-washing 2 x 30 min. in TBS-Tween 20 0.15% for 60 min.
-rabbit antihuman factor XIII subunit S (Behering) diluted 1:1000 in TBS-Tween 20 0.15%.
-washing as above.
-goat antirabbit immunoglobulin, heavy and light chain specific, horseradish peroxidase conjugated (Calbiochem) diluted 1:2000 in TBS-Tween 20 0.15% for 60 min.
-washing as above.
-development with a solution of 10 mg of 3-3' diaminobenzidine in 40 ml TBS and 40 µl 10% H2O2. The reaction was stopped with running tap water.

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FXIIIB RESULTS

MOTHER	2-1
CHILD 1	4-1
CHILD 2	2-1
CHILD 3	4-2
P.FATHER	4-2

CALCULATION OF THE PATERNITY PROBABILITY PERCENTAGE

	without FXIIIB frequency	with FXIIIB frequency
CHILD 1	99.87 %	99.999 %
CHILD 2	99.99 %	99.993 %
CHILD 3	99.81 %	99.998 %

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FACTOR XIIIB (FXIIIB) IN DISPUTED PATERNITY
COMPLETE ANALYSIS OF A FAMILY WITH THE RARE PHENOTYPES 4-1 AND 4-2

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SUMMARY

Paternity testing was performed on a family of five persons (mother, three children and putative father); 28 blood group systems have been tested.
While typing FXIIIB, the rare phenotypes 4-1 and 4-2 were found in three members of the family.
The paternity probability was calculated with and without FXIIIB frequency.

FXIIIB DETERMINATION

The factor XIIIB phenotyping is carried out by isoelectric focusing on thin-layer agarose gel, with pH gradient from 4 to 7 and subsequent immunofixation.
The method of LEIFHEIT and collaborators is used with several modifications.

Sample preparation and agarose gel composition

The sera are pretreated with neuraminidase Typ VIII (SIGMA). The agarose gel has the following composition : 0.20 g Agarose IEF (PHARMACIA), 0.12 g ACES, 2.00 g D-Sorbitol, 18.5 ml bidist. water, 750 µl Ampholine pH 4.0-6.5 and 750 µl Ampholine pH 5.0-7.0 (LKB).

Isoelectric focusing

Cathodic solution : 0.25 M NaOH; anodic solution : 0.25 M CH_3COOH; the temperature is maintained at 8°C during the IEF.

A prefocalisation is carried out during 30 minutes at 1200 V, 50 mA, 8 W.
8-10 µl of pretreated serum samples are applied on the gel, 1 cm from the cathodal site.
For salt removal, IEF is first conducted at 150 V, 50 mA, 8 W for 30 minutes, then pursued at 1200 V, 50 mA, 8 W for 165 minutes.

Immunofixation

Immunofixation is performed by applying 0.75 ml of 1:2 with bidistilled water diluted FXIIIB antiserum (BEHRING). The gel is left for incubation at 37°C for 90 minutes, then treated with NaCl, washed, stained (Coomassie Blue) and destained as usual.

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TABLE 2. F13A PHENOTYPE AND GENE FREQUENCY DISTRIBUTION

Phenotypes			Gene Frequencies		
	White (No. 1527)	Black (No. 975)		White	Black
1	1001	636	F13A*1 =	0.8098	0.8062
1,2	469	285	F13A*2 =	0.1896	0.1846
2	55	37	F13A*7 =	0.0003	0.0067
1,7	1	11	V =	0.0003	0.0026
2,7	0	1			
1,V	1	5			
7,V	0	1			

Chi Square			
White	0.456	p >.99	(df6)
Black	1.379	.99 >p >.90	(df6)

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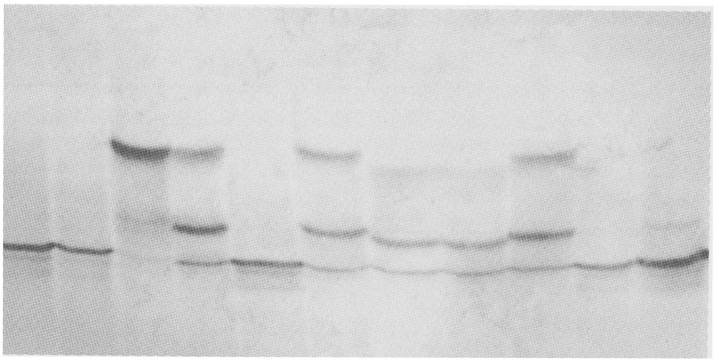


Fig. 1. F13A phenotypes blotted onto Nytran membrane. The cathode is on the top. From left to right: F13A 1, 1, 2, 1-2, 1, 1-2, 1-7, 1-7, 1-2, 1, 1.

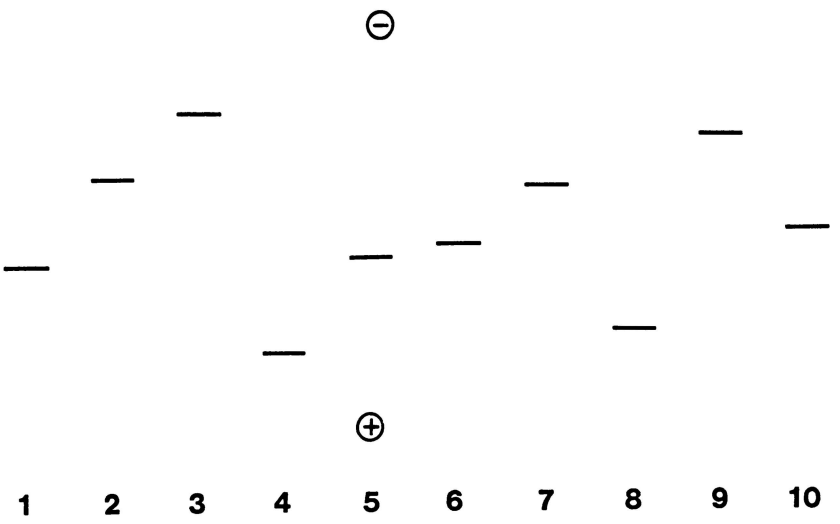


Fig. 2. Positions of F13A variants on IEF. The F13A 7 band is slightly anodal to the F13A 2 band.

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TABLE 1. IEF TECHNIQUE FOR F13A PHENOTYPING

Agarose IEF (Pharmacia)		0.225 gm
Sucrose		2.7 gm
LKB Ampholyte 5-7		0.8 ml
LKB Ampholyte 4-6.5		0.8 ml
Electrolytes:	Anode	1.0 M H_3PO_4
	Cathode	0.2 N NaOH
Sample Wicks		Whatman # 1 (4X4mm)
Focusing:	Volts:	2000
	Milliamps	unlimited
	Watts	5 (15 minutes) remove wicks
		7 (15 minutes)
		9 (15 minutes)
		10 (15 minutes)

1. Passively blot to Nytran, 5 X 20 cm, presoaked in TBS (20mM Tris-HCl, pH 7.5, 0.5 M NaCl). Place Nytran on gel, cover with piece of filter paper presoaked in TBS and 1 cm of paper towels. Blot for 30 minutes.

2. Air dry

3. Block membrane 20-30 minutes with 1% HiPure Gelatine, 5% Tween-20 and 0.25% Hammarsteen Casein in TBS.

4. To membrane add 10-15 ml of 1:500 diluted antibody 1(rabbit anti-human F13A) in block solution. Gently agitate for 60 minutes at room temperature.

5. Wash membrane 3 X 5 minutes in TBS.

6. To membrane add 10-15 ml of 1:1000 diluted antibody 2 (ALP conjugated goat anti-rabbit IgG) in block solution. Gently agitate for 60 minutes at room temperature.

7. Wash membrane 3 X 5 minutes in TBS.

8. Place membrane in clean dish with ALP substrate mixture:

   50 ml TBS (pH 9.1)
   25 ul NBT (75 mg in 700 ul DMF and 300 ul H_2O)
   75 ul BCIP (50 mg in 1 ml DMF)

   Place in dark at room temperature for 10-30 minutes.

9. Wash membrane briefly in tap water and blot dry in dark.

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Identification of F13A*7 as a Population Specific Marker

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INTRODUCTION

Factor XIII(F13), a precursor of fibrinoligase, is a tetramer with 2A and 2B subunits. F13B functions as a carrier and regulator of the enzymatically active F13A subunit. The two most common alleles are F13A*1 and F13A*2 with all other alleles appearing as rare or uncommon in most population groups reported in the literature.(Board, 1979; Olaissen et al., 1983; Dykes et al., 1986). Ten different genetic variants of F13A have been reported using isoelectric focusing (IEF) techniques (Dykes et al, 1988). F13A can only be detectable in plasma samples, therefore it cannot be used in blood stain analysis. In parentage testing the exclusion probability of F13A=0.15. For this study we tested F13A on Whites and Blacks using IEF separation detected by immunoblotting. This report identifies the allele F13A*7 to be more specific for Black populations.

METHODS

Blood samples for parentage testing submitted to Analytical Genetic Testing Center were tested for GM, KM, ACP1, PGM1, ESD, GLO1, GC, PLG, F13A, F13B and 3-4 DNA probes. Plasma samples for F13A phenotyping were tested by isoelectric focusing on agarose gels at a pH range 4-7. Nytran was used for blotting gels and bands were detected by ALP conjugated antibodies, Table 1.

RESULTS

F13A phenotyping by IEF followed by ALP immunoblotting, in our study, permitted us to identify seven of the ten documented variants. Figure 1 shows a membrane after detection. As can be seen the F13A*7 gene product focuses slightly anodal to F13A 2. Figure 2 shows a diagram of the ten variants. The phenotyping results on 1,527 Whites and 975 Blacks are seen in Table 2. Except for F13A*7, all rare variants were simply classified as "V" for statistical purposes.

Gene frequency distribution of F13A demonstrated very similiar frequencies for both F13A*1 and F13A*2. In contrast the frequency for F13A*7 was 0.0067 in Blacks and 0.0003 in whites. We also identified more rare variants in Blacks (0.0026) versus Whites (0.0003). Although the data indicates a similiar exclusion probability for both populations, the gene frequency data tends to support the presence of F13A*7 as a Black marker.

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F13B phenotypes		F13B allele frequencies	
1	299 (59%)	*F13B*1*	0.7623
2-1	62 (12%)	*F13B*2*	0.0815
3-1	111 (22%)	*F13B*3*	0.1513
4-1	5 (<1%)	*F13B*4*	0.0049
2	4 (<1%)	Σ	1.0000
3-2	13 (3%)		
3	15 (3%)		
Σ	509		

The numbers of the phenotypes observed show no significant difference to the figures expected under Hardy-Weinberg equilibrium (F13A: X^2 = 2.47, 1 df, p = 0.12 and F13B: X^2 = 2.69, 6 df, p = 0.85).
The allele frequencies for F13A and F13B are also in good agreement with data published for other Caucasoid populations (see e.g. Dykes et al. 1986 or Kreckel et al. 1983).
The chances of paternity exclusion in cases of disputed parentage by using F13A and F13B are 12.6% and 20.3%, respectively.

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POLYMORPHISMS OF COAGULATION FACTORS 13A AND 13B IN WEST GERMANY

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AIMS OF THE STUDY

- definition of the phenotypes of the coagulation factors 13A (F13A) and 13B (F13B) in a population sample of West Germany,
- population genetics of F13A and F13B,
- use of F13A and F13B in paternity testing.

MATERIALS AND METHODS

- blood samples: from healthy, unrelated inhabitants of Aachen (West Germany)

- F13A and F13B typing: method according to Dykes et al. (1986) by isoelectrofocusing in polyacrylamide gel pH 5 - 6.5; detection of the gene products by Western blotting (rabbit anti-F13A and anti-F13S [Behring] respectively, and peroxydase-labelled pig anti-rabbit-immunoglobulin-serum [Dakopatts]).

RESULTS

The methods used for typing F13A and F13B are fast and simple techniques giving reliable results.
The F13A and F13B phenotype and allele frequencies are given in the Table.

Table F13A and F13B phenotype and allele frequencies in West Germany

F13A phenotypes		F13A allele frequencies	
1	307 (66%)	*F13A*1*	0.8194
2-1	148 (32%)	*F13A*2*	0.1806
2	10 (2%)	Σ	1.0000
Σ	465		

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In Fig. 1 the FXIIIB banding patterns by PAGIF and subsequent semidry electroblotting are illustrated. From left to right:

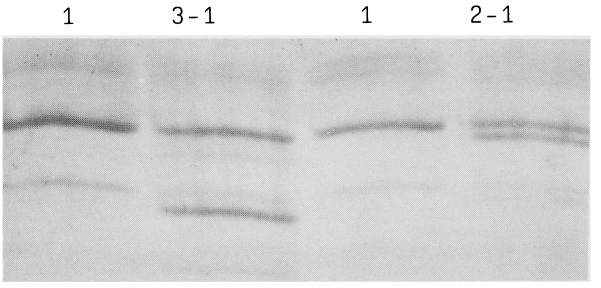


Fig. 1: FXIIIB phenotypes by PAGIF and semidry electroblotting, visualisation by POD development.

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RESULTS AND DISCUSSION

Both AGE followed by immunoblotting technique and PAGIF followed by semidry electroblotting are useful for FXIIIA and FXIIIB phenotyping. There are no differences in banding pattern of FXIIIA detectable by these methods. In table 1 the FXIIIA phenotype distribution and gene frequencies are demonstrated.

Table 1: FXIIIA phenotypes and allele frequencies in Northern Germany (Mecklenburg area)

Phenotypes	observed	expected	allele frequencies
FXIIIA 1	201	200.82	FXIIIA*1 = 0.7803
2-1	111	111.55	FXIIIA*2 = 0.2166
2	16	15.50	FXIIIA*3 = 0.0015
3-1	1	0.77	FXIIIA*4 = 0.0015
4-1	1	0.77	
Total	330	329.51	Chi^2=0.193 0.9<p<0.95 2df

In table 2 the FXIIIB phenotype distribution and gene frequencies in a population sample of Northern Germany are presented.

Table 2: FXIIIB phenotypes and allele frequencies in Northern Germany (Mecklenburg area)

Phenotypes	observed	expected	allele frequencies
FXIIIB 1	209	209.17	FXIIIB*1 = 0.7676
2-1	47	42.18	FXIIIB*2 = 0.0774
3-1	80	84.40	FXIIIB*3 = 0.1594
2	0	2.12	
3-2	8	8.51	
3	11	8.52	
Total	355	354.90	Chi^2=3.430 0.5<p<0.7 5df

Gene frequencies of both systems are in accordance to other Caucasoid populations. The single exclusion chance for nonfathers is calculated to be 14.0% in FXIIIA- and 19.5% in FXIIIB-system.

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Coagulation Factor XIIIA and XIIIB polymorphisms in the North of Germany (Mecklenburg area)

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INTRODUCTION

Coagulation factor XIII consists of two A and two B subunits and circulates in plasma and thrombocytes. The A subunit has transglutaminase activity forming cross links between fibrin monomers in the final step of coagulation. Both subunits show geneticly determined polymorphisms described first by Board (1979,1980). Subsequent investigations in Japanese,European and American populations identified two common alleles in the FXIIIA and three common alleles in the FXIIIB-system.

MATERIALS AND METHODS

FXIIIA polymorphism was studied on plasma or thrombolysates. For FXIIIB phenotyping plasma or serum samples were treated with Neuraminidase. Agarose gel electrophoresis (AGE) with subsequent passiv immunoblotting was carried out mainly according to the method of Olaisen et al. (1983) and Hjalmarson (1986). Polyacrylamide gel isoelectric focusing (PAGIF) was conducted on 0.35 mm thin gels (T=4,6%,C=3%) with 3% carrier ampholytes and 12% glycerine. For FXIIIA typing a mixture of 0.4 ml Servalyt 5-7 and 0.4 ml Servalyt 5-8 was used. The anode- and cathode solutions were Anode fluid 3 and Cathode fluid 10 (Serva). Samples were applied on 8x5 mm Whatman 3 papers at the cathodal side. For FXIIIB typing a mixture of 0.6 ml Pharmalyte 4-6.5 and 0.2 ml Servalyt 5-7 was employed. Samples were applied on 10x5 mm Whatman 3 paper strips at the anodal side. Focusing was carried out at 2000V, 8 W and 10 mA at 10°C for 4 hrs. including 40 min. prefocusing.

Transfer to NC-membranes (Schleicher & Schuell) was conducted by semidry electroblotting in 25 mM Tris, 192 mM Glycine, 20% Methanol buffer for 1 hr. at 0.8 mA/cm². After transfer NC-membranes were blocked for 2 hrs. in PBS-Tween. Clotimmun FXIIIA/S (Behring Werke) was used in a 1:400 dilution as first antibody. After a washing procedure in PBS-Tween the membranes were soaked in a 1:800 dilution of POD conjugated anti rabbit globulin for 4 hrs. Visualisation of banding patterns was performed in a mixture of 20 mg 3 Amino 9 ethylcarbazole (Sigma), 50 ml PBS and 30 µl 30% H_2O_2.

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Surprinsingly the concentration of the sialidase seems to have no or only a minimal influence on the reliability of the PLG group determination. Pretreatment of 18ul serum with 0,4 U down to 0,08 U of CLPF sialidase leads always to a good separation of the bands of all PLG groups (Fig.6).

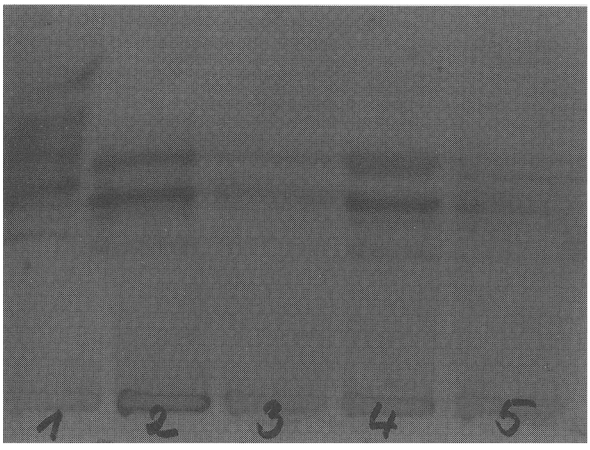


Fig.6:Pretreatment of a PLG 2-1 serum with different concentrations of CLPF sialidase.

Legend: 1:Untreated.
2:Room temp 18 hours;0,4U
3: 37°C of CLPF sialidase
4:Room temp 18 hours;0,4U
5: 37°C of CLPF sialidase

Conclusion.
In order to reliably determine PLG groups in the cellulose acetate foil electrophoresis it is necessary to take care that the current during the run increases from 15mA to 32mA by filling up the electrophoresis chambers with buffer solution. The sera should be pretreated with at least 0,08 U of CLPF sialidase per 18ul of serum for 18 hours at room temperature.

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acetate foils are used and the electrophoresis chambers are filled up with buffer without paper bridges within the chambers (Fig.3).

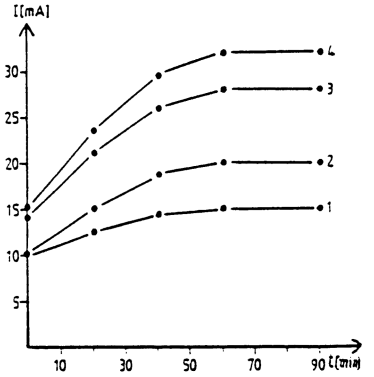


Fig.3: Change of current during electrophoresis.

Legend:1.with paper bridges 2.Chamber filled up 3.With paper bridges 4.Chambers filled up

Cellogel foil cellulose acetate foil

It is very important to use special sialidases. We have found out that the pretreatment of sera with CLPF sialidase gives excellent results. Pretreatment with VCH or IVI sialidases leads independent of the incubation time and temperature to completely other and partially misleading patterns. E.g. PLG 1 looks like PLG 1-F (Fig 4) and PLG 2 looks like PLG 1-M (Fig.5).

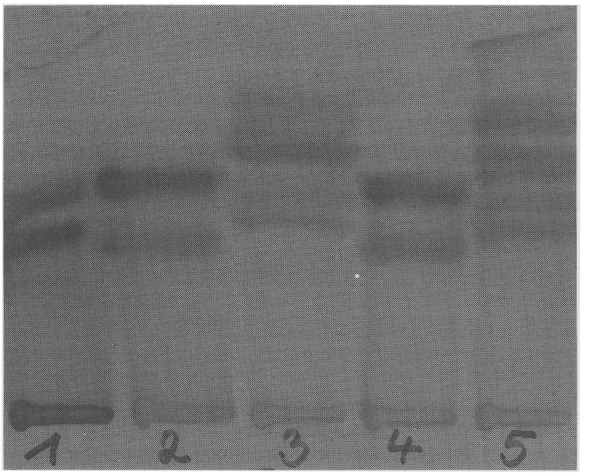


Fig.4:Pretreatment of a PLG 1 serum with different sialidase.

Legend:1: PLG 2-1 reference. Sialidase from. 2:CLPF 1. 3: VCH.4:CLPF 2. 5:IVI.

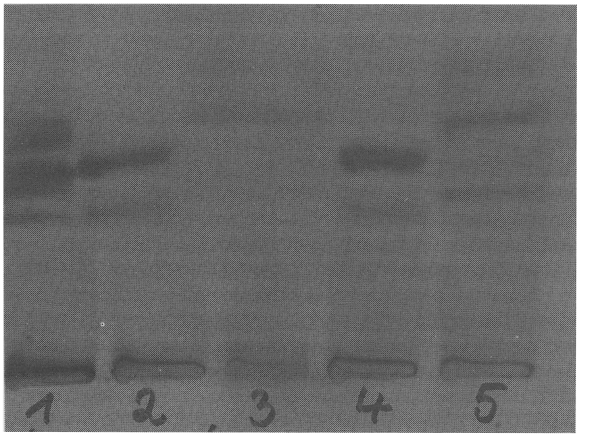


Fig.5: Pretreatment of PLG 2 serum with different sialidase

Legend:1:PLG 2-1 reference.Sialidase from 2:CLPF 1. 3:VCH. 4:CLPF 2. 5:IVI.

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THE SIGNIFICANCE OF CURRENT AND OF ORIGIN AND CONCENTRATION OF SIALIDASES FOR THE DETERMINATION OF PLASMINOGEN GROUPS

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Plasminogen (PLG) groups have been presumed first from Sachs and Szirmai (1972) on the basis of difficult diffusion-absorption tests. They were detected by Hobart (1979) and Raum et al. (1980) with an isoelectric focusing technic. Jaeger et al. (1983) have shown that PLG groups can also be determined with a simple foil electrophoresis. With a chance of exclusion of 16% the PLG groups system is highly suited for parentage testing. Until now three frequent phenotypes (PLG1, 2-1 and 2) and six rare (PLG 1-F, 1-M, 1-S, 2-F, 2-M and 2-S) have been observed (Fig.1).

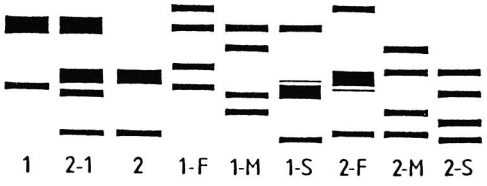


Fig.1:

In order to test the reliability of the PLG group determination in the cellulose acetate foil electrophoresis it appeared that a safe determination can be affected by various influences.

Material and methods.

Sera of the types PLG 1, 2-1 and 2 were investigated in principle according to Jaeger et al (1983) with the following variations:

First the electrophoresis chambers were filled up with tris-barbital-glucine buffer (pH 9.5)(Fig.2) and on the other hand they were filled only to a third. The partition walls were bridged within the chambers with filter paper.

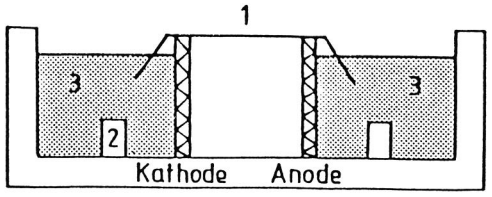


Fig.2: Electrophoresis chamber

Legend: 1:Cellulose acetate foil.2:Partition wall.3:Electrophoresis Chambers filled up without paper bridges within the chambers.

The sera were pretreated with sialidase from clostridium perfringens (CLPF)(Sigma and P-L-Biochemicals) from vibrio cholerae (VCH)(Behring) and from influenza virus (IVI) (Calbiochem), 0,4 U in phosphate buffer (pH 7.0) and 18ul serum by incubation for 18 hours at room temperature.

Finally pretreatment of sera with CLPF sialidase was carried out with 0,4 U down to 0,08 U.

Results.

If the current at a constant terminal voltage of 320 is lower than 15 mA in the beginning and 32 mA at the end of the electrophoresis the bands are not sufficiently separated. This can only be reached if cellulose

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The comparison of the functional assay (caseinolytic overlay) with the immunoblotting technique gave no indication for the existence of a *PLG*Q0* allele in the samples tested.
The numbers of the phenotypes observed show no significant difference to the figures expected under Hardy-Weinberg equilibrium (X^2 = 4.82, 3 df, p = 0.19).
Furthermore, the allele frequencies are in good agreement with data published for other Caucasoid populations (see e.g. Weidinger et al. 1985).
The chance of paternity exclusion in cases of disputed parentage by using the PLG polymorphism is 18.3%.

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POLYMORPHISM OF PLASMINOGEN IN WEST GERMANY

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AIMS OF THE STUDY

- definition of the plasminogen (PLG) phenotypes in a population sample of West Germany,
- population genetics of PLG,
- use of PLG in paternity testing.

MATERIALS AND METHODS

- blood samples: from 510 healthy, unrelated inhabitants of Aachen (West Germany),

- PLG typing: method according to Skoda et al. (1986), isoelectrofocusing in polyacrylamide gel pH 3.5 - 9.5 (LKB); detection of the gene products by using a caseinolytic overlay and by Western blotting (rabbit anti-PLG and peroxydase-labelled pig anti-rabbit-immunoglobulin-serum, Dakopatts).

RESULTS

The technique used for PLG typing is a fast and simple method giving reliable results.
The products of the following alleles could be defined: *PLG*A*, *PLG*B*, *PLG*A1*, PLG*A2, *PLG*A3* and *PLG*M2*.
The phenotype and allele frequencies are given in the Table.

Table PLG phenotype and allele frequencies in West Germany

PLG penotypes		PLG allele frequencies	
A	245 (48%)	*PLG*A*	0.6823
A B	196 (38%)	*PLG*B*	0.3059
B	57 (11%)	*PLG*A1*	0.0010
A1 A	1 (<1%)	*PLG*A2*	0.0010
A3 A	6 (1%)	*PLG*A3*	0.0069
A M2	3 (<1%)	*PLG*M*	0.0029
A2 B	1 (<1%)	Σ	1.0000
A3 B	1 (<1%)		
Σ	510		

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associated with the Tf C2 gene was provided by Auconi et al. (1982) who also observed a significant increase in the C2 frequency among extremely premature infants. Explanations for these observations have generally involved also the possible protective effects of certain Tf alleles against microbial infection or differences in the fetal growth-promoting effects of transferrin. Recently a special function of transferrin as a general growth factor in mammalian cells and particularly in the regulation of proliferation of cells of the immune system were recognized. Bryan and Leech (1983) have demonstrated the immunregulatory effects of Tf. Lum et al. (1986) suggest that Tf, synthesized by inducer T lymphocytes, acting in concert with the Tf receptor system, represents an autocrine pathway of potential importance in embryonic development and in immunregulation.
The further definition of the role of the Tf-Tf receptor system in the regulation and stimulation of the immunological system and in cellular proliferation and embryonic development generally should stimulate investigation into the biological function of transferrin genetic variation.

Table II Distribution of transferrin C subtypes in women with and without spontaneous abortion

	our study				Beckman et al. 1980			
	women with previous abortion		Controls Weidinger 1982		women with previous abortion		Controls	
Tf	N	%	N	%	N	%	N	%
C1	46	61.33	330	73.33	88	61.1	248	71.5
C1-2	24	32.00	101	22.44	45	31.3	88	25.4
C2	5	6.67	11	2.45	11	7.6	11	3.2
C1B1			1	0.22				
C1B1-2			2	0.45				
C1B2			4	0.89				
C1D1			1	0.22				
	N=75		N=450		N=144		N=347	
RR for C1 =0.54	TfC1=0.7733		TfC1 =0.8544		TfC1=0.7670		TfC1=0.841	
RR for C2-1=1.59	TfC2=0.2267		TfC2 =0.1367		TfC2=0.2330		TfC2=0.159	
RR for C2 =2.80			TfB1 =0.0011					
			TfB2 =0.0045					
			TfB2-1=0.0022					
			TfD1 =0.0011					

Differences between women with previous abortion and controls were high significant. $\chi^2 = 7.47$, df=1, $0.01 > p > 0.001$

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Table I Distribution of transferrin allele frequencies in different populations

Population	N	Tf allele frequencies C1	C2	C3	C4	D1	Dchi	others	
EUROPE									
Southern Germany	1108	0.79	0.13	0.07	-	-	-	<0.01	Weidinger et al 1984
Northern Germany	869	0.84	0.16	-	-	-	-	-	Jansen et al 1981
Hessen	876	0.77	0.16	0.06	-	-	-	<0.01	Kühnl et al 1981
Sweden Umeá	1084	0.82	0.16	-	-	-	-	<0.01	Beckmann et al 1980
Italy Rome	1352	0.76	0.18	0.05	-	-	-	<0.01	Pascali & Auconi 1983
NORTH AMERICA									
USA Whites	947	0.77	0.16	0.05	-	-	-	0.01	Dykes et al 1982
USA Blacks	386	0.84	0.12	<0.01	-	0.03	-	-	Dykes et al 1981
Amer-indians Apache	140	0.81	0.01	-	0.18	-	-	-	Dykes et al 1982
AFRICA									
Black Nigeria	131	0.86	0.05	-	-	0.08	-	-	Beckmann et al 1980
Pygmy Bi-Aka	337	0.82	0.03	-	-	0.15	-	-	Constans et al 1981
South Africa	426	0.90	0.09	0.01	-	-	-	-	Walter et al 1983
Mocam-bique	82	0.82	0.10	-	-	0.07	-	-	Rodewald et al 1988
ASIA-INDIA									
North I. Vaish	62	0.55	0.34	0.11	-	-	-	-	Kamboh 1984
South I. Konda Kammara	118	0.65	0.25	0.01	-	-	0.09	-	Walter et al 1981
N.W.I. Kotwalia	92	0.85	0.15	-	-	-	-	-	Walter et al 1983
Indonesia Bali	290	0.50	0.34	-	-	-	0.01	0.14	Brequet in Kamboh and Ferell 1987
Filip-pines	107	0.81	0.16	-	-	-	0.03	-	Walter et al 1983
China	118	0.74	0.24	-	-	-	0.01	-	Kamboh and Kirk 1983
AUSTRALIA									
Whites Adelaide	400	0.82	0.15	0.03	-	-	-	0.01	Nicholls & Mulley 1982
Aborigines Central	366	0.90	0.03	-	0.01	0.05	-	0.01	Kamboh and Kirk 1983
Polynesia Samoa	227	0.90	0.09	0.01	-	-	-	<0.01	Kamboh and Kirk 1983
Micro-nesia	411	0.95	0.04	<0.01	-	-	-	-	Kamboh and Kirk 1983
Papua New Guinea	196	0.88	0.08	-	-	0.04	-	-	Kamboh and Kirk 1983

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Beckmann 1985); they proposed a hypothesis that the Tf C2 gene confers an increased risk for cellular damage by hydroxyl radicals.
A review of the literature shows that Tf is one of the most intensively studied systems by IEF and data are available from more than 125 populations from quite diverse geographical areas (Beckmann et al. 1980; Kamboh and Ferrell 1987; Rodewald et al. 1988).
The purpose of our study was to re-examine the relationship between Tf subtypes and spontaneous abortion. In addition to the Tf C alleles there are some other Tf alleles whose distribution is of significance in anthopologigal genetics and therefore we will discuss them too.

MATERIAL AND METHODS

Transferrin subtypes were studied in 75 women from Western Germany with a history of previous abortion. The serum samples were desialylated and subjected to IEF in the pH-range of 4-6.5 (Kühnl and Spielmann 1978).

RESULTS AND DISCUSSION

The distribution of Tf allele frequencies in different populations are given in Table 1. The two common alleles Tf C1 and Tf C2 are present in all populations studied. Generally, the Tf C1 is higher in Blacks, Micronesians, Polynesians, Melanesians and Australian Aborigines, ranging from 80% to 95%, the maximum being found in Micronesian groups. Europeans and American whites have similar frequencies and their distribution falls in the range of 75-78%, but is slightly higher in Australian whites (82%) and in Northern Germany (84%). The minimum value in South Asian populations have been recorded of Bali (50%).
The distribution of the Tf C2 allele frequencies is somewhat reciprocal to that of the Tf C1 allele. The lowest values of Tf C2 have been found in some Amerindian groups (1%), Pygmies in Africa (3%) and Micronesians and Melanesians (4-5%). In European populations the range of Tf C2 frequencies is 13-19%, and in Asian populations the range is extended to 15-34% (Table 1). The maximum values (34%) have been reported in one Indian tribal population and in two genetically isolated groups from Indonesia, and these high C2 frequencies are probably due to genetic drift or founder effects.
Tf C3 appears to be very usefull in population-genetic studies. It is present in 3-7% of European, American and Australian whites and with a slightly lower frequency in 1-4% of Asiatic populations. The discovery of the Tf C4 allele at higher frequencies in New World Amerindian populations clearly demonstrated that this is a marker of these indigenous groups. Most of the remaining Tf C alleles , C5 - C13, are rare and show restricted ethnic or geographic distribution.
The two most frequent variants of Tf D allele were D1 (in Africans, range to 3-15% and in Aborigines 5%) and D chi (in Chinese, Filippinos, and South Indian populations), as a marker of Mongoloid populations.
Table 2 shows the transferrin C subtypes of women with previous abortions and the controls. Among these women significantly increased frequency of the C2 allele and especially of the C2 phenotype was found. There was also a significant departure from the expected Hardy-Weinberg equilibrium in the women as indicated by chi square value. The results of this study were in agreement with previous observations of the study of Beckmann et al. (1980). There appears to exist a selection mainly against fetuses with the C2 phenotype. It is obvious that the mechanism behind a selection mainly against the C2 type can be due to an immunological incompatibility between mother and fetus. Evidence in support of this possible selective mechanism

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Transferrin C subtypes in different ethnic groups and spontaneous abortion

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INTRODUCTION

Schade and Caroline discovered transferrin (Tf), one of the most important members of the iron-binding class of proteins of human plasma, in 1946 (Schade and Caroline 1946). Other members of this class include lactoferrin and ovotransferrin or conalbumin. Tf, sometimes also referred to as sirophilin or serotransferrin is part of the ß-globulin fraction of serum proteins. Tf is synthesized predominantly in the liver and its vital function is to tansport iron from the intestinal sites from cells in which the metal is absorbed, stored, or recovered from senescent red cells, to the cells in which iron-contained molecules are synthesized, such as the proerythroblasts of the bone marrow (Cleve et al. 1988). Transferrin has two binding sites for iron, with slightly different physical and chemical properties. Normally, serum Tf is only one-third satured with iron. Under these conditions it may protect against infections by a variety of microbial species by successfull competing for ferric iron essential for their growth: the free iron-binding sites compete with the siderophores of invading bacteria and fungi (Weinberg 1974). In addition to its function in nutrient binding and transport, Tf has also been suggested to act as a circulation growth factor in proliferation during embryogenesis (Ekblom et al. 1983). Genetically determined electrophoretic variation in the Tf present in human serum was first reported by Smithies (1957) using stach gel electrophoresis for separation. The common Tf type was designated Tf C, and 9 anodal variants and 12 cathodal D variants had been distinguished by 1975 (for review see Walter 1975). Until 1978, Tf C was considered to be a single variant. However, using isoelectric focussing, (IEF) Kühnl; Spielmann and Thymann (1978) independently described two common subtypes, Tf C1 and Tf C2. One year later Kühnl and Spielmann (1979) described another common C variant among Europeans, Tf C3. Family studies indicate an autosomal codominant inheritance of these alleles and recently the Tf locus has been assigned to human chromosome 3q21-q26 (Yang et al. 1984). Subsequently, IEF has revealed some additional heterogenety in Tf C and the total number of C variants has now reached 16 (Kamboh and Ferrell 1987; Kornhuber and Kühnl 1986). Nothing is known so far about the selective forces which may maintain the C1-C2-Cx polymorphism. With the discovery of the Tf C subtypes this problem attracted renewed interest, and various interesting observations were made: Beckmann et al. (1980) observed an increased frequency of the Tf C2 subtype in mothers with spontaneous abortion; Auconi et al. (1982) found a significant increase of the Tf C2 allele among premature newborn infants born before the 32nd week. Beckmann et al. (1985) reported the association of the Tf C2-1 and C2 phenotypes in factory workers afflicted with an occupational photodermatosis presumed to be due to toxic substances released unintentionally from a powder paint with epoxypolyester resign. The same group also reported a significant association of the C2 type with rheumatoid Arthritis (Dahlqvist and

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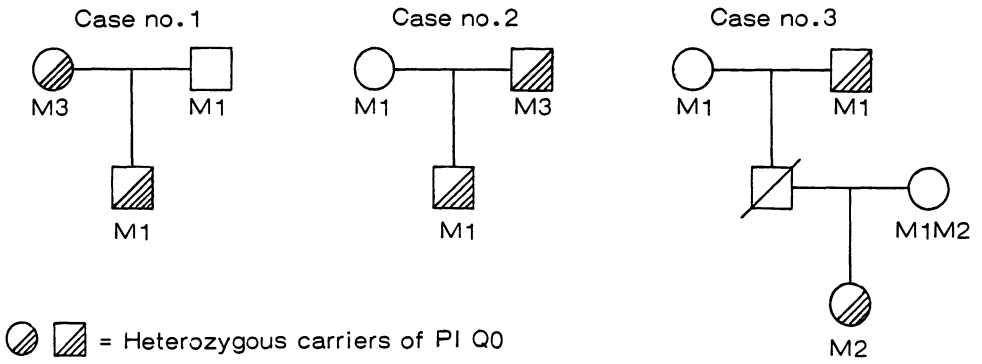


Fig. 2. Transmission of the PI*Q0 allele in three cases of disputed paternity

an incompatibility was observed between the child (PI M1) and the alleged father (PI M3). The man could not be excluded as the father of this child in 26 other genetic systems. Biostatistical evaluation of the combined data yielded a paternity probability of W>99.99%. In case no. 3 the alleged father was deceased. The grandparents were classified as PI M1, the mother PI M1M2, and the child PI M2. Family study has shown no inconcistencies in other systems. In all three cases the existence of a PI null allele was assumed. The band patterns of heterozygous PI Q0 carriers were clearly reduced and the α1AT-concentrations in sera were only approximately 50% of the normal range.

At present the three PI Q0 cases will be investigated at the DNA level. Former molecular studies have shown that α1AT genes were present in PI Q0 carriers but without or with faulty production of mRNA transcripts (Garver et al. 1986; Nukiwa et al. 1987). A possible explanation for faulty α1AT gene transcription could be the presence of a single mutation, deletion, addition, or rearrangement of DNA in the exons as well as introns of the gene.

We indicate that a null allele should be considered in cases of paternity with high probabilities if an exclusion is found only in the PI system. The haplotypic frequency of PI*Q0 in European populations is estimated to 0.1-0.2%, which is approximately one-tenth of the deficient PI*Z allele frequency. The PI Q0 heterozygotes of our paternity cases are apparently healthy, but individuals with certain deficient haplotypes such as PI Z or PI Q0 have a high risk for development of emphysema (Talamo et al. 1973; Muensch et al. 1986).

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MATERIALS and METHODS

Serum samples were obtained from apparently healthy individuals for paternity testing. For PI typing the samples were used without previous treatment. Isoelectric focusing (IEF) with pharmalytes pH 4.2-4.9 was carried out in 0.5 mm thin flat bed polyacrylamide gels (dimension 250 x 115 mm) which were fixed on silanized glass plates (Weidinger et al. 1985). In some cases PI M subtypes and PI variants were classified by hybrid-IEF with a very narrow immobilized pH gradient (Weidinger and Cleve 1986). Alpha$_1$-antitrypsin concentration in sera was determined by single radial immunodiffusion using NOR-Partigen plates from Behringwerke, Marburg.

RESULTS and DISCUSSION

Figure 1 presents the band patterns of several PI phenotypes including the PI M subtypes of individuals involved in paternity cases no.2 and 3. In case no. 1 an apparent maternal exclusion and in case no. 2 an apparent paternal exclusion were found in the course of paternity examinations (Fig. 2). The phenotype constellation of case no. 1 was: child PI M1, mother PI M3 and father PI M1. The inverse homozygosity between the mother and her son would mean an exclusion of maternity, but there was no evidence for an exchange of blood samples and any other exceptions in the study of 26 red cell antigens, serum proteins and red cell enzyme systems. In case no. 2

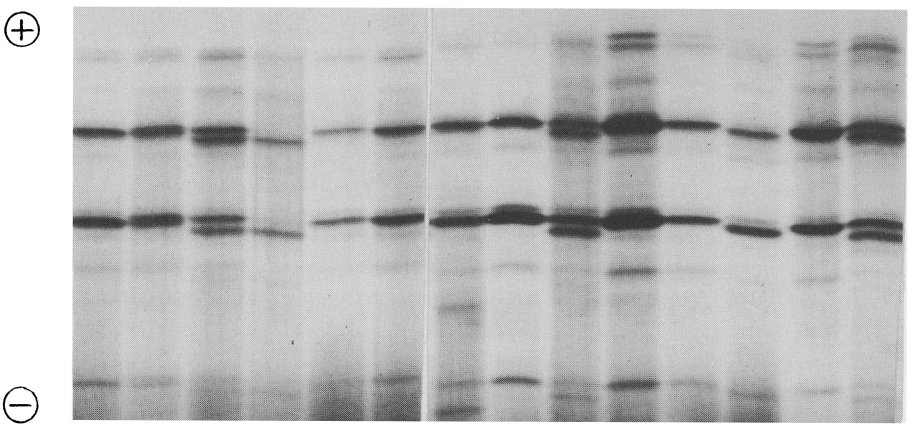


Fig. 1. Band patterns of different PI types obtained by isoelectric focusing in polyacrylamide gel, pH range of 4.2-4.9. The phenotypes were: (1) M3, (2) M1M3, (3) M1M2, (4) M2-Q0, (5) M1-Q0, (6) M1, (7) M1 Z, (8) M1, (9) M1M2, (10) M1, (11) M1-Q0, (12) M3-Q0, (13) M1, and (14) M1M2. Lanes 3,4,5, and 6 are samples from the mother, child, grandfather and grandmother of case no. 3. Lanes 10, 11, and 12 are samples from the mother, child and alleged father of case no. 2

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PI M Subtyping: Indication for an Alpha$_1$-Antitrypsin Null Allele in three Paternity Cases

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INTRODUCTION

Alpha$_1$-antitrypsin (α1AT), or α_1-protease inhibitor (PI), is a 52-kDa glycoprotein in human plasma with an unusual microheterogeneity. Its major physiological function appears to be the inhibition of neutrophil elastase (Travis and Salvesen 1983). Genetic variation in the PI system was first described by Laurell and Eriksson (1963), and Fagerhol and Braend (1965). The technique of isoelectric focusing (IEF) has permitted a division of the most common type PI M into the polymorphic subtypes M1, M2 (Constans and Viau 1975; Frants and Eriksson 1976), M3 (Klasen et al. 1977; Genz et al. 1977), and M4 (Constans et al. 1980; Weidinger et al. 1982). More recently, three further PI M variant types, tentatively named M5, M6 and M7 were revealed by IEF with immobilized pH gradients (Weidinger et al. 1985; Görg et al. 1985; Weidinger and Cleve 1986). Sequence analysis of the protein coding region has permitted the distinction of two M1 haplotypes (M1 Ala^{213} and M1 Val^{213}) in the PI*M1 gene (Nukiwa et al. 1987). Hitherto approximately 75 alleles were identified at the protein and/or gene level (Fagerhol and Cox 1981; Brantly et al. 1988). The PI locus has been assigned to chromosome 14q31-32.3 (Long et al. 1984).

The most common deficient allele in the PI system is PI*Z which has a frequency of 1-2% in most European populations. The molecular basis of the deficient haplotype PI Z has been identified at the protein and gene level (Kidd et al. 1983; Cox et al. 1985). In contrast to PI Z only little is known about the rare deficient PI null state. Existence of PI*Q0 was first described in a proband who had severe emphysema with almost complete absence of α1AT in serum (Talamo et al. 1973). Heterozygous carriers cannot be detected by subtyping if there is not an apparent isolated maternal or paternal exclusion constellation in the PI system which is not confirmed by the other marker systems.

In this paper we report indication for a silent PI allele in three different cases of disputed paternity.

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Table 1

Phenotype	Obs.	Exp.
M1	1,018	1,022.154
M2M1	407	426.563
M2	53	44.503
M3M1	403	381.694
M3M2	78	79.644
M3	28	35.633
M1S	143	142.290
M2S	33	29.690
M3S	26	26.567
SZ	4	3.893
FS	1	1.241
SS	3	4.952
M1Z	57	55.933
M2Z	14	11.671
M3Z	9	10.443
FZ	0	0.488
ZZ	0	0.765
FM1	23	17.825
FM2	2	3.719
FM3	1	3.328
FF	0	0.078
M1Mvar	4	4.610
M2Mvar	1	0.962
M3Mvar	1	0.861
MvarS	1	0.321
MvarZ	0	0.126
FMvar	0	0.040
MvarMvar	0	0.005
TOTAL	2,310	2,309.999

Gene Frequencies

M1	=	0.6652
M2	=	0.1388
M3	=	0.1242
S	=	0.0463
Z	=	0.0183
F	=	0.0058
Mvar	=	0.0014

DISCUSSION

Figure 1 shows the focusing patterns of common and rare Pi phenotypes.

The clarity of resolution compared to other IEF methods is due largely to the thorough reduction/alkylation of the Pi molecule. Incorporation of ACES into the gel mixture flattens the pH gradient, particularly in the M8 region where Pi M subtypes are most readily distinguished. This also helps keep the middle of the gel clear of proteins with similar isoelectric points such as haptoglobin and group specific component.

Table 1 summarizes the population analysis of over 2,000 unrelated caucasians received at the London Hospital Medical College in the course of paternity investigations. In such cases no distinction is made between Pi M3 and M4 subtypes.

This method demonstrates that a simple, rapid procedure can produce high resolution focusing.

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Serum Pretreatment

30ul serum
10ul 7.5mg/ml dithiothreitol (Sigma)

20 minutes at room temperature, followed by

10ul 9mg/ml iodoacetic acid (BDH)

30 minutes at room temperature (during prefocus)

IEF Details

Samples inserted on 5 x 5mm^2 Whatman No. 1 filter paper, 1cm from cathode edge.

Whatman No. 17 electrode strips soaked in 1M sodium hydroxide (cathode), 1M orthophosphoric acid (anode). Gels run at 10^{o}C.

Stepwise voltages applied as follows:

550 volts	40 minutes
1,100 volts	60 minutes
2,000 volts	30 minutes
2,500 volts	15 minutes
3,000 volts	5 minutes

Visualization

Gel fixed for 5 minutes with 12.5% trichloroacetic acid, then washed briefly in tap water.

Gel stained with 0.04% Serva Blue W (Serva) at 50^{o}C for 10 minutes and destained thoroughly under running tap water.

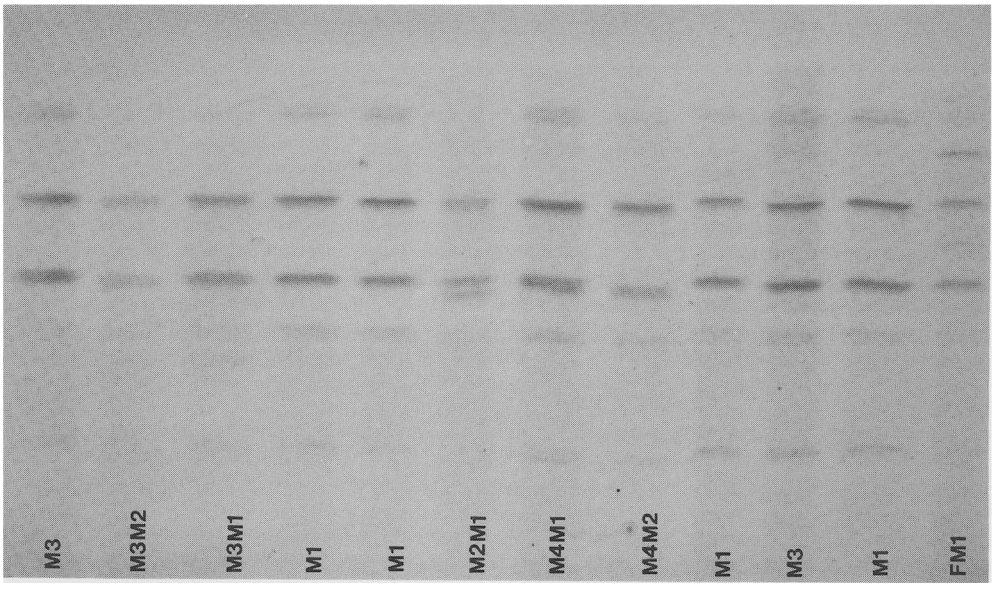


Figure 1

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A Rapid High Resolution Seperator IEF Technique for Phenotyping Alpha-1 Antitrypsin

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INTRODUCTION

1 antitrypsin (Pi) ranks amongst the most informative of the Polymorphic serum proteins used in forensic science (exclusion rate 31% in parentage testing, discriminatory index 74% in criminal investigations). While complete Pi phenotyping usually requires the use of intricate immobilized pH gradients, this presentation outlines a straightforward, rapid, high-resolution separator isoelectric focusing (IEF) technique. Focused bands are often sharp enough to distinguish Pi M subtypes with similar mobilities such as M3 and M4.

With the incorporation of ACES into an ultrathin gel to produce a pH 4.2 - 4.9 gradient flattened in the middle, and by pretreating serum samples with both a reducing and an alkylating agent, Pi M 1, 2 and 3 are fully resolved. The Pi bands are differentiated clearly in the middle of the gel allowing confident phenotyping of the reduced concentration alleles Pi Z and Pi S, even though a general protein stain is used to visualize the bands.

MATERIALS AND METHODS

Gel Composition

The following mixture produces 6 gels with a polyacrylamide matrix of T 5.3%, C 3.8%.

30ml acrylamide premix (26.7g acrylamide, 40.0g Acrylogel (BDH) in 1L H_2O)
5.2ml glycerol (BDH)
2.5ml Pharmalyte 4.2 - 4.9 (Pharmacia)
60mg ACES* (Sigma)
0.3ml 10mg% riboflavin (Sigma)

38ml

Gels measure 230 x 115 x 0.25mm^3 using one layer of Dymo tape to set gel thickness.

* ACES (N-[2-Acetamidol]-2-aminoethanesulfonic acid)

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Family 2 - single indirect EsD exclusion

Father 1	Paternity index on 21 other polymorphisms
Mother 2.1	including HLA = 20,000 times
Child 2	(relative chance of paternity 99.995%)

The isozyme pattern of the dilution series obtained after IEF is shown in Figure 2. Visual assessment of the relative band strengths was as follows:

	1/2	1/4	1/8	1/16	1/24	1/36
Homozygous 1 control	++++	+++	++/+++	++	+/++	+
Sample of father	+++	++	+/++	+	+	(+)
Homozygous 2 control	++++	+++	++	+	(+)	(+)
Sample of child	+++	++	+	(+)	-	-

DISCUSSION

The example results from two single exclusion families demonstrate that this method is efficient at showing reduced enzyme activity in PGM and EsD. Enzyme activity is visibly lower at each stage of the dilution series in the family samples compared to the nOrmal activity homozygous controls. Using IEF to focus the polymorphic enzymes before assessing relative activity ensures the non-polymorphic enzymes are seperated out and do not obscure the true activity levels of interest. In laboratories lacking the resources for enzyme assay or scanning densitometry this method could provide the means to objectively assess reduced activity in any of the routinely used enzyme grouping systems.

Single-exclusion families occur with a regular although very low frequency; the average incidence of silent alleles in enzyme systems is estimated to be 0.001 (Polesky et.al. 1983). PGM and EsD are both close to this general figure with an incidence of 0.001 and 0.0007 respectively. Where it is not possible to investigate a single indirect exclusion with an enzyme assay the method outlined here is a straightforward but effective approach for highlighting the presence of null alleles or activity variants.

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RESULTS

Two widely used polymorphic enzyme systems which, when assayed, are masked by non-polymorphic enzyme activity are phosphoglucomutase (PGM) and esterase D (EsD). Examples are given of the dilution method for both of these grouping systems.

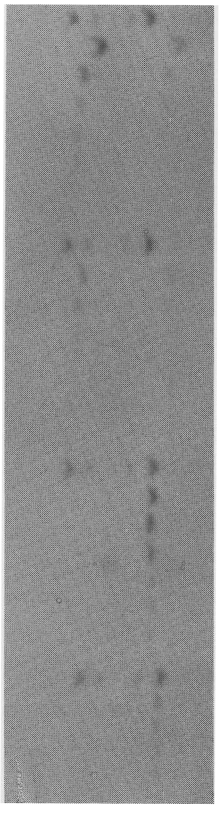
Family 1 - single indirect PGM exclusion

Father 2+
Mother 1+1-
Child 1+

Paternity index of 19 other polymorphisms including HLA = 5,000 times (relative chance of paternity 99.98%)

The isozyme pattern of the dilution series obtained after IEF is shown in Figure 1. Visual assessment of the relative band strengths was as follows:

	1/2	1/4	1/8	1/16	1/24	1/36	1/60
Homozygous 2+ control	++++	+++	++	+	(+)	-	-
Sample of father	+/++	+	(+)/+	(+)	-	-	-
Homozygous 1+ control	++++	+++	+/++	+	(+)	-	-
Sample of child	++	+	(+)	(+)	-	-	-



control
1-2
1-4
1-8
1-16
1-24
1-36
1-60
control
1-2
1-4
1-8
1-16
1-24
1-36
1-60
control
1-2
1-4
1-8
1-16
1-24
1-36
1-60
control
1-2
1-4
1-8
1-16
1-24

PGM 1+ control

1+ child

PGM 2+ control

2+ father

◄ Figure 1

EsD 2 control 1/2
1/4
1/8
1/16
1/24
1/36
Child 1/2
1/4
1/8
1/16
1/24
1/36
EsD 1 control 1/2
1/4
1/8
1/16
1/24
1/36
Father 1/2
1/4
1/8
1/16
1/24
1/36



Figure 2 ►

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A Simple Dilution Technique for Assessing Red Cell Enzyme Activity in Cases of Single System Incompatibilities

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INTRODUCTION

Laboratories using a large battery of blood group polymorphisms for paternity investigations will find a very small proportion of families give a single indirect exclusion which is inconsistent with the results obtained in the other grouping systems; sometimes these even appear to exclude the mother. It is important in such cases to distinguish between true exclusions and the presence of null alleles or activity variants which could provide additional evidence of parentage.

Single indirect exclusions occuring in red cell enzyme polymorphisms can be analysed by scanning the electrophoresis or isoelectric focusing (IEF) gel with a densitometer, to produce objective values for the relative band strength in each sample. Alternatively, enzyme activity can be assayed. Unfortunately certain red cell enzymes share activity with a wide range of non-polymorphic enzymes so that the reduced/altered-activity is masked and becomes undetectable.

This presentation details a very simple dilution technique which, when applied with IEF, shows reduced enzyme activity not detectable by enzyme assay.

MATERIALS AND METHODS

Samples of 3x saline washed, packed cells from each of the family members and the appropriate control homozygotes were diluted with distilled water to the following concentrations:

1/2, 1/4, 1/8, 1/16, 1/24, 1/36, 1/60

Standard IEF techniques were used to phenotype the samples (Divall & Ismail 1983, Divall 1984). The strength of the primary isozyme band of each sample in the dilution series was then visually assessed and scored on a ++++ to (+) scale. The dilution concentrations used give the optimum contrast in band strength between normal and reduced activity phenotypes following IEF.

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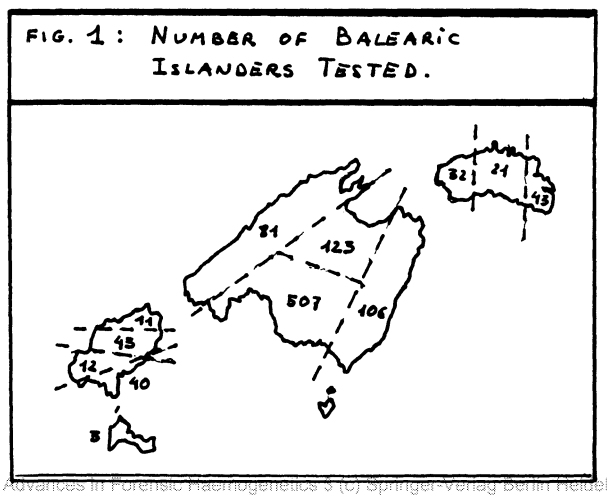
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TABLE 3: Statistical analyses of differences in Hp, GLO and Gc gene frequencies between the Balearic Islands and several Mediterranean populations.

Communities compared	Hp X^2 (2 d.f.)	P
Balearic vs.Sardinia	.1577	NS
Balearic vs.Sicily	2.8105	p<0.1
Balearic vs.Morocco	1.5695	NS
Balearic vs.Barcelona	10.6179	p<0.005
Balearic vs.Greece	9.9333	p<0.005
Balearic vs.Adriatic coast	.2908	NS
	GLO X^2 (2 d.f.)	P
Balearic vs.North Africa	4.1984	NS
Balearic vs.Turkey	.1213	NS
Balearic vs.Bulgary(Balcans)	7.1751	p<0.005
Balearic vs.Israel(ashkenazi)	13.1632	p<0.001
Balearic vs.Italy	47.4656	p<0.001
Balearic vs.Barcelona	93.2035	p<0.001
	Gc X^2 (5 d.f.)	P
Balearic vs.Albanian	9.6603	NS
Balearic vs.Serbia-Croatia	.7933	NS
Balearic vs.Bulgary	41.0834	p< 0.001
Balearic vs.North Africa	167.2431	p< 0.001
Balearic vs.Greece	192.8765	p< 0.001
Balearic vs.Barcelona	33.8536	p< 0.001



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Gc	1F-1F	30	2.94	28.55	2.8
Gc	1S-1S	309	30.35	308.6	30.31
Gc	2-2	81	7.95	80.91	7.94
Gc	2-1F	95	9.33	96.13	9.44
Gc	2-1S	317	31.13	316.03	31.04
Gc	1F-1S	186	18.27	187.74	18.44
TOTAL		1018		1017.96	

Hp 1*	.4151	Hp 2*	.5848	χ^2 .0105 (1 df) p >.70
GLO 1*	.3379	GLO 2*	.5848	χ^2 .0086 (1 df) p >.90

Gc 1F*	.1674	Gc 1S*	.5505	Gc 2*	.2819	χ^2 .10664 (3 df)p >.99

TABLE 2 : Distribution of Hp^1, GLO^1, Gc^{1F} and Gc^2 gene frequencies in several Mediterranean populations.

Population	Number tested	Hp^1	Reference
Italy (Adriatic coast)	119	.408	Ritter, 1980
Italy (Sardinia)	440	.401	Ritter, 1980
Italy (Sicily)	107	.397	Ritter, 1980
Greece	370	.45	Kaplanoglou, 1982
Greece (Corfu)	507	.342	Ritter, 1980
Morocco	123	.401	Moullec,1960
Spain (Barcelona)	317	.38	Moral, 1983
Balearic Islands	1014	.415	this study

		GLO^1	
Italy (Napoli)	606	.389	Ranzani, 1979
Bulgary (Balkan)	145	.31	Golan, 1979
North Africa	202	.316	Golan, 1979
Turkey	151	.334	Golan, 1979
Israel(ashkenazi)	191	.301	Golan, 1979
Spain (Barcelona)	625	.445	Huguet,1986
Balearic Islands	1012	.337	this study

Population	Number Tested	Gc^{1F}	Gc^2	Reference
Italy (Serbian)	34	.161	.279	Lindberg,1986
Italy (Albanian)	154	.185	.298	Lindberg,1986
Italy (Sardinia)	316	.187	.259	Petrucci,1986
Greece	300	.13	.20	Kouvatsi,1987
Bulgary	217	.138	.33	Nevo, 1987
North Africa	---	.20	.177	Nevo, 1987
Spain(Barcelona)	839	.131	.32	Gené, 1986
Balearic Islands	1018	.167	.281	this study

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(v/v). Plate dimensions were 200*115 cm. A mixture of Ampholine and Pharmalite pH 4-6, 4-6.5, 4.5-5.4 was used. Polymerization was carried out with ammonium persulfate 1% solution and TEMED.

IEF was performed in Pharmacia equipment (ECPS 3000/150,VH- 1). Focusing was carried out until 4.500 volthours, removing paper after 30 min, with the following maximal conditions: 1500V, 8W (prefocusing) and 5W (focusing), T +8ºC. Gc bands were read after simple precipitation with sulphosalicylic acid.

RESULTS AND DISCUSSION

Table 1 show the distribution of Hp, GLO and Gc phenotypes, respectively, among 1014, 1012 and 1018 auchtouchtonous islanders from Balearic islands. The gene frequencies are the following: Hp 1 .4151, Hp 2 .5848; GLO 1 .3379, GLO 2 .5848; Gc 1F .1674, Gc 1S .5505, Gc 2 .2819. There is a good agreement betwween the observed and the expected frequencies of phenotypes, assuming Hardy-Weinberg equilibrium.

Table 2 show the distribution of Hp, GLO, Gc and Gc gene frequencies in several Mediterranean populations. Contrary to what may be expected, the gene frequencies observed for Hp, GLO and Gc in the Balearic islands are very different to those from other Spanish populations (Barcelona). For Hp: Frequencies similar to those found in Sardinia, as well as in certain Italian populations on the Adriatic coast, and also in the coastal areas of Morocco. For GLO: Frequencies similar to those found in the coastal areas of Northern Africa, as well as in certain areas of Western Turkey. For Gc: Frequencies similar to those in Albanian and Serbo-Croatian populations of Italy.

Table 3 show the statistical analyses (χ^2) of differences in Hp, GLO and Gc gene frequencies between the Balearic islands and several Mediterranean populations.

TABLE 1: Hp, GLO and GC distribution in Balearic Islands

Phenotypes		Observed		Expected	
		n	%	n	%
Hp	1-1	174	17.15	174.79	17.23
Hp	2-1	494	48.71	492.41	48.56
Hp	2-2	346	34.12	346.79	34.2
TOTAL		1014		1013.99	
GLO	1-1	115	11.36	115.57	11.42
GLO	2-1	454	44.86	452.84	44.74
GLO	2-2	443	43.77	443.57	43.83
TOTAL		1012		1011.99	

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Hp, GLO and Gc Polymorphisms in the Balearic Islands (Spain): The Mediterranean Blueprint

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INTRODUCTION

Anthropological and historical studies about the Balearic Islands show us that the origin do not come from the Iberian Peninsula,the origin is in the oriental civilization. The Balearic Islands was the point from the comercial routes extends to Europe; in the same way as the others islands in the West Mediterranean. The isolate of the population in the islands, his cultural independence, the high relations among the population even the middle of this century, and his particular historic evolution make interesting to study the hemogenetic origin of the population.

The Balearic Islands are formed by 194 islands. The main are:Mallorca (534.512 p.), Menorca (57.243 p.), Ibiza (59.993 p.) and Formentera (4.222 p.).There are a high density of people near the main city in each island, and the rest are diferent because there are not many peopple;in the main city of the Islands, Palma de Mallorca, are 290.372 p.

The distribution of Hp, Glo and Gc Polymorphisms in the population wich are shown in this work is important as the known of the genetic balearic population as for forensic medicine (paternity testing).

MATERIALS AND METHODS

Fresh blood samples were obtained from 1022 healthy donors who were clasified according to their ancestral origin as pure Balearic islanders(3rd. previous generation). Individual characteristics to have autochthonous islanders were assigned to the different geographical areas selected in the map (Fig.1). After arrival at the laboratory, the serum was separated from blood cells by centrifugation and stored at - 40ºC until tested. The phenotypes Hp, GLO and Gc were determined twice or more.

Hp phenotypes were determined in polycrylamide gels electrophoresis according to Raimond (1964), with a staining technique according to Compton (1976).

GLO phenotypes were determined in agarose-starch gel electrophoresis according Wetterling (1981), with a staining technique according to Parr (1977) with minor modifications.

Gc subtypes were carried out 0.4 mm polyacrylamide gels (PAGIF), T=6.2%, C=3.2% and an ampholine concentration of 2%

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Inheritance of Some Electrophoretic Phenotypes of Human Hair

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Materials and Methods

The method used was a one-dimensional SDS electrophoresis of low sulfur proteins in the presence of urea which was developed by Gerhard (1987). Hairs of 5 families with 3 or 4 generations and 6 to 56 members were examined. Hair fragments were extracted in a solution of 50 mM DTT, 8 M urea, and 2 % w/v SDS. Polypeptids were stained with CBB-R 250.

Results

Four electrophoretic phenotypes could be distinguished. They were named K1, K1m, K3 and K3m. The distribution of these phenotypes is listed in table 1.

Discussion

We think that the four phenotypes observed are inherited in an autosomal dominant-recessive way, and that they are controlled by two independent loci *K* and *m*. Data from our random sample do not contradict to this hypothesis. The pedigrees indicate that the genotype of K1 is $*K^1/*K^1$, whereas the genotype of K3 is $*K^3/*K^1$ in this series. The genotype of non-m is *non-m/*non-m and the genotype of m is *m/*non-m. K3 is dominant and K1 is recessive. The modifying gene *m is dominant, while *non-m can be called recessive. The statistical evaluation of observed and expected data supports our assumption concerning the mode of inheritance. Although the sample size was very small (n=33) and K3m occured only once in the entire random sampling, the X^2 of 0,072 indicates the goodness of fit of our model.

Table 1. Distribution of Phenotypes

	observed		expected	
Phenotype	n	%	n	%
K1	23	69.70	23.27	70.51
K1m	6	18.18	5.85	17.73
K3	3	9.09	3.10	9.40
K3m	1	3.03	0.78	2.36
Σ	33	100.00	33.00	100.00

$X^2 = 0.072$; 1 d.f.; $p = 0.794$

Reference

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of salivary amylase(Amy_1) and salivary acid phosphatase(s-Acp) observed were 0.011 for Amy_1 and 0.217 for $s\text{-}Acp^A$,respectively. The gene frequencies of parotid salivary polymorphisms observed were 0.214 for Pa^+, 1.000 for Pb^1, 0.753 for Db^+, 0.394 for PmF^+, 0.028 for Ph^+, 0.733 for PIF^+, respectively.The *Pst 1* and *Bam H1* restriction site polymorphism was studied. Distribution of allele frequencies were 0.487 for 5.7 Kpb fragment and 0.531 for 6.5 Kpb fragment. Comparison of gene frequencies of genetic markers in parotid saliva of Japanese with those of other racial groups. The frequency of Pa^+ allele in Japanese was higher than those in Negroes and lower than those of other Orientals. The Pb^2 and Db^+ alleles in Negroes were higher than those in Caucasians and Orientals. The PIF^+allele in Japanese was somewhat higher than that in Caucasians and the frequency of the PIF^+ allele in Negroes was much lowerthan that of the other racial groups. Furthermore, the PmF^+ allele in Orientals was higher than that of Caucasians. The frequency of the Ph^+ allele in Japanese was lower than that of Malays and Chinese, and somewhat higher than that of Indians. The frequency of the Amy_1^V allele in Japanese was similar to that in Caucasians, but much lower than that in Negroes. In the salivary protein and enzyme polymorphisms there were differences within the Japanese population, and the salivary polymorphisms appear to be a valuable personal identification and anthropological markers.

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Distribution of Twelve Human Salivary Polymorphisms in Japanese Population

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Introduction

This report presents the data on the gene frequencies of twelve polymorphic systems in human salivary protein [salivary acid protein(Pa), parotid basic proteins(Pb), proline-rich proteins (Pr), double-band proteins(Db), parotid middle-band protein (PmF), parotid isoelectric focusing variant(PIF),parotid heavy protein(Ph)] parotid and whole salivary enzymes [parotid salivary acid phosphatase(s-Acp), salivary amylase(Amy_1)] The blood groups of whole saliva [secretor character(se), Lewis type(Le^a)] and the RFLP(human amylase cDNA) were studied in Japanese population.

Experimental materials and methods

Examination of Se, Lewis and Amy_1 systems:Whole saliva was used. Examination of Pa, Pb, Pr, Db, PmF, Ph, PIF and s-Acp systems: Parotid saliva was used, samples were collected with double-chamber cup of Curby type. Se, Lewis and Amy_1 typings of whole saliva were performed according to the methods of Ikemoto and Merritt. Pa, Pb, Pr, Db, PmF, Ph, PIF and s-Acp typings of parotid saliva were performed according to the methods of Azen, Friedman, and Ikemoto. RFLP of human salivary α-amylase after cleavage with restriction endonucleases *Pst 1* and *Bam H1* was examined according to the methods of Ishizaka and Tsuchida.

Gene frequencies of Japanese population

The gene frequencies of Se and Lewis blood groups observed were 0.516 for *Se* and 0.455 for Le^a, respectively. The gene frequencies

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Conditions

Electrode solutions	+ 10 mM glutamic acid
	- 10 mM NaOH
Sample application	+ PGM, ACP
	- ESD, Pi, ORM, Tf

Focusing conditions: 7 W, 4 mA, 3500 V for 3 h
Staining: appropriate substrates for enzymes and silver staining for proteins

RESULTS AND DISCUSSION

Pi, Tf C, ORM and PGM phenotypes are much better distinguishable by HIEF than by IEF with CA since pH range can be considerably reduced .
Bands are sharper and straighter using HIEF particularly with contaminated bloodstains.
HIEF gave good results to the final extract solution of 1:64 for Pi and 1:32 for Tf, ORM, PGM, ACP and ESD .
For ACP and ESD, HIEF has serious handicaps: ACP typing by HIEF is expensive (five different Immobilines must be used), the ACP*A allele is not perfectly separated from Hb, and although the ESD*5 allele can be better separate with HIEF, the alleles ESD*1 and ESD*2 are not well distinguished .
HIEF is clearly the method of choice for the detection of Pi, Tf, ORM and PGM1 in bloodstains.
The resolution power of HIEF (and IPGs) is illustrated with the demonstration of a new PGM variant, which appeared to be slightly cathodal to PGM1 a8 and anodal to PGM1 a1, assuming as lineal the pH range of Dykes and Polesky (1981), but without repetitions in the cathodic side. Family studies confirms the codominant mode of inheritance of this variant.

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Formation of Immobilized pH gradients

Composition of the acidic and basic components of the gradient mixer for the separation of the ACP, ESD, PGM1, Pi, Tf and ORM phenotypes (see * for specific immobilines volumes)

Basic light	mL	Acidic dense	mL
Acrylamide (29.1%) + bis-acrylamide (0.9%)	1.25	Acrylamide + bis-acrylamide	1.25
Glycerol 87%	0.00	Glycerol	2.10
H2O	X	H2O	Y
Total volume	7.50	Total volume	7.50
TEMED (10 mL%)	0.01	TEMED	0.01
Ammonium persulfate (10 g%)	0.01	Ammonium perfulfate	0.01

* Volume (µl) 0.2 M Immobiline pK

Proteins pK	Acidic dense solution			Basic light solution		
	Pi	Tf	ORM	Pi	Tf	ORM
3.6	-	527.5	-	-	142	-
4.6	377	-	354	374	-	433
6.2	-	582.5	-	-	347	-
9.3	121.5	-	131.5	207	-	350

pH range: Pi 4.2-4.6, Tf 5.3-6.3, ORM 4.3-5.1

* Volume (µL) 0.2 M Immobiline pK

Enzymes pK	Acidic dense solution			Basic light solution		
	ACP	ESD	PGM1	ACP	ESD	PGM1
3.6	351	176	338	87.5	-	91
4.6	127	375	-	61.5	431.5	-
6.2	208	431.5	408.6	65.5	431.5	353.5
7.0	66.5	-	-	172.5	-	-
8.5	173	-	-	173	-	-
9.3	-	-	-	-	101	-

pH range: ACP 5-8, ESD 4.8-5.8, PGM1 5.6-6.6

HIEF gel casting

Polymerization: 15 min at room temperature + 1 h at 50 °C
Washing: 6 x 10 min with distilled water. Drying
Rehydration (mold method) more than 1 h

	ACP	ESD	PGM	Pi	Tf	ORM
Ampholine	4-6/6-8	4-6	5-7	3.5-5	5-7	4-6
%	1%	2%	1%	0.5%	0.5%	1%

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The Use of Hybrid Isoelectric Focusing for the Detection of Polymorphic Proteins and Enzymes in Bloodstains Analysis

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INTRODUCTION

Immobilized pH gradients (IPG) show a number of advantages over IEF with carrier ampholytes (CA). Its power of resolution is 10 times greater, any pH gradient can be created, sample of up to 10 times grater can be loaded and cathodic drift is eliminated (Righetti et al. 1988).
Nevertheless, previous experience with immobilized pH gradients gels has shown them to be insensitive compared to Ampholine gels (Altland et al. 1986). This is a great inconvenience for the detection of polymorphism in minute bloodstains.
Incorporating CA into IPG gels (HIEF) produces improvement of sensitivity because the CA improves the conductivity and prevents the precipitation of protein before separation can be achieved (Altland et al. 1987).
In this paper we report the advantages and disadvantages of HIEF for the analysis of certain proteins (Pi, Tf, ORM) and enzymes (PGM, ACP, ESD) in bloodstains.

MATERIAL AND METHODS

Samples: 30 µL bloodstains (2 weeks old) on cotton cloth.
Extraction: 30 µL 0,05 M DTT.
Pretreatment: Neuraminidase (1U/mL). 12 h. 4 °C for ORM
0.5 M ferrous ammonium sulphate. 12 h. 4 °C for Tf
Dilutions: Doubling dilutions of the bloodstain extracts we prepared to a final dilution of 1:64

HIEF: IPG gels were cast according to Bjellqvist et al. (4) on a GelBond PAG films.
Gel dimensions: 260 x 100 x 0.5 mm.

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CONCLUSIONS

- SIEF using miniaturized polyacrylamide gels as well as HIEF in small IPG matrices (focusing distance: 50-55 mm) have been shown as reliable and reproducible isofocusing methods with a resolution capacity similar to that achieved by IEF in large gels (focusing distance: 100-120 mm).

- The use of small gels is a fast and economic method that allows the simultaneous analysis of several polymorphic proteins because it is possible to run up to 4 different gels (45 x 60 x 0.25 - 0.5 mm) at the same time using only one flat bed apparatus.

- Immunoblotting, which has been shown as a specific and sensitive method, is recomended for routinary subtyping of GC, ORM and Tf.

- The distribution of phenotypes found in Madrid for the proteins analyzed in this study are in good agreement with the H-W equilibrium.

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Table 2. Distribution of GC phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
1S	116	114.14	GC * 1S = 0.5794
2-1S	112	109.49	GC * 2 = 0.2779
1S-1F	48	55.40	GC * 1F = 0.1397
2	25	26.25	GC * $1A_{VAR}$= 0.0029
2-1F	27	26.30	
1F	10	6.63	
$1s-1A_{VAR}$	2	1.14	
TOTAL	340	339.35	

$\Sigma\ I^2 = 3.51367$; df = 3; P > 0.30

Table 3. Distribution of PI phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
M1M1	114	120.61	PI * M1 = 0.5956
M1M2	73	63.14	PI * M2 = 0.1559
M1M3	48	45.28	PI * M3 = 0.1118
M1S	48	45.28	PI * M4 = 0.0132
M2M3	13	11.85	PI * S = 0.1118
M2	6	8.26	PI * Z = 0.0073
M2S	5	11.85	
M1M4	5	5.34	
M3S	7	8.49	
S	7	4.25	
M3	4	4.25	
M2Z	3	0.77	
M4	2	0.059	
M1Z	2	2.95	
$M1VAR_{anod}$	1	1.78	
$SVAR_{anod}$	2	0.3	
Others	0	5.42	
TOTAL	340	340	

$\Sigma\ I^2 = 7.28$; df = 4; P > 0.10. Phenotypes with n(exp) below 5 were combined for I^2 calculation.

Table 4. Distribution of Tf phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
C1	218	213.59	Tf * C1 = 0.7926
C1C2	78	86.39	Tf * C2 = 0.1603
C1C3	24	23.77	Tf * C3 = 0.0441
C2	13	8.74	Tf * C6 = 0.0029
C2C3	4	4.81	
C3	1	0.66	
C1C6	1	1.56	
C2C6	1	0.32	
C3C6	0	0.086	
C6	0	0.0028	
TOTAL	340	339.93	

$\Sigma\ I^2 = 3.0118$; df = 2; P > 0.2. Phenotypes with n(exp) below 5 were combined for I^2 calculation.

Table 5. Distribution of ORM-1 phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
F1S	157	146.46	ORM-1 * F1 =0.6206
F1	117	121.32	ORM-1 * F2 =0.0047
S	38	44.20	ORM-1 * S =0.3746
F2S	3	1.109	
F1F2	0	1.83	
F2	0	0.006	
TOTAL	315	314.92	

$\Sigma\ I^2 = 1.7829$; df = 1; P > 0.10. Phenotypes with n(exp) below 5 were combined for I^2 calculation.

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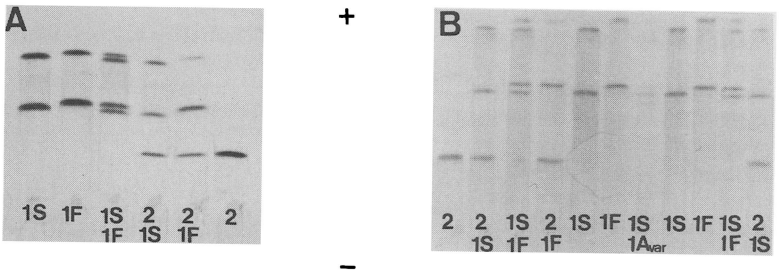


Fig. 1. GC phenotypes analyzed by A) SIEF and B) HIEF in miniaturized polyacrylamide gels.

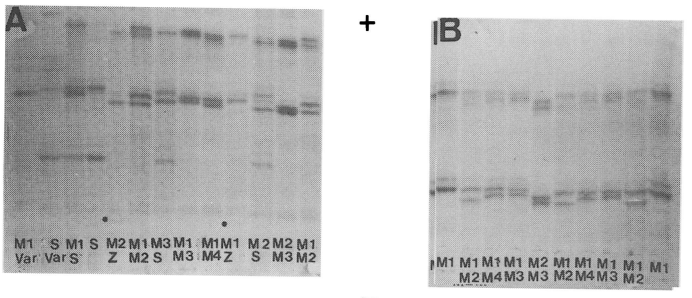


Fig. 2. Pl phenotypes analyzed by HIEF in miniaturized polyacrylamide gels: A) IPG=4.35-4.75 B) IPG=4.35-4.55.
p

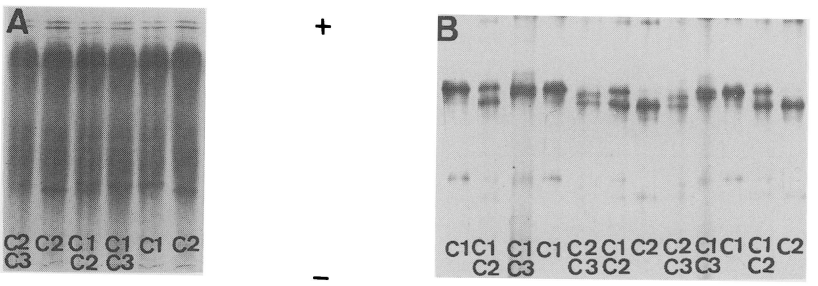


Fig 3. Tf phenotypes analyzed by A) SIEF and B) HIEF in miniaturized polyacrylamide gels.

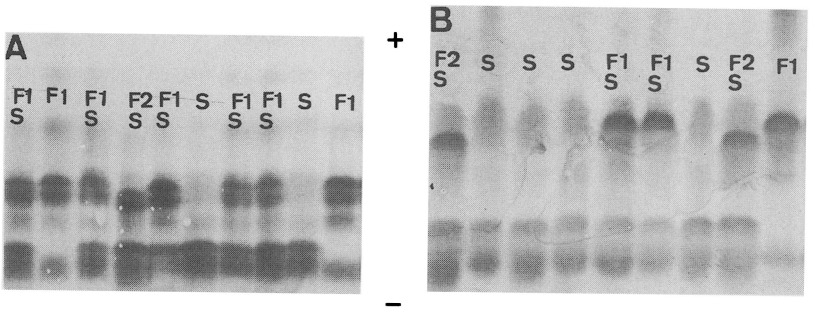


Fig.4. ORM-1 phenotypes analyzed by A) SIEF and B) HIEF in miniaturized polyacrylamide gels.

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TABLE 1

IEF PROCEDURES (1)

PROTEIN	SAMPLE TREATMENT	SIEF	HIEF	DETECTION METHODS (2)
GC	Plasma samples diluted 1:50 with 6 M urea	2% Pharmalyte 4.5-5.4 1.2 % HEPES, 0.6 % ACES	IPG: 4.8-5.2 Rehydration solution: 2% Pharmalyte 4.5-5.4 0.6 % HEPES,20 % sucrose	- Immunoblotting: * 1st Ab: rabbit anti-human GC (1:500) * 2nd Ab: swine anti-rabbit IgG HRP or AP conjugated (1:1000)
PI	Plasma samples without previous treatment	---	IPGs: 4.35-4.75 4.35-4.55 Rehydration solution: 2 % Pharmalyte 4.2-4.9 0.8 % ACES,20 % sucrose	- Coomasie briliant blue
Tf	Plasma samples diluted 1:7 with 0.15 % ferrous amonium sulfate and incubated 16 h at 4o C	2 % Pharmalyte 4-6.5 1.2 % HEPES	IPG: 5.2-5.7 Rehydration solution: 2 % Pharmalyte 4-6.5 20 % sucrose	- Coomasie briliant blue - Immunoblotting: * 1st Ab: rabbit anti-human Tf (1:1000) * 2nd Ab: swine anti-rabbit IgG HRP
ORM	10 µl of plasma + 10 µl of neuraminidase (C. perfringens, 1 U/mg) at 37o C for 16 h.	1 % Pharmalyte 4.5-5.4 1 % Pharmalyte 4.2-4.9 1 % ACES	IPG: 4.4-5.2 Rehydration solution: 1 % Pharmalyte 4.5-5.4 1 % Pharmalyte 4.2-4.9 0.8 % ACES, 20 % sucrose	- Immunoblotting: * 1st Ab: rabbit anti-human ORM (1:1000) * 2nd Ab: swine anti-rabbit IgG HRP conjugated (1:1000)

(1) The composition of the miniaturized polyacrylamide gels (50-100 x 60 x 0.25-0.5 mm) was 6.2 % T and 3.2 % C. Gels for SIEF and IPG matrices were cast as previously described (Alonso, 1988; Alonso, 1989). SIEF was performed using Pharmacia equipment (FBE 3000, RCPS 3000/150 and VH-1) and HIEF was performed using LKB equipment (2117-003 Multiphor II, 2297 Macrodrive 5 and 2219 Multitemp II). The platinized electrodes rested directly on the gel surface with an interelectrode distance of 55 mm. Gels were prefocused at a low voltage (SIEF: 30-60 V/cm; HIEF: 90-120 V/cm) during 20 min. Samples were applied on the prefocused gels using pieces of Whatmann Nº 3 filter paper (4 x 3 mm) at 0.5 cm from the cathode and IEF was continued for 20 min. with the same settings as prefocusing. Sample applicators were then removed and gels were focused adjusting the power settings to obtain 130-180 V/cm (SIEF) or 300-380 V/cm (HIEF). The total focusing time was 120-140 min. for SIEF and 200-360 min. for HIEF.

(2) Immunoblotting was carried out by simple difussion from the gel to PVDF membranes (Millipore) and focused bands were detected on themembrane by a two-step enzyme-immuno-assay as previously described (Alonso 1988). Antiserums were purchased from Dakopatts (Denmark).

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ISOELECTRIC FOCUSING IN MINIATURIZED GELS: APPLICATION TO GC, PI, Tf AND ORM SUBTYPING IN CENTRAL SPAIN

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INTRODUCTION

Since the introduction of isoelectric focusing (IEF) for the analysis of protein polymorphisms of forensic science interest, several technical modifications have been developed to improve its reproducibility and resolution capacity. These include: IEF in a mixture of "separators" and carrier ampholytes (separator-IEF; SIEF) (Caspers et al. 1977; Gill et al. 1984); IEF in immobilized pH gradients (IEF-IPG) (Bjellquist et al. 1982; Cleve et al. 1982) and IEF in IPG-CA supplemented gels (hybrid-IEF; HIEF) (Atland et al. 1985).

On the other hand, the detection of focused bands by enzyme-immunoassay after blotting to immobilizing matrices (immunoblotting), has become a useful tool in forensic science practice due to its high sensitivity and feasibility (Tamaki 1986).

In this study the GC, PI, Tf and ORM-1 genetic variants were investigated by SIEF and/or HIEF using miniaturized gels. The subtypes were detected by immunoblotting in all cases but PI. The distribution of subtypes and the allele frequencies found in Central Spain are shown.

MATERIAL AND METHODS

Plasma samples were obtained from unrelated individuals living in Madrid (n=340 for GC, Tf and PI subtyping and n=315 for ORM subtyping). Samples were stored at - 40o C until used for a maximun period of 6 months. Sample preparation, IEF procedures and detection methods are shown in Table 1.

RESULTS

Figures 1 to 4 show the banding patterns of the GC, PI, Tf and ORM phenotypes respectively as observed by SIEF and/or HIEF.

Tables 2 to 5 show the distribution of GC, PI, Tf and ORM phenotypes respectively observed in Madrid (Central Spain). Allele frequencies are also shown.

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Separator method for Native-PAGE

Sample application down at				1.2	0	Vh
Sample application up at				1.3	0	Vh
SEP 1.1	600 V	12.5 mA	3.5 W	15 °C	60	Vh
SEP 1.2	600 V	1.0 mA	3.5 W	15 °C	2	Vh
SEP 1.2	600 V	12.5 mA	3.5 W	15 °C	60	Vh

Staining with o-dianisidine (0.2% w/v in 5% acetic acid and 5% H_2O)

DISCUSSION

Miniaturized IEF gels, run and stained with the PhastSystem, offer a number of advantages over conventional IEF in normal size gels. First, faster separations are possible due to short separation distances and the high field strength applied. Second, the separations generally have a good resolution due to efficient cooling and the high number of V/cm. In general, results are much more easily reproduced since all variables can be programmed (e.g. number of V/h, temperature, staining).
Two gels (24 samples) can be run simultaneously in the separation unit while at the same time two other gels can be stained in the development unit.
Although PGM, ACP and ESD phenotypes can be clearly distinguished, the resolution for enzymes is not as good as that for proteins, because diffusion is proportionally higher than in gels of normal size and bands which are close together can appear as a single, broader band. Only those enzymes with fast detection methods and well-separated isoenzymes can be typed with this method. However, the small amount of substrate needed for staining should be kept in mind. Nevertheless IPGs gels or HIEF can be used and in some cases this problem solved.
The system is much more useful for proteins, especially those systems needing immunofixation because only a minimal amount of expensive antisera is needed. Transfer to nitrocellulose is also possible with good results . An other advantage is the fast Coomassie and silver staining due to automation and elevated temperatures. Nevertheless, we recommend developing the last step of silver staining outside the development unit for better control of band intensity. After staining, gels can be dried with a hair-dryer and so provided a permanent record of the results. Gels can also be mounted in frames and projected directly as slides.
Native-PAGE for Hp typing with the PhastSystem gave good and reproducilble results, being an advantageous method over conventional normal size gels.
This system has potential advantages not only for the study of polymorphic proteins in paternity cases and population genetics research, but also in criminal investigations (e.g. bloodstains analysis). Thus, only 0,3 fL of sample need be applied over the gel, and with silver staining methods as little as 1 ng/fL of protein can be detected, thus permitting typing of polymorphic proteins in very minute bloodstains

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	Ampholine pH 6-8 (1:1)
Esterase D (ESD)	Ampholine pH 4-6;
	Ampholine pH 5-7 (1:1)
Plasminogen (PLG)	Ampholine pH 3-9
Properdin Factor B (Bf)	Ampholine pH 5-7;
	Pharmalyte pH 4-6.5 (1:1)
Factor XIII A (FXIIIA)	Pharmalyte pH 4-6.5;
	Ampholine pH 5-7 (2:1)
Factor XIII B (FXIIIB)	Pharmalyte pH 4-6.5;
	Ampholine pH 5-7 (1:1)
Complement C3 (C3)	Ampholine pH 4-8

Sample application (0.5 µL)

+ : PGM, ACP, Tf
- : ORM, Pi, Gc, ESD, Bf, PLG, FXIII A,FXIII B, C3

Optimized method for IEF program into the separation method file of PhastSystem.

Sample application down at			1.2		0 Vh
Sample application up at			1.3		0 Vh
Extra alarmed to sound at			1.1		70 Vh
SEP 1.1	2000 V	2.0 mA	3.5 W	15 °C	15 Vh
SEP 1.2	200 V	2.0 mA	3.5 W	15 °C	15 Vh
All markers except ACP and Pi					
SEP 1.3	2000 V	5 mA	3.5 W	15 °C	450 Vh
For ACP					
SEP 1.3	2000 V	5 mA	3.5 W	15 °C	350 Vh
For Pi					
SEP 1.3	2000 V	5 mA	3.5 W	15 °C	500 Vh

For ORM, Bf, PLG, FXIIIA, FXIIIB and C3 the gels were immunofixed according to (2) or transfer to Immobilon membranes, according to (3), washed 5h with saline and then stained with CBB.
The enzymes were stained outside the development unit by laying on the gel 2 mL of 1% agarose containing the appropriate substrate. CBB and silver staining were carried out as described in the Phastsystem Manual (Pharmacia. Uppsale. Sweden)

Native PAGE for Hp typing

Gel characteristics	Stacking T = 5% C = 3%	Separation gel zone T = 7% C = 2%
Buffer in gels 0.112 Acetate, 0.112 Tris, pH 0.5		
Buffer in strips (0.2% agarose)	0.88 M L-alanine, 0.25 M Tris; pH 8.8	

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Fast IEF of some polymorphic proteins and enzymes in bloodstains using the PhastSystem

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INTRODUCTION

Although the use of miniaturized gels in electrophoresis was proposed many years ago (Radola 1980), such use did not become widespread until the recente introduction of automated systems. PhastSystem (Pharmacia Fine Chemicals, Uppsala, Sweden) is an automated system wich consists of one separator and one development unit, an a control unit containing the microprocessor, wich controls and monitors both separation and development processers according to programmed methods.
In this paper we describe optimal programs for the separation of polymorphic proteins and enzymes using miniaturized polyacrylamide gels, and discuss the potential advantages and disadvantages of the method.

MATERIAL AND METHODS

Polyacrylamide gels were cast over GelBond PAG films and cut from larger slabs to an appropriate size (5x5 cm).

Gel characteristics

T = 5%
C = 3%
Ampholyte concentration = 2% w/v
Photopolymerization with riboflavin and UV light

pH ranges and ampholyte composition

Marker	Ampholyte composition
α1-antitrypsin (Pi)	Pharmalyte pH 2.5-5; Ampholine pH 3.5-5 (1:1)
Group-specific component (Gc)	Pharmalyte pH 4-6.5; Ampholine pH 4-6 (1:1)
Transferrin (Tf)	Ampholine pH 5-7
Orosomucoid (ORM)	Ampholine pH 4-6
Phosphoglucomutase (PGM1)	Ampholine pH 5-7
Acid phosphatase (ACP)	Ampholine pH 4-6;

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mination of three different antibodies shows Alpha-2 antiplasmin and PLG-bands in the same pI-positions. The interpretation of these results are causes in the high binding affinity of alpha-2 AP to plasmin or other complex formations. Alpha-2 AP shows 23 - 28 % homology to plasmin and to other protease inhibitors.

A polymorphism also correlated to plasminogen is observed in the Kallikrein system. The polymorphism is observed in the cathodic part of the band pattern. We excluded a cross reaction of the antibody with highly purified kallikrein and plasminogen. The genetic interpretation suggests a linkage of the genes of Plasminogen and Kallikrein on chromosome 6.

The polymorphism of plasminogen was first described by Hobart 1979 and Raum 1980. Besides the three common phenotypes A, AB and B, a number of variants are observed. The allelefrequences are

PLG*1 = 0.7150
PLG*2 = 0.2742
PLG*V = 0.0012.

The theoretical exclusion rate is calculated to be 18.7 %.

The genetically determined polymorphism of Orosomucoid (ORM) was also determined by agarose isoelectric focusing. We observed the following allele frequencies:

ORM1*F1 = 0.6463
ORM1*F2 = 0.0145
ORM1*S = 0.3387.

The theoretical chance for the exclusion of non- fathers was calculated to be 18.07 %.

In conclusion agarose isoelectric focusing offers many opportunities. Especially in the search for new systems which are applicable in parentage testing.

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Three common phenotypes 1, 2-1 and 2 are observed. The allele frequencies for the two common allels are

FXIIIA*1 = 0.8021
FXIIIA*2 = 0.1959.

Variants are found in less than 0.2 %. For the theoretical exclusion rate we calculated a value of 14.8 %.

The B-subunit of coagulation factor XIII acts as a carrier molecule. After cleavage of the subunit A, factor XIIIB is detectable in serum. The genetic polymorphism was first described by Board in 1980. The common phenotypes FXIIIB 1, 2-1, 2, 3-2, 3-1 and 3 as well as products of the rare alleles FXIIIB*4 and B*6 are found in the southern German population. The allele frequencies for FXIIIB are

FXIIIB*1 = 0.7603
FXIIIB*2 = 0.0843
FXIIIB*3 = 0.1534
FXIIIB*V = 0.0019.

The theoretical exclusion rate for the FXIIIB system is calculated to be 22.7 %.

In agarose focusing coagulation factor XII (Hageman-factor) shows a heterogenous band pattern in the pH-range from 6.4 - 7.3. Four main bands and several minor bands are observed. FXII can be demonstrated with plasma and serum. Coagulation factor VII (proconvertin) shows a uniform two band pattern in the pH-range from 5.6 - 6.4. We examined 250 healthy unrelated persons and didn't find in factor XII as well as factor VII a polymorphism useful for parentage testing.

Alpha-2 antiplasmin shows a band pattern identical with plasminogen. Untreated as well as neuraminidase treated sera and the exa-

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THE APPLICATION OF AGAROSE ISOELECTRIC FOCUSING IN PATERNITY TESTING

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Agarose as carrier medium for isoelectric focusing is a practicable alternative to polyacrylamid in routine application in parentage testing. Since the introduction of highly purified agarose 10 years ago, most of the new systems have been described with the agarose technique.

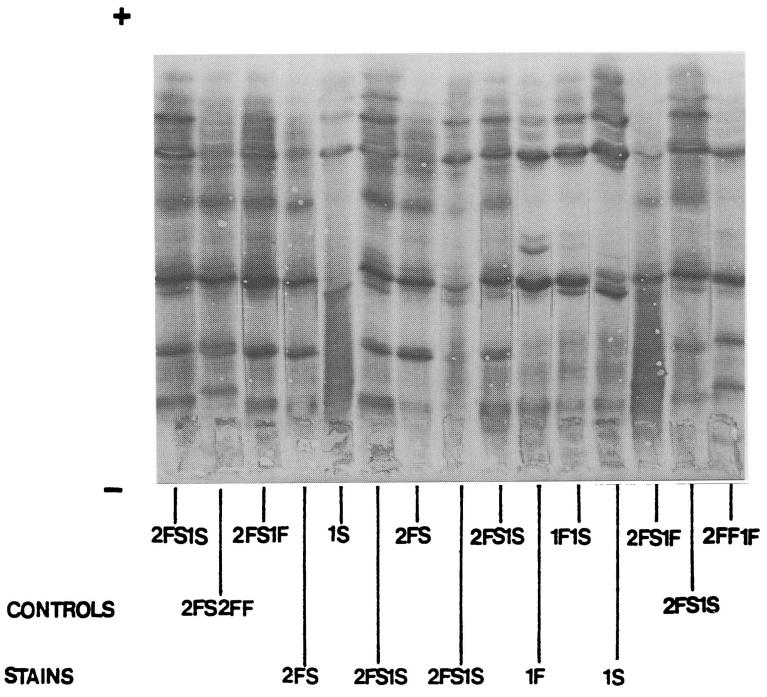
Agarose has the following advantages. In comparison to polyacrylamid it is non toxic. There is no limitation by gel pore size for larger proteins to penetrate the gel (for example native IgM-molecules with a molecular weight of 900,000). Immunological detection can be performed directly on the surface of the gel (immunofixation). Proteins with a low concentration in sera can be blotted with high transfer rates, an electroblot is not necessary.

The disadvantages of agarose are caused by its sensitivity to overloading, early cathodic drift and separation problems in narrow pH-gradients.

With a standardized agarose technique several proteins were examined regarding there usefulness in parentage testing. The gels were prepared with 0.8 % agarose, 10 % sorbitol and 2.5 % carrier ampholytes. Additional sulfonic acid derivates (ACES, MES, MOPS) were used in concentration of 0.8 %. Depending upon their concentration in serum or plasma immunofixation or capillary blotting were used with monospecific antibodies.

A genetically determined polymorphism of the coagulation factor XIII is known for the A- and B-subunit.
The enzymatic subunit (FXIIIA) is found in plasma. FXIIIA was first described by Board in 1979 with agarose gel electrophoresis.

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Hp subtype pattern of case work stains

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blotting performed for 1.5 h at 130 mA using a Semi-Dry Electroblotter (JKA-Biotech). The anode buffer was 0.3 M tris, 20% ethanol, pH 10.4, and the cathode buffer 0.04 M 6-amino-n-hexanoic acid, pH 9.4.

Visualization

The technique was as described for the detection of orosomucoid (Thymann and Weidinger 1988) using the following concentrations and incubation times: rabbit anti human Hp 1/2000, over night; biotinylated swine anti-rabbit IgG 1/2000, 2 h; alkaline phosphatase conjugated avidin 1/6000, 2 h.

RESULTS AND DISCUSSION

The Hp subtype patterns obtained with bloodstains from case work are shown in fig.1. The common subtypes were easily recognizable. The 2FF bands were more distinct than by agarose gel electrophoresis (Thymann et al. 1989), but it was difficult to distinguish the type 2SS-2FS from the type 2FS. This has to be taken into consideration when type 2FS stains are encountered. The method is more sensitive than Hp subtyping by agarose gel electrophoresis and therefore better suited for the analysis of stains. The technique is also useful for reexamination of weak or doubtful Hp patterns found by our routine agarose method for Hp subtyping of serum samples. Normal serum samples could be subtyped in dilutions of 1 in 64. Hp subtyping of 26 control stains of various age was compared to Hp typing by polyacrylamide gradient gel electrophoresis. No discrepancies were found. Stains up to 24 months old were succesfully subtyped. In a blind trial comprising 14 one month old bloodstains correct results were obtained for 13 stains, for the last one the type (disclosed as 2SS-2FS) could not be determined. 30 stains from case work were examined. Subtyping and grouping by conventional Hp typing was possible with 22 stains and the results were in agreement. 4 stains could be classified within the main groups whearas subtyping was doubtful. 4 stains could not be typed by any of the methods

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HAPTOGLOBIN SUBTYPE DETERMINATION OF BLOODSTAINS

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INTRODUCTION

The high discriminating power (78%) of the haptoglobin subtypes makes it desirable to include this system in the panel used for analysis of bloodstains in criminal cases. Hp subtyping by isoelectric focusing of neuraminidase treated serum was introduced by Teige et al. (1985, 1988) and appeared to be a very suitable procedure for Hp subtyping in paternity investigations. The procedure was modified to make it applicable to analysis of blood stains. Urea was added to the gel, and electroblotting was used for the transfer of protein to the membrane.

MATERIAL AND METHODS

Bloodstains and extraction

Bloodstains from individuals with known Hp subtypes were made on cotton cloth and stored at room temperature for up to thirty months. Case work material was kept at room temperature until examined. Bloodstains (2.5 x 5 mm) were cut into small pieces and extracted at room temperature over night with 30 µl neuraminidase (Clostridium perfringens, Boehringer 0.5 U/ml 0.1 M sodiumacetate pH 5.5). The stains were centrifuged, 25 µl of urea-mercaptoethanol-ampholine (Teige et al. 1988) was added and the extracts frozen at -20°C for 18 h.

Polyacrylamide gel

The gel (120x250x0.5 mm) contained 3.0 ml acrylamide/bisacrylamide (BioRad T=30, C=3.3), 9.5 ml dest. water, 1.6 ml glycerol, 1.0 g urea, 300 µl Resolyt pH 4-8 (BDH), 250 µl Ampholine pH 5-7, 550 µl Ampholine pH 4-6 (LKB), 40 µl TEMED and 110 µl 10 % ammoniumpersulphate. The gel was cast on GelBond PAG film.

Isoelectric focusing

This was performed at 5°C with the electrodes placed directly on the gel surface (Multiphor II, LKB). Prefocusing was carried out for 1 h (max. 500 V, 15 mA, 10 W). The samples were applied 10 mm from the cathode on 4x10 mm filter paper (Whatman nr. 17), and focusing was performed for 15 min. The filter paper was removed and the focusing continued for 1.5 h (max. 2000 V, 15 mA, 15W).

Electroblotting

The membrane (Immobilon-P, 45 µm, Millipore) was soaked in ethanol and subsequently in dest. water. The gel was removed from the Gel Bond-film by means of a Film Remover (LKB) and the membrane was placed on the gel. The gel/membrane sandwich was covered with dry filterpaper (AGF 138 15 x 25 cm) and electro-

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Table 1. Distribution of Hp phenotypes among 500 unrelated blood donors from south western Switzerland.

phenotype	observed n°	%	expected n°		allele frequencies
1F	12	2.4	10.8	0.13	1F = 0.147
1F-1S	37	7.4	36.8	0.00	1S = 0.249
1S	28	5.6	31.3	0.34	2FF = 0.003
1F-2FF	0	0.0	0.3	0.30	2FS = 0.567
1S-2FF	0	0.0	0.5	0.50	2SS = 0.034
2FF-2FS	3	0.6	1.15	2.97	
2FF-2SS	0	0.0	0.05	0.05	
2FF	0	0.0	0.0	0.00	
1F-2FS	81	16.2	84.65	0.15	
1S-2FS	147	29.4	144.0	0.06	
2FS-2SS	17	3.4	14.45	0.45	
2FS	161	32.2	165.65	0.13	
1F-2SS	5	1.0	3.7	0.45	
1S-2SS	9	2.2	6.3	1.15	
2SS	0	0.0	0.3	0.30	

The common Hp subtype pattern obtained by agarose IEF of immunoprecipitated and cleaved Hp are shown in Fig. 1. The observed and expected phenotype frequencies and the corresponding gene frequencies among 500 unrelated blood donors from south western Switzerland are reported in table 1. Our gene frequencies are in good agreement with those established in other European countries [Thymann et al. 1977; Olaisen et al. 1981; Shibata et al. 1982; Patzelt and Schröder 1986, 1988; Teige at al. 1986, 1988; Zischler et al. 1987, 1988; Bertrams et al. 1988; Hjalmarson 1988]. Furthermore, we have determined the Hp subtypes in 100 corpse bloods and in 100 hemolyzed blood samples stored for five months and containing 50-300 mg/100 mL alcohol. The corpse blood samples were tested in two ways : first, we have determined the Hp subtypes in the plasma samples, secondly, the hemolyzed corpse blood were analyzed after freezing at -20 °C. The patterns of the hemolyzed corpse blood were the same as those obtained from the corresponding plasma samples.

Hp subtypes can be determined in hemolyzed blood samples as far as free hemoglobin is removed with methylenchlorid precipitation (Fig. 2). Without this pretreatment, the differentiation of Hp pattern becomes difficult as an additional band at 1S localisation appears.

In conclusion, the agarose IEF method presented here can be recommended for the determination of Hp subtypes in plasma and hemolyzed blood samples in forensic practice.

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Electrophoresis at 8 °C

Electrode solutions :	anode 1 M H_3PO_4 cathode 1 M NaOH
Samples :	20 uL on 10x5 mm filterpaper applied 0.5 cm from the cathode after drying 10 min
Prefocusing :	30 min at 1600 V, 10 mA, 5 W
Focusing with samples :	30 min at the same setting
Focusing without samples :	60 min at 1600 V, 10 mA, 10 W
Fixation and staining :	60 min with formaldehyde-ethanol and Serva Blue G solution according to Steck et al (1980)
Destaining :	according to need

RESULTS AND DISCUSSION

We have determined the Haptoglobin phenotypes in 500 fresh plasma samples and in 100 corpse plasma samples by agarose isoelectric focusing and by polyacrylamide electrophoresis [Heredero et al. 1974]. No discrepancies between the two methods were noticed.

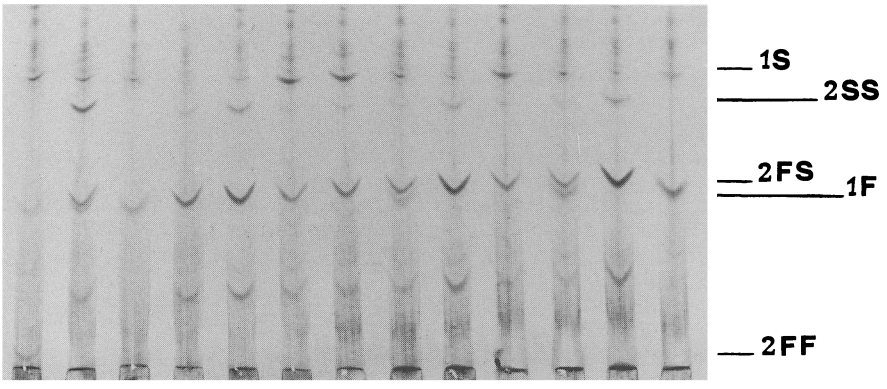


Fig. 1. Hp in plasma as obtained with IEF in agarose. From left to right : 1F-2FF;2FS-2SS;1F;2FS;2FS;1S-2FS;1S-2FS;1F-2FS;2FS;1S-2FS;1F-2FS;2FS;1F.

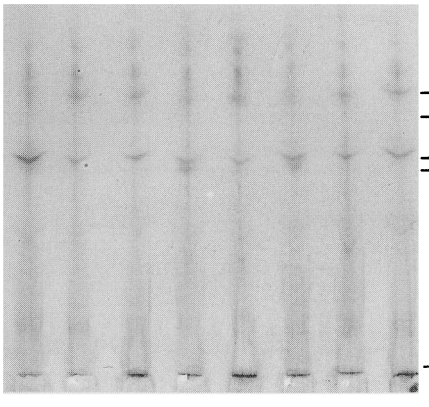


Fig. 2. Hp in hemolyzed corpse blood as obtained with IEF in agarose. From left to right : 2FS;1S-2FS; 1S-2FS;1F-2FS;1S-2FS; 1F-2FS;1S-2FS;1S-2FS.

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HAPTOGLOBIN SUBTYPING BY AGAROSE ISOELECTRIC FOCUSING

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INTRODUCTION

Some isoelectric focusing methods have been described for Haptoglobin subtyping on polyacrylamide gels [Olaisen et al. 1981, Shibata et al. 1982, Teige et al. 1985, Patzelt et al. 1985]. The aim of the present investigation was to develop an alternative method for Hp subtyping in a forensic laboratory.

We used an immunoprecipitation with subsequent separation of reduced and alkylated Hp [Patzelt et al. 1986, Teige et al. 1988] in agarose isoelectric focusing. We here described the band patterns for the common phenotypes and the gene frequencies of Hp-subtypes in a south-western Swiss population. Furthermore, we investigated the possibility of Hp determination in corpse plasma, in hemolyzed corpse blood and in stored hemolyzed blood samples containing between 50-300 mg/100 ml alcohol.

MATERIALS AND METHODS

Preparation of plasma samples

20 uL plasma
12 uL rabbit anti-human Hp (Dako)
80 uL polyethylenglycol solution (8% PEG 6000, 1.7% NaCl, 0.2% NaN3)
incubate overnight at 4 °C
centrifuge and wash with 1 ml NaCl
add 20 uL of a solution for reductive cleavage and mix with the immunoprecipitate [1 g urea and 22 mg dithiothreitol in 1.2 ml of a borate buffer pH 8.8 (0.1 M boric acid, 0.04 M sodium hydroxyde)]
incubate 45 min at 37 °C
add 8 uL of a iodacetamide solution (100 mg/mL)
incubate 15 min at room temperature
immediately freeze at -20 °C

Pretreatment of hemolyzed blood samples

60 uL hemolyzed blood
150 uL methylenchlorid
mix, centrifuge and collect the aqueous supernatant
then, prepare like plasma samples

Agarose gel 260x125x0.5 mm (16 mL)

0.2 g agarose Pharmacia
2.0 g sorbitol
1000 uL Ampholine pH 5-6.5 (LKB)
400 uL Ampholine pH 3.5-9.5 (LKB)

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Table 1. Phenotype and Gene Frequencies of Haptoglobin in the Swiss Population

Hapto Subtypes	observed N	%	expected N	%	chi-sq.	Hapto Genefreq.
1F	21	1.66	20.10	1.59	0.0403	0.1260
1F-1S	70	5.53	76.22	6.02	0.5076	
1F-2FF	2	0.16	3.15	0.25	0.4198	
1F-2FS	192	15.16	185.96	14.69	0.1962	
1F-2SS	13	1.03	13.48	1.06	0.0171	
1S	64	5.05	72.28	5.71	0.9485	0.2389
1S-2FF	2	0.16	5.97	0.47	2.6400	
1S-2FS	386	30.49	352.68	27.86	3.1480	
1S-2SS	19	1.50	25.57	2.02	1.6881	
2FF	0	0	0.12	0.01	0.1200	0.0099
2FF-2FS	18	1.42	14.57	1.15	0.8075	
2FF-2SS	3	0.24	1.06	0.08	3.5506	
2FS	407	32.15	430.21	33.98	1.2522	0.5829
2FS-2SS	66	5.21	62.37	4.93	0.2113	
2SS	3	0.24	2.26	0.18	0.2423	0.0423
Total	1266	100	1266	100	15.7894	1
d.f. = 10	$0.10 < p < 0.20$					

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IEF:
- Settings of power supply (max.): 1600 V, 10 mA, 15 W, constant throughout the whole procedure, temperature of cooling plate: 10^{o} C
- Prefocusing: 30 min.
- Sample application: transfer the total of prepared samples on pieces of Whatman No 1 filter paper 5 x 17 mm which have been prealigned on application tape / apply 1.5 cm from cathode
- Focusing: use same setting of power supply / focus for 45 min. / remove application tape with filter paper pieces / continue focusing under same conditions for another 90 min.
- Staining: immerse gel for 90 min. in staining solution at room temperature on a rocking table / destain with changes of destaining solution until a colourless background is achieved / for preservation the gel may be dried at room temperature and covered with a transparent membrane.

RESULTS AND DISCUSSION

The Hp subtype pattern is shown in figure 1. Phenotype and gene frequencies are presented in table 1. They are in good agreement with the expected ones according to the Hardy-Weinberg law.

Gene frequencies correspond rather well with observations in other European populations (1-5). In a total of 248 mother/child pairs from cases of disputed paternities no "impossible" phenotype combinations were observed.

The isolated chance of exclusion of a falsely accused father almost doubles (from about 18% to 33%) by subtyping compared to the classical typing. This is also illustrated by our observation of 22 exclusions in the Hp subtypes during the last few months among our routine paternity cases, where only 10 among them were also excluded by the classical Hp typing. All of these 22 exclusions were corroborated in one or more other systems.

Hypohaptoglobinemia due to clinical reasons occur quite often and are usually not readily typable. With immunoprecipitation such samples can be typed by simply increasing the amount of sample volume in the sample preparation step.

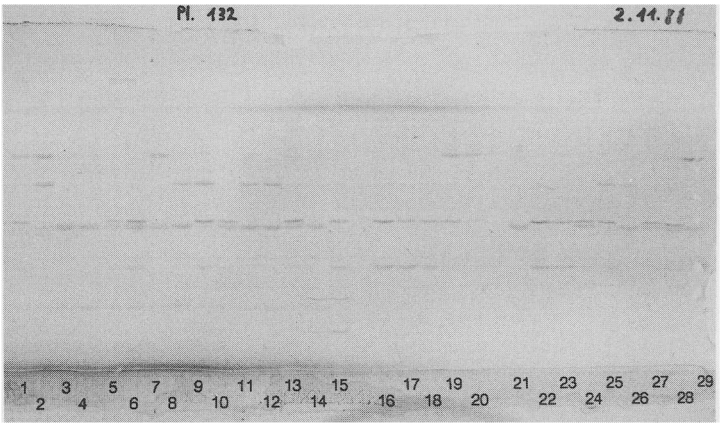


Figure 1. IEF gel of Hp subtypes. 1S-2FS (1, 19, 20); 1S-2SS (2); 1F (3, 4, 21); 1F-2FS (5, 6, 10, 13, 24, 27, 28); 1F-1S (7, 29); 1F-2SS (8, 11, 12, 26); 2FS-2SS (9, 25); 1F-2FF (14); 2FF-2FS (15); 2FS (16, 17, 18, 22, 23)

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Haptoglobin Subtypes in the Swiss Population: Phenotype and Gene Frequencies - Description of an Easy Method for Routine Typing

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INTRODUCTION

We describe a technique which proved to be reproducible and easy to perform in routine testing.

In principle:
1. Isolation of haptoglobin (Hp) by immunoprecipitation
2. Reduction and alkylation in presence of urea
3. Isoelectric focusing
4. Protein staining

MATERIAL AND METHODS

Serum of 699 blood donors and EDTA-plasma of 567 unrelated individuals and 248 children engaged in cases of disputed paternity

Reagents:
- Acrylamide / Bis Solution: 14.55 g Acrylamide / 0.45 g N,N'Methylenediacrylamide dissolved in distilled water ad 100 ml
- Anolyte: 1 M H_3PO_4 - Catholyte: 1 M NaOH
- Staining Solution: 0.5 g Serva Blue R dissolved in 90 ml Ethanol / 50 ml Formaldehyde 36.5 % and 210 ml distilled water added
- Destaining solution: 250 ml Ethanol / 10 ml Formaldehyde 36.5 % and 750 ml distilled water added
- Anti-Haptoglobin: Rabbit-Anti-Human Haptoglobin, IgG fraction, Dakopatts, Code Q 330
- Precipitating aid: 8 g Polyethyleneglycol 6000 / 1.7 g NaCl / 0.2 g NaN_3 dissolved ad 100 ml in distilled water
- Reducing agent (freshly prepared): 1 g urea dissolved in 1.2 ml Borate Buffer (0.1 M Boric acid adjusted with 0.04 NaOH to pH 8.8) / 20 µl 2-mercaptoethanol or alternatively 22 mg DL-Dithiothreitol added
- Alkylating agent: 100 mg Iodoacetamide dissolved in 1 ml distilled water

Gel:
- Cassette: 260 x 125 x 0.5 mm / support: Gelfix for PAGE membrane (Serva) / cover: glass plate treated with Repel Silane
- Gel: Acrylamide / Bis solution 5.6 ml / Glycerol 87 % 2.25 ml / LKB Ampholine pH 5 - 7 675 µl / pH 6 - 8 225 µl / pH 3.5 - 5 225 µl / pH 3.5 - 9,5 113 µl / Servalyte pH 5 - 6 225 µl / distilled water 8.1 ml / degas in vacuum, then add TMED 56 µl / 1% Ammoniumpersulfate, freshly prep. 560 µl / pour mixture into precooled (4° C) cassette / polymerize at room temperature for at least 3 hours.
- Sample preparation: to Eppendorf centrifuge tube successively add precipitating aid 80 µl / EDTA plasma or serum 20 µl (if Hp conc. is low, add more sample, max. 160 µl) / anti-Hp antiserum 12 µl / vortex and keep at 4° C over night / centrifuge (8000 g) for 4 minutes / discard supernatant / wash residue with 500 µl saline / remove washing fluid completely / dissolve residue in 20 µl of reducing agent and mix / incubate at 37° C for 45 minutes / add 8 µl of alkylating agent and mix / after 15 min. samples may be applied on IEF gel or they may be frozen at -20° C for later separation

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This paper is from the thesis of Laura J. Mendoza submitted as partial fulfillment of the requirements for the degree of Master of Science in Criminalistics in the Graduate College of the University of Illinois at Chicago, 1989.

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at least 1 hour. Gels are then rinsed in distilled water for 5 minutes before staining in Coomassie Brilliant blue R-250. (Staining is the same as with cellulose acetate strip for the polyacrylamide gel according to Edwards).

RESULTS AND DISCUSSION

Narrow range (pH 4.5-5.4) ultrathin-layer agarose IEF with MOPS and HEPES proved to differentiate the common and rare GC phenotypes tested. Results were comparable to those described by Edwards (1986) using narrow range ultrathin-layer polyacrylamide gel IEF with chemical spacers. Both of these methods separated the GC 1S and 1F bands by approximately 2 mm, while the narrow range agarose IEF gel without the chemical spacers separated these bands by only about 1 mm. An increase in distance between the cathodal and anodal bands by 1-2 mm for the gels using the MOPS and HEPES was also observed. Figure 1 shows the separation of several GC variants by all three methods. The improvement in separation can be emphasized by looking at the rare GC variants GC 1S-1C6, GC 1S-1C32, and GC 1S-1C33 on each gel. These variants appear identical on the agarose gel without the chemical spacers, but are clearly delineated on the other gels.

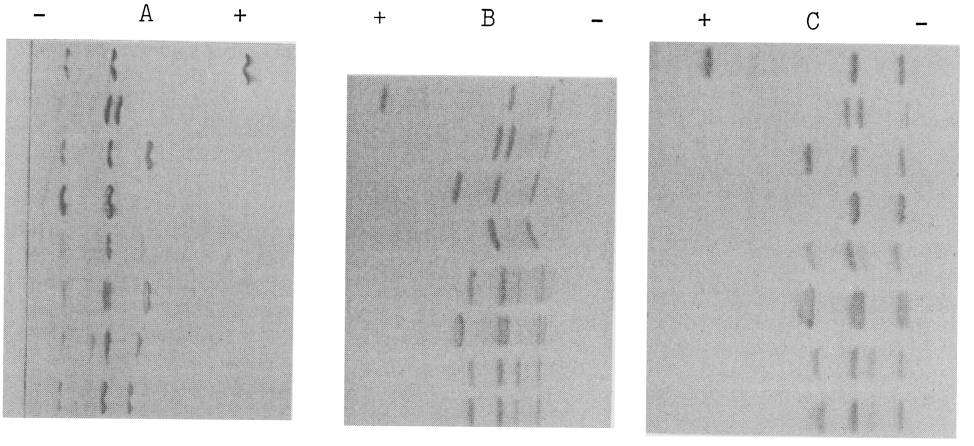


Figure 1. From top: 1S-2C14;1F-2A3;2-1S;1S;1S-1C6;2-1S1F control; 1S-1C32;1S-1C33. A)Polyacrylamide gel with MOPS and HEPES, B)Agarose gel, C)Agarose gel with MOPS and HEPES.

As a blind trial, 71 laboratory personnel of Rush Medical Center were tested by all three methods and found to be easily subtyped. Identical phenotypes were obtained for all specimens by each method.

A new improved method using agarose IEF has been presented. Results by this method compared favorably with those obtained by polyacrylamide gel IEF. The advantages of agarose being non-toxic, less expensive, and easy to work with make this technique highly desirable for routine paternity and forensic casework.

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Table 1. Known Rare GC Variants Used

GC 1 Variants	GC 2 Variants
1F-1C10	1S-2C14
1S-1C6	1F-2A3
2-1A10	
1S-1C32	
1S-1C33	
1F-1C16	

Gel Preparation

Agarose IEF gels (225x114x1 mm) were cast on GelBond^{R} film (FMC). Add 0.2g Agarose IEF, 2.5g sucrose, 0.16g HEPES, 0.5g MOPS to 20 ml distilled water and dissolve by boiling. (This is sufficient for two gels.) Cool to 75°C and add 1.5 ml Pharmalyte 4.5-5.4. Aspirate molten agarose solution into a 25 ml syringe and quickly dispense into a preheated LKB capillary gel casting kit with constant pressure on the syringe barrel and the glass plates at about a 45° angle. Permit to gel for 20 minutes. Store at 4°C for a minimum of 1 hour in a humidity chamber. Gels may be stored for up to 1 month. Agarose gels were also made in the same manner without the MOPS and HEPES. Acrylamide gels were made according to Edwards (1986).

Sample Preparation

All serum samples were treated with 6 M Urea in a 1:5 dilution to dissociate any GC that may be bound to actin. The GC-actin complexes alter the pI; therefore, incomplete dissociation may produce additional banding making it more difficult to interpret the banding pattern (Emerson et al 1984). To the gel at a position 3 mm from the cathode, approximately 5 ul of sample solution with sample application pieces (3 x 3 mm).

Running Conditions

An LKB Multiphor 2117 tank was used having the electrodes separated by 9.5 cm. A 0.5 N NaOH solution was used on the cathodal wick and a 1 M H_2PO_4 solution was used on the anodal wick. Agarose IEF gels were focused at a maximum of 1650 V, unlimited W, and unlimited mA for 90 minutes at a temperature of 4°C. Acrylamide IEF gels were focused according to Edwards (1986).

Immunofixation

At the completion of the run, the GC proteins were identified by immunofixation using anti-GC globulin. The acrylamide gels were immunofixed according to Edwards (1986). For the agarose gels, anti-GC was diluted 1:6 with 0.9% saline and poured directly on the gel 2 cm anodal to the sample application pieces. Cover gel with a saline moistened filter paper. Place gel in a humid chamber for 1 hour at room temperature. Remove filter paper, then press and dry gel with layers of paper towels, a glass plate, and a 5 kg weight. Rinse gel in 0.9% saline for

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Group Specific Component Phenotyping by Ultrathin-layer Agarose Isoelectric Focusing Using Chemical Spacers

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INTRODUCTION

Polyacrylamide gel isoelectric focusing has become the method of choice for classification of the common GC phenotypes, primarily because of improved resolution. Isoelectric focusing in immobilized pH gradients provided far superior results in delineating the phenotypes but has not proven practical for routine testing (Cleve et al 1982). With the introduction of narrow pH range carrier ampholytes by Pharmacia, there was improved resolution of the common GC phenotypes for both polyacrylamide and agarose IEF using a pH range of 4.5-5.4 (Lizana and Olsson 1984). Since the common GC phenotypes fall within a pH range of 4.8-5.1, further methods have been used to flatten the pH gradient curve in this region to improve resolving power. Edwards (1986) used ultrathin-layer IEF in polyacrylamide gel containing a combination of chemical spacers with narrow range Pharmalytes (pH 4.5-5.4) to improve the separation of the GC 1F and GC 1S bands. The results compared favorably with those obtained in immobilized pH gradient gels.

A problem associated with using polyacrylamide is that it is a potential health hazard due to it toxic properties. Agarose, on the other hand, is virtually non-toxic and improvements in resolution with this media should be considered. The method described here utilizes agarose as the media and employs a modification of the method described by Edwards (1986).

MATERIALS AND METHODS

Reagents

Agarose IEF, Pharmalyte 4.5-5.4, and Repel-Silane were from LKB-Pharmacia. Acrylamide, Bis-acrylamide, Sucrose, MOPS, HEPES, and Coomassie Brilliant Blue R-250 were obtained from SIGMA. Rabbit Anti-GC-globulin was obtained from Atlantic Antibodies. All other chemicals used were of analytical grade.

Serum samples were obtained from healthy laboratory personnel at Rush Medical Center and from the AABB's Paternity Specimen Program (PSP). Included in these samples were 8 known rare variants (see Table 1). The samples were aliquoted and stored frozen at either -20°C or -70°C until tested.

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Table 2. AHCY gene frequencies (%) in various populations

Population	N	AHCY*2	AHCY*3	Reference
Japan	214	4.7	0.0	Akiyama et al. (1984)
SW Germany	114	4.0	0.0	Bissbort et al. (1983)
W Germany	647	2.5	0.5	Scheil and Borner (1985)
England	166	2.4	0.6	Arredondo-Vega et al.(1989)
Italy	386	2.3	0.9	Corbo et al. (1987)
Italy	374	2.4	0.7	Scozzari et al. (1987)
Sardinia	93	1.1	1.6	Scozzari et al. (1987)
NW Portugal	248	1.4	0.0	this work

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Arredondo-Vega et al.(1989) on the basis of an unsuccessful separation of AHCY variant allozymes by this kind of technique.

Population genetics

In Table 1 we present the observed distribution of AHCY phenotypes and the corresponding expected values according to Hardy-Weinberg formalism.
Table 2 shows some of the available data on AHCY gene frequencies.
The frequency of the AHCY*1 allele found in our sample is the highest reported so far. Unlike all other European populations no evidence for the occurrence of the AHCY*3 allele was found.

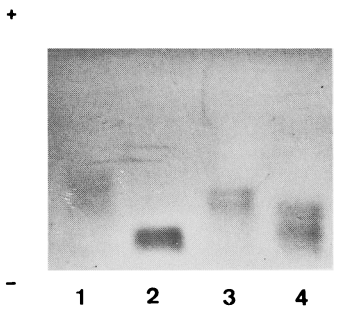


Fig. 1. AHCY phenotypes demonstrated by isoelectric focusing.
Lane 1: AHCY 2-1, pretreated with IACH
Lane 2: AHCY 1, pretreated with DTT
Lane 3: AHCY 1, pretreated with IACH
Lane 4: AHCY 2-1, pretreated with DTT

Table 1. Distribution of AHCY phenotypes in a sample from NW Portugal

	phenotypes		
Sample size	1	2-1	2
248	241 (241.10)	7 (6.85)	0 (0.05)

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not support the occurrence of any sulfhydryl variants within AHCY*1 or AHCY*2 electromorphs.

MATERIAL AND METHODS

Blood samples from 248 unrelated individuals living in Porto district (N.W. Portugal), were collected by venous puncture and used after storage up to 7 years.
Haemolysates were prepared by sonication, treated with toluene and diluted 1:1 in solutions containing either dithiothreitol (20 mM) or iodoacetic acid (20 mM). The mixtures were incubated at 37°C for 30 minutes and left overnight at room temperature.
Separation of AHCY allozymes by isoelectric focusing and activity staining were performed according to Rocha et al. (1988).

RESULTS AND DISCUSSION

Biochemical genetics

In our sample only the AHCY 1 and AHCY 2-1 phenotypes were found. Figure 1 depicts a zymogram showing the differences between these phenotypes after pretreatement with dithiothreitol (DTT) or iodoacetic acid (IACH).
All the samples reduced with DTT and identified as AHCY 1 clearly showed a more anodal pattern when alkylated with IACH. This observation is in accordance with the findings of Corbo et al. (1987) and Scozzari et al. (1987) and confirm their suggestions on the occurrence of at least one freely reactive -SH group in the AHCY*1 gene product. Moreover, it seems to rule out the possibility of occurrence in our sample of sulfhydryl variants within the AHCY*1 electromorph.
Likewise, the AHCY 2-1 pattern presents an anodal displacement upon alkylation with iodoacetic acid indicating the presence of at least one free sulfhydryl group also in the AHCY*2 allozyme. Since this displacement was found in all 2-1 individuals, it also excludes heterogeneity in the sulfhydryl reactivity of the AHCY*2 gene products analysed.

The satisfactory distinction between the AHCY phenotypes obtained by means of isoelectric focusing after sample pretreatement with DTT or IACH under the conditions here described seems to remove some of the difficulties in working with the AHCY polymorphism at the protein level reported by

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Biochemical and population genetics of S-adenosylhomocysteine hydrolase in NW Portugal

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INTRODUCTION

Human S-adenosylhomocysteine hydrolase (AHCY; EC 3.3.1.1) exhibits a genetic polymorphism first described by Bissbort et al. (1983) who demonstrated the occurrence of two common electrophoretic phenotypes (AHCY 1 and AHCY 2-1) determined by two codominant alleles (AHCY*1 and AHCY*2) at an autosomal locus.

More detailed studies on the electrophoretic patterns of AHCY, have shown convincing evidence of the presence of at least one freely reactive sulfhydryl group in the AHCY*1 gene product; Corbo et al. (1987) found a net increase in the anodal mobility of the AHCY 1 phenotype when haemolysates were submitted to prolonged storage or pretreated with negatively charged thiol reagents prior to electrophoretic analysis. Scozzari et al. (1987), on the other hand, also described similar changes in the behaviour of AHCY from stored samples and have shown that they could be reversed upon treatment with mercaptoethanol. In general such changes have been recognized as complicating factors in the correct identification of AHCY phenotypes since they lead to the confusion between the AHCY 1 and AHCY 2-1 electrophoretic patterns.

More recently we described an improved technique for AHCY typing by means of isoelectric focusing using haemolysates pretreated with dithiothreitol (Rocha et al., 1988). We also reported an heterogeneity in the modification of the AHCY 1 patterns of untreated samples, possibly due to the occurrence of sulfhydryl variants within the AHCY*1 electromorph.

In the present work we investigate this hypothesis studying the effects of pretreating haemolysates with dithiothreitol and iodoacetic acid on the separation of AHCY 1 and 2 allozymes by means of isoelectric focusing. The results obtained from the analysis of 248 unrelated individuals indicate that AHCY*2 gene product also has at least one freely reactive -SH group but do

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Table 1 *PHENOTYPE AND GENE FREQUENCIES OF PGP IN THE DÜSSELDORF POPULATION*

	observed		*expected*	
Phenotype	*n*	*%*	*n*	*%*
PGP ^{1}	1172	74.08	1171.7	74.07
PGP $^{2-1}$	261	16.50	262.5	16.59
PGP ^{2}	18	1.14	14,7	.93
PGP $^{3-2}$	8	.51	13.1	.83
PGP $^{3-1}$	118	7.46	117.0	7.40
PGP ^{3}	5	.32	2.9	.18
total	1582	100.01	1581.9	100.00

GENE FREQUENCIES: PGP^{1} .8606 PGP^{2} .0964 PGP^{3} .0424

Table 2 *PGP PHENOTYPES IN 688 MOTHER-CHILD PAIRS IN DÜSSELDORF*

	Child						
Mother	*1*	*2-1*	*2*	*3-2*	*3-1*	*3*	*n*
1	431	44			16		491
2-1	50	57	6	3	2		118
2		10					10
3-2		1			5		6
3-1	27	5		2	28		62
3					1		1

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Adli Tip Dergesi (in press)

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The PGP phenotypes were demonstrated by means of starch gel electrophoresis. The method was described by *Barker and Hopkinson, Martin et al.* (References see 3).

RESULTS

The patterns of PGP seem to be more indistinct than zymograms of other enzymes - similiar to the isozyme patterns of GPT after starch gel electrophoresis. However, a reliable determination is possible.
Table 1 shows the distribution of phenotypes in our study. We found a good agreement between the observed and expected values according to the *Hardy-Weinberg* law, the gene frequencies were calculated as: PGP^1 .8606 PGP^2 .0964 and PGP^3 .0424 .We analysed the distribution of phenotypes in 688 mother child pairs. As expected we did not find any mother child exclusion. This study includes 502 "critical" mother child pairs (Tab.2)
We combined this study and the study of *Henke et al.* (2). Now the combined studies on PGP polymorphism in Düsseldorf include 2145 individuals
Combining 9 studies on PGP polymorphism in Germany the combined studies include 10524 individuals and show the distribution of phenotypes in Germany. There is a good agreement between observed and expected values. The combined gene frequencies were calculated as: PGP^1 .8551 PGP^2 .1059 and PGP^3 .0390.

DISCUSSION

Though the PGP typing by means of starch gel electrophoresis may not have been optimal there are no difficulties for a proficient examiner to distinguish the different phenotypes. But it is absolutely necessary to use a wellknown PGP^{1-3} as control .
When adding the studies of other authors data of 10524 examined individuals have been published in Germany. Because of its AVACH-value (more than 12 %) PGP is a useful marker in paternity testing
Our investigation of 688 mother child pairs agrees with the data reported by other authors (Referances see 3). Thus our results confirm the formal hypothesis, that PGP polymorphism is controlled by three codominant alleles at an autosomal locus. The analysis of the "critical" mother child pairs did not show any deviation from *Mendelian* proportions - such as reported by other authors. Only in one case a "silent" allele was found (*Weber*, 1986).
Henke (2) reported about a low frequency of PGP^3 in Düsseldorf, significantly lower than in Bonn (1). We cannot support this suggestion. The PGP^3 frequency reported by *Brink* et al. is high - possibly due to the small number of investigated cases - but the PGP frequencies in Düsseldorf are very similiar to those in other countries of Germany.

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Additional Data on PGP (EC 3.1.3.18) Polymorphism in Düsseldorf

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SUMMARY

Blood samples from 1582 unrelated German individuals in the Düsseldorf area were studied for phosphoglycolate phosphatase polymorphism. The number of the observed phenotypes did not diverge from the expected values (Hardy-Weinberg).
The gene frequencies were calculated as: PGP^1 .8606 PGP^2 .0964 PGP^3 .0424 . The assumed autosomal codominant mode of inheritance was confirmed by the analysis of 688 mother child data, including 501 "critical" mother child pairs. The assumption that there is a lower PGP^3 frequency in the Düsseldorf area could not be confirmed.
Several other PGP studies are discussed and compared.

INTRODUCTION

Phosphoglycolate phosphatase (PGP) was first described in plant tissues (*Richardson and Tolbert, 1961; Randall, Tolbert and Gremel, 1971*).
In 1977 *Badwey* described the enzyme activity in human erythrocytes. It remains conceivable that PGP activity plays some part in the regulation of oxygen transfer to and from haemoglobin (*Rose, 1976; Rose and Liebowitz, 1970*).
There is no evidence to suggest that different electrophoretic variants of PGP are associated with different levels of activity but day-to-day variation in PGP activity even in the same individual has been reported (*Turner and Hopkinson, 1981*).
The results of several authors indicate the localization of the gene for human PGP on the chromosome 16 (*Weil et al., 1979; Povey et al., 1980; Gal et al., 1986*).
Six phenotypes - due to three alleles PGP^1, PGP^2 and PGP^3 - can be separated by electrophoresis. A rare allele was described as $PGP^{Sumatra}$ (*Henke et al., 1985*). *Weber* reported about a "silent" allele PGP^0.
Close linkage between PGP and the markers ABO, acP, ADA, PGM_1, PGM_3, GLO and HLA could be ruled out. Close linkage to other markers could not be proved (*Siebert et al., 1980*)(References see 3).

MATERIALS AND METHODS

Blood samples of 1582 unrelated and apparently healthy Germans living in the Düsseldorf area were investigated. 688 mother child pairs were analysed.

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Results

Unambigious results could be obtained with 58 % of the bloodstains, 51 % of the seminal stains, and 5 % of the hair roots; doubtful results were found with 6 % of the bloodstains, 12 % of the seminal stains, and 3 % of the hair roots. Due to small sample size or advanced inactivation of the enzymes, clearly readable zymograms could not be obtained with 36 % of the bloodstains, 37 % of the seminal stains, and 92 % of the hair roots. Seminal stains sometimes showed a mixture of the victim's and the accused's phenotype. 6 bloodstains showed additional bands being either in the region of the 1- or the 2+ band. These stains simultaneously exhibited misleading results in the ABO-system (aquired B).

Discussion

Concerning the bloodstains the percentage of unambigious results in the present study is in good agreement with those of other investigations (Budowle et al. 1986). With seminal stains Budowle et al. (1986), however, found considerably less usable results (16 %), whereas Oya et al. (1984) obtained positive zymograms in 40 %. It is remarkable that Budowle et al. (1986) and Oya et al. (1984) in their examinations could not find one single false result. Berg et al. (1979, 1981), Schwerd and Hein (1983), Rittner et al. (1981) and Oya et al. (1984) had observed additional bands in the 1- and 2+ region with 1+ phenotypes, additional bands in the 2+ region with 1+ 2- phenotypes, and a disappearing of the 1- band with 1+ 1- phenotypes with artificially made bloodstains, respectively blood samples stored for months as well as with cadaver tissue.

We could show by an additional electrophoretic separation of stored blood samples in agarose gel the 2+ band of 1+ phenotypes as degradation artefact.

Finally it should be pointed out that these additional bands may even pose difficulties to persons experienced in PGM subtyping. In our material such additional bands, however, occurred in less than 2 % of the examined bloodstains; therefore PGM 1 subtyping of stains can be recommended for routine cases as a reliable method.

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Practical Value of PGM 1 Subtyping in Blood- and Semen Stains and Hair Roots

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Introduction

There is no doubt about the value of PGM 1 typing of bloodstains. The first investigations using starch gel electrophoresis were done by Culliford (1967), Heidel (1968) and Brinkmann (1969). The amount of stain needed was extremely large (between 10 and 50 mg of dried blood). With the introduction of agarose gel electrophoresis (Monn 1968; Culliford 1971; Grunbaum 1974) and cellulose acetate electrophoresis (Zajac and Sprague 1975) the quantity of stain could be reduced considerably. Isoelectric focusing allowed subtyping of PGM 1 of fresh blood and stains with a significantly higher value of information than before (Bark et al. 1976). Especially Berg et al. (1979, 1981), Schwerd and Hein (1983), Rittner et al. (1981), Oya et al. (1984) and Lawton and Kerr (1984) pointed out that additional or conversional bands occur with old blood samples respectively artificially prepared bloodstains as well as with cadaver tissue, thus leading to a false zymograms interpretation in some cases. Such conversional bands were not reported on in the few field studies published so far (blood- and seminal stains: Budowle et al. 1986; seminal stains: Oya et al. 1982). Therefore further examinations with practical cases seemed to be desirable.

Material and Method

The results of 370 blood- and 197 seminal stains as well as 40 hair roots in PGM 1 subtyping were interpreted. They were obtained from 81 different forensic cases from the Erlangen-Nürnberg Institute of Forensic Medicine which in their majority meant rape (46) and homicide (29).

The preparation of the samples was carried out depending on the carrier material. Whereas we did not pretreat bloodstains on cloth, cotton wool, and paper, we transferred blood- and seminal stains on other carriers (e.g. glass, metal, and others) to cotton tissue after having them partially dissolved. Bloodstains on cotton cloth and the like having a size of 0,1 to 1 cm2 were cut into small strips or fibrillated and then incubated with 1-2 drops of dithiothreitol solution (8 mg dithiothreitol + 1 ml aqua bidest) in a filter sampler (Porex Medical, no. FS 410) at room temperature for 2 hours. The sampler was put into an Eppendorf tube and centrifuged at 2000 g. The eluate at the bottom of the tube was placed onto the gel using an applicator strip (Serva, no. 42989) in a distance of 1 cm from the anode. Focusing was done in commercial gels with immobilised pH gradients (LKB Immobiline Dry Plate, pH 5,6-6,6). Gel's rehydration was carried out according to the manufacturer's recommendation. Focusing at 5000 V, 4 mA, and 12 W; prefocusing 30 min, focusing 3 h. Enzyme specific agarose overlay staining as described by Spielmann and Kühnl (1982).

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Phenotypes	1A2B	1B2A	Sum
Mothers	2ps x N=1325.3	2qr x N=840.7	2166.0
Inform. families	2ps x N x (q+r)=423.7	2qr x N x (p+s)=571.0	994.7

Recombination freq.=recomb.children/tot.children=5/994.7=0.005

All cases where there is a risk for a false paternity exclusion due to recombination in the oogenesis are listed in Table 2 and the excluding capacity of each such case is also shown. Thus if PGM_1 is used as a single excluding system in paternity testing, it is advisable not to exclude fathers in these combinations. In this way the exclusion capacity of the PGM_1 system in paternity testing in Sweden will be reduced from 33.6 % to 30.8 %. However, one must have in mind that recombination can also occur during the spermatogenesis, though more unusual.

With the knowledge of the above mentioned limitations, the conclusion must still be that the PGM_1 system is a valuable tool in paternity investigations.

Table 2. Parent-child combinations where a recombination causes false exclusions

Mother	Child	Father	E %
1A2B	1A1B	1A	0.0050
		1A2A	0.0024
		1A2B	0.0011
1A2B	1A2A	1A	0.0047
		1A1B	0.0025
		1A2B	0.0010
1A2B	1B2B	2B	0.0001
		1A2B	0.0011
		2A2B	0.0003
1A2B	2A2B	2B	0.0001
		1A2B	0.0010
		1B2B	0.0003
1B2A	1A1B	1B	0.0008
		1B2A	0.0016
		1B2B	0.0007
1B2A	1A2A	2A	0.0008
		1B2A	0.0016
		2A2B	0.0006
1B2A	1B2B	1B	0.0001
		1A1B	0.0007
		1B2A	0.0002
1B2A	2A2B	2A	0.0001
		1A2A	0.0006
		1B2A	0.0002
		Tot.	0.0276

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MATERIAL AND METHODS

Blood samples from 16 463 parent-child pairs involved in paternity cases in Sweden during 1980-1988 have been typed for PGM_1 by isoelectric focusing according to an previously published method by Wetterling (1986).

RESULTS AND DISCUSSION

Eight cases of recombination were observed in the material shown in Table 1. Five of these were found because none of the PGM_1 alleles of the heterozygote mother had been inherited by the child. Three cases were observed as a result of single paternity exclusions in the PGM_1 system despite very high probability of paternity. They are not included in the calculation of the recombination fraction.

In all these cases the recombination has occurred during the oogenesis. A cross-over during the spermatogenesis seems to give less viable sperms. However, one such case could be found in Dr Bertrams material (Bertrams 1988).

Table 1. Isolated PGM exclusions during 1980-1988 (16 463 families tested). The recombinant haplotype marked with an asterisk

Mother-child exclusions				
Case	Mother	Child	Father	Probability of maternity
1	1B2A	1A*1A	1A1B	95.3
2	1A2B	1B*2A	1A2A	99.9
3	1B2A	1A*1A	1A1B	85.5
4	1A2B	1B2A*	1A1B	98.4
5	1B2A	1A2B*	1A1B	83.4
Father-child exclusions				
Case	Mother	Child	Father	Probability of paternity
6	1A2B	1A1B*	1A	99.8
7	1A2B	1A1B*	1A	>99.9
8	1A2B	1A1B*	1A2A	99.8

The recombination frequency is calculated as follows (J. Valentin, Stockholm, personal commun.):

Number of mother-child pairs (N)=16 463, number of recombinants (n)=5

PGM_1 1A(p)=0.6136 1B(q)=0.1642 2A(r)=0.1555 2B(s)=0.0656

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Intragenic recombination within the PGM_1 locus

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INTRODUCTION

The formal genetics of the PGM_1 system has been questioned due to unexplained maternity exclusions and single paternity exclusions (Wetterling 1986, 1988; Bertrams 1988).

In 1979 Carter et al described a phylogeny theory of the PGM_1 locus, which later was further developed by Takahashi et al (1982). They proposed that two point mutations and one intragenic recombination had produced the four common PGM_1 alleles defined today. From gene frequency studies and PGM_1 typings on higher primates the PGM_1 1A allele was considered to be the ancestral gene. Two point mutations at this locus gave the PGM_1 2A and 1B alleles. In addition a cross-over between the 1B and 2A alleles resulted in the PGM_1 2B allele (Fig. 1).

Thus the PGM_1 locus can be divided into two subloci and it would be more correct to refer to the PGM_1 system as haplotypes rather than alleles. The exclusions reported are examples of cross-overs in indivuduals with the phenotypes 1A2B and 1B2A. These two types are the only genotypes that can give rise to recombinant gametes, and this verifies the theory.

The aim of this report is to describe the consequences of the PGM_1 system as an excluding system in paternity cases.

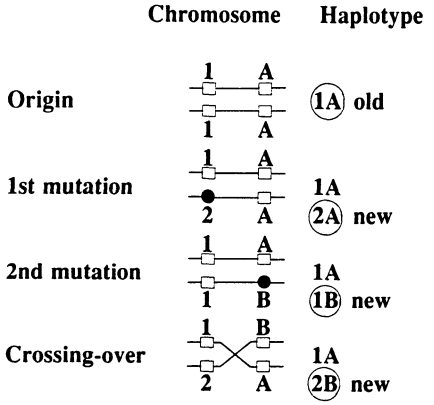


Fig. 1. Phylogeny theory of the PGM_1 locus

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Table 1 shows enzyme activities of the partial deficient genotype ADA 1/ ADA Q0 in comparison to normal ADA 1 homozygotes. The ADA activity in erythrocytes from the mother and her child is approximately 50% of normal controls.

Table 2 gives the ADA phenotype distribution and allele frequencies in a sample from Southern Germany. The observed and expected values are in good agreement with the Hardy-Weinberg equilibrium. ADA*1 was found as the most frequent allele in many populations (Prokop and Göhler 1986). In Caucasian populations the frequency varies between 0.91 and 0.96. The occurrence of variant and silent alleles seems to be very infrequent in the ADA system. Family studies have shown no exception from an autosomal transmission of ADA alleles in 614 parents with a total of 627 children (Table 3).

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Table 1. Enzyme activities of normal and partial deficient ADA genotypes

	Genotype	Enzyme activity (nMol/h x mg Hb)	
		ADA	PNP
Controls (n = 11)	ADA 1/ADA 1	41.7 ± 7.0	1751 ± 163
Mother	ADA 1/ADA Q0	17.9	1756
Child	ADA 1/ADA Q0	23.3	1798

Table 2. ADA phenotypes and allele frequencies in a population sample from Southern Germany

Phenotype	Observed		Expected		Allele
	n	%	n	%	frequencies
ADA 1	1738	88.09	1740.38	88.21	ADA*1= 0.9392
2-1	229	11.61	224.59	11.38	ADA*2= 0.0606
2	5	0.25	7.25	0.37	ADA*6= 0.0002
6-1	1	0.05	0.78	0.04	
Total	1973	100.00	1973.00	100.00	

$\Sigma\chi^2 = 0.8503$; df = 2; P> 0.20

Table 3. Distribution of ADA phenotypes in 614 parents with 627 children

Matings		Children's phenotype					Total
	n	1	2-1	2	6-1	1-Q0	
ADA 1 x 1	497	508	-	-	-	-	508
1 x 2-1	99	53	48	-	-	-	101
1 x 2	2	-	2	-	-	-	2
2-1 x 2-1	14	6	8	0	-	-	14
1 x 6-1	1	0	-	-	1	-	1
1 x 1-Q0	1	0	-	-	-	1	1
Total	614	567	58	0	1	1	627

daughter from Eggenfelden, Bavaria. There was no alteration in the electrophoretic pattern if samples were treated with reducing agents. This rare variant was first observed in two individuals of a family from Schwerin (Radam et al. 1974) and later in a family from Düsseldorf (Manz et al. 1979). It has been found also in an Arab Moslem family from Northern Israel (Nevo 1977) and in a Japanese population (Komatsu et al. 1987).

Figure 2 demonstrates the band pattern of ADA 1-Q0 side by side with common phenotypes ADA 1 and ADA 2-1. The silent ADA*Q0 allele was found in a mother and her son from Augsburg, Bavaria. Both individuals are apparently healthy.

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substrate concentrations were 0.25 mM adenosine or 0.5 mM inosine for ADA and PNP, respectively. Twenty mU of milk xanthine oxidase (Boehringer) were added in both tests. Formation of uric acid in both assays was followed at 293 nm with $\varepsilon = 12.5\ cm^2/\mu Mol$. Activities were routinely tested at two different hemolysate concentrations to check for linearity.

RESULTS and DISCUSSION

In Fig. 1 the electrophoretic patterns of the three common phenotypes ADA 1, 2-1 and 2 are shown in comparison to a rare ADA phenotype. The faster migrating variant, which is similar to ADA 6, is characterized by two main bands anodal to ADA 1. It was observed as phenotype ADA 6-1 in a mother and her

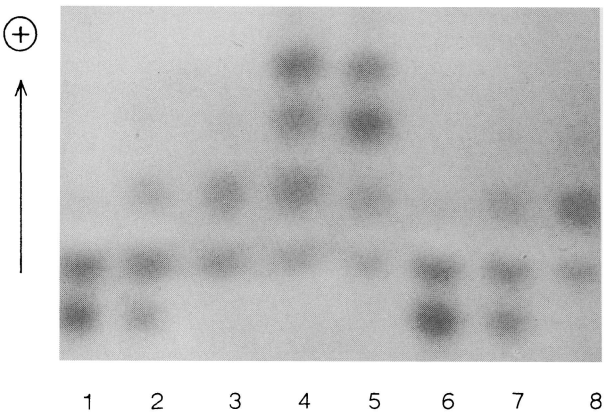


Fig. 1. Patterns of erythrocyte ADA isozymes obtained by starch gel electrophoresis. The phenotypes were: (1,6) ADA 2, (2,7) ADA 2-1, (3,8) ADA 1, (4) ADA 6-1, mother and (5) ADA 6-1, daughter

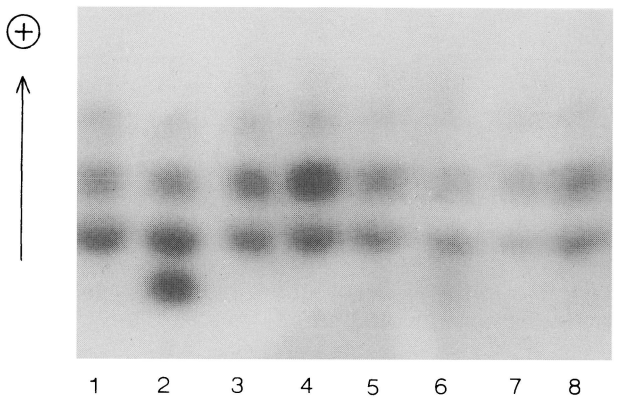


Fig. 2. Pattern of the partial deficient ADA 1-Q0 types from a mother (6) and her son (7) in comparison with normal phenotypes

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Further Evidence for a Silent Allele and a Rare Variant in the ADA System

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INTRODUCTION

Genetic polymorphism of human erythrocyte adenosine deaminase (ADA, EC.3.5.4.4) was discovered by Spencer et al. (1968) using starch gel electrophoresis. Three common phenotypes, designated ADA 1, 2-1 and 2 were due to two autosomal codominant alleles ADA*1 and ADA*2. Several rare electrophoretic variants, ADA 3(Hopkinson et al. 1969), ADA 4 (Dissing and Knudsen 1969), ADA 5 (Detter et al. 1970; Renninger and Bimboese 1970), ADA 6 (Radam et al. 1974), ADA 7 (Berg et al. 1975), ADA 8 (Jenkins et al. 1976), and ADA 9 (Nenkov et al. 1981; Henke et al. 1986) were subsequently found by family and population studies. There is also evidence for the existence of a silent allele (Giblett et al. 1972; Brinkmann et al. 1973). Deficiency of ADA with very low or no activity in red and white cells is associated with severe combined immune deficiency disease (Giblett et al. 1972; Dissing and Knudsen 1972; Chen et al. 1974). A point mutation in the ADA sequence appears to be responsible for the loss of function in this allele (Bonthron et al. 1985). Heterozygotes for ADA*Q0 are apparently healthy. The ADA locus has been assigned to the long arm of chromosome 20 (Tischfield et al.1974; Mohandas et al. 1984).

In this paper we present further evidence for the existence of a silent allele and a rare variant in the ADA system. Population and family data from a study in Southern Germany are given.

MATERIALS and METHODS

Blood specimens were collected from individuals involved in paternity cases. Erythrocytes were washed twice in physiological saline and hemolysed by sonification. For ADA phenotyping, horizontal starch gel electrophoresis was carried out at 5°C and a field strength of 10 V/cm for 15 h with a sodium phosphate buffer, pH 6.5 (0.01 M for gels and 0.15 M for electrode chambers). The gels were stained according to the method of Spencer et al. (1968).

Hemolysates for the enzyme assays were prepared by lysis 1 vol of washed packed erythrocytes in 3 vol of distilled water, two additional 15 sec pulses with a sonifier and removal of the membranes by centrifugation at $48000 \times g_{max}$ for 30 min. Hemoglobin was determined by the cyan-methemoglobin method using the Merckotest® reagent.

Adenosine deaminase and purine nucleoside phosphorylase (PNP) were assayed in coupled tests. In a total volume of 1 ml 50 mM potassium phosphate, pH 7.4,

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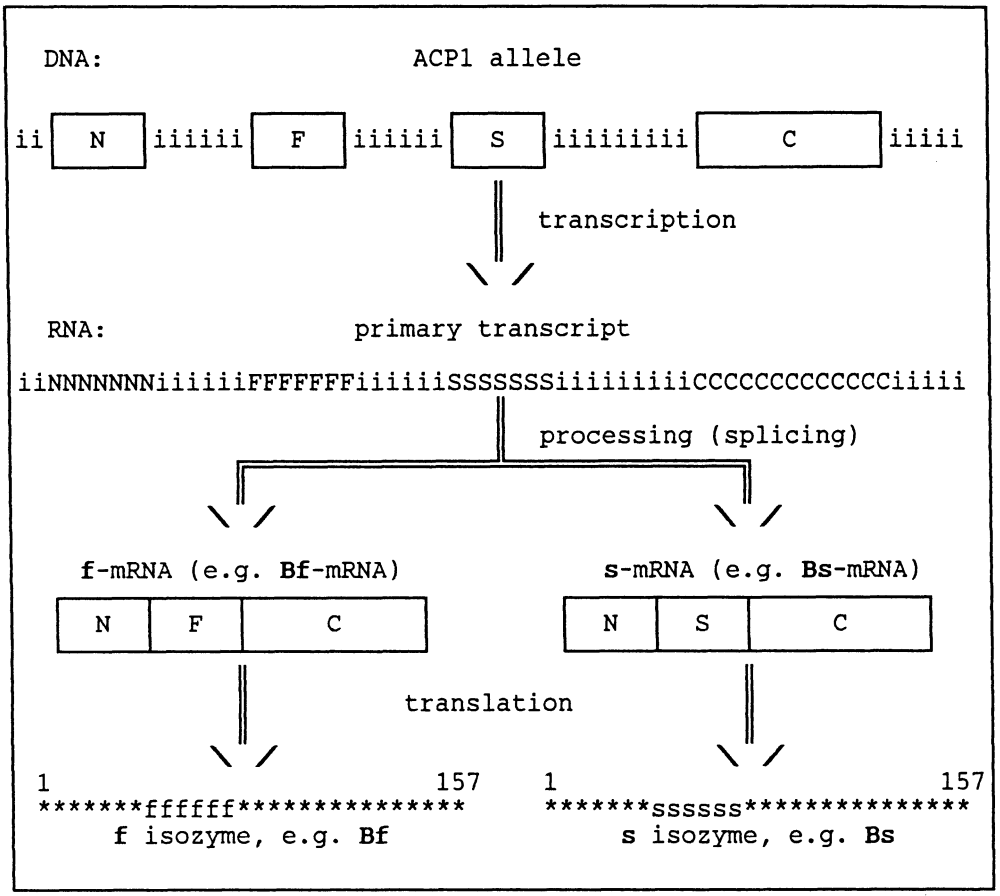


Fig. 2. Hypothetical mechanism for the synthesis of acid phosphatase **f** and **s** isozyme. N, F, S, C = ACP1 exons; i = ACP1 introns.

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molecule contains one more basic amino acid (histidine) than the **Bf** molecule. This is consistent with the lower anodal mobility of the **Bs** isozyme.

The finding of a **Bf** or **Bs** specific sequence within the acid phosphatase molecule rules out both the conformer and the post translational modification hypotheses. On the other hand it is consistent with the hypothesis of alternative splicing. Assuming that the results obtained with the **B** isozymes are general for ACP1 isozymes, it may be predicted that ACP1 alleles consist of at least 4 exons, one of which codes for the **f** specific region, another for the **s** specific region, yet another for the N-terminal (N) region and the remaining exon(s) for the C-terminal (C) region (fig. 2). A mutational event occurring in a N or a C exon would alter both the **f**-mRNA and the **s**-mRNA, and the effect on the amino acid sequence of the **f** and the **s** isozyme determined by the new allele would be the same. The hypothesis of alternative splicing therefore offers a simple explanation for the pair wise variation in electrophoretic properties observed for most allozymes. The genotypic variation of the **f/s** ratio may be due to allelic differences near to the splice sites e.g. within the introns.

The finding of specific **f** and **s** sequences offers the biochemical basis for the different catalytic and molecular properties of **f** and **s** isozymes, and it suggests that **f** and **s** isozymes may serve different biological functions in the cell.

Bf isozyme (Mw; 17,916):

Ac-Ala[1]-Glu-Gln-Ala-Thr-Lys-Ser-Val-Leu-Phe-Val-Cys-Leu-Gly-
Asn-Ile-Cys-Arg-Ser-Pro-Ile-Ala-Glu-Ala-Val-Phe-Arg-Lys-Leu-
Val-Thr-Asp-Gln-Asn-Ile-Ser-Glu-Asn-Trp-**Arg[40]-Val-Asp-Ser-Ala-**
Ala-Thr-Ser-Gly-Tyr-Glu-Ile-Gly-Asn-Pro-Pro-Asp-Tyr-Arg-Gly-
Gln-Ser-Cys-Met-Lys-Arg-His-Gly-Ile-Pro-Met-Ser-His-Val[73]-
Ala-Arg-Gln-Ile-Thr-Lys-Glu-Asp-Phe-Ala-Thr-Phe-Asp-Tyr-Ile-
Leu-Cys-Met-Asp-Glu-Ser-Asn-Leu-Arg-Asp-Leu-Asn-Arg-Lys-Ser-
Asn-Gln-Val-Lys-Thr-Cys-Lys-Ala-Lys-Ile-Glu-Leu-Leu-Gly-Ser-
Tyr-Asp-Pro-Gln-Lys-Gln-Leu-Ile-Ile-Glu-Asp-Pro-Tyr-Tyr-Gly-
Asn-Asp-Ser-Asp-Phe-Glu-Thr-Val-Tyr-Gln-Gln-Cys-Val-Arg-Cys-
Cys-Arg-Ala-Phe-Leu-Glu-Lys-Ala-His[157]-(?)

Bs isozyme (Mw; 17,851), specific sequence:

-Val[40]-Ile-Asp-Ser-Gly-Ala-Val-Ser-Asp-Trp-Asn-Val-Gly-Arg-
Ser-Pro-Asp-Pro-Arg-Ala-Val-Ser-Cys-Leu-Arg-Asn-His-Gly-Ile-
His-Thr-Ala-His-Lys[73]-

Fig. 1. Amino acid sequence of the **Bf** and **Bs** isozymes encoded by the ACP1*B allele. The sequences printed in boldface are **Bf** or **Bs** specific. The N-terminal alanine residue is blocked (acetylated) and the underlined sequence was deduced from the amino acid composition of an endoproteinase Glu-C peptide and a tryptic peptide containing the 2 first and the 6 first amino acid residues, respectively, and by FAB mass spectrometry[1] of the latter peptide. The C-terminal has yet to be confirmed.

[1]Kindly performed by Dr. P. Højrup, University of Odense

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Human Red Cell Acid Phosphatase (ACP1): The Primary Structure of the two Isozymes **Bf** and **Bs** Encoded by the ACP1*B allele

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Being one of the good informative genetic markers, red cell acid phosphatase has served the forensic scientist for a quarter of a century. Over the years this enzyme has been the subject of many studies, yet some important genetic and biochemical questions still remain to be answered.

One of these questions concerns the mechanism by which each allele generates two electrophoretically different isozymes, **f** and **s** (Hopkinson et al. 1964). This isozyme pair is produced in allele specific ratios (Dissing 1987). The **f** isozymes differ from the **s** isozymes with respect to a number of catalytic and molecular properties, while the **f** isozymes are indistinguishable from one another, as are the **s** isozymes (Fenton and Richardson 1971, Dissing 1986, Dissing and Svensmark). The genotypic difference in the relative content of **f** and **s** isozyme may account for the different properties of the various acid phosphatase phenotypes (Dissing 1986).

We have recently detected major chemical differences between the **Bf** and **Bs** isozymes (Dissing and Sensabaugh 1987). Digestion of **Bf** and **Bs** with trypsin yielded 17 and 15 peptides, respectively. Five peptides were unique to the **Bf** isozyme and three to the **Bs** isozyme. This excluded the possibility that the two isozymes are conformational isomers, which has been generally assumed for almost two decades (Harris 1980). It also argued against that one isozyme is the result of a simple post-translational modification of the other. It was suggested that the **f** and **s** isozymes may be the result of alternative splicing of the primary RNA transcript (Dissing and Sensabaugh 1987).

To test this hypothesis the tryptic peptides as well as peptides generated by cleavage by two other proteinases (endoproteinase Glu-C and endoproteinase Asp-N) have been sequenced. Overlapping sequences from the various peptides allowed the construction of the primary structure of the **Bf** and **Bs** isozymes (fig. 1).

Both isozymes consist of a single peptide chain. The **Bf** and **Bs** chains are identical with respect to both the N-terminal and the C-terminal regions representing approximately 4/5 of the molecule. However, starting with amino acid residue 40 each chain contains an internal section of 34 residues that is peculiar either to the **Bf** or to the **Bs** molecule. It is noted that while the two isozymes contain the same number of acidic amino acid residues the **Bs**

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Martell RW, Oudshoorn M Arendse B, du Toit ED (submitted for publication) Polymorphism of DRw52 and its association with DRw11 and DRw12 in South African blacks (Negroes) and individuals of mixed ancestry (Cape Coloureds). Hum Immunol

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were observed for the various DRw53 haplotypes. The 14.5 and 2.8 kb fragments were seen in all DRw53 haplotypes and are associated with the DRB4 gene. In addition, four restriction fragments associated only with DRw53 haplotypes occurred in an allelic pattern (Table 2). The first pattern, provisionally designated DRw53a, was characterized by the 7.5 and 2.6 kb fragments and the second, DRw53b, was characterized by 5.8 and 2.7 kb fragments. The patterns clearly segregated in several families (Martell et al. 1989).

CONCLUSION

This study has demonstrated the existence of another allele of the DRB3 gene provisionally designated DRw52d as well as polymorphism of the HLA-DRw53 specificity which is encoded by the DRB4 gene. We have provisionally called these alleles DRw53a and DRw53b. The two allelic DRw53 patterns have previously been described in association only with DR7, and were used to split this specificity into two subtypes (Bidwell 1988). We have observed the same two RFLP patterns in DR4, DR7 and DR9 haplotypes of Negroid origin, indicating these to be as a result of a split of DRw53. It should, however, be noted that several published sequences of DRw53 have not shown polymorphism, and it may be that the RFLPs defining the DRw53 alleles in this study are due to sequence variations in a non-coding portion of the gene, and would thus not be detectable at a product level.

This study demonstrates the importance of the new histocompatibility typing techniques, in order to reveal polymorphic variants not previously detectable and which appear to be specific to populations of SA Negroid origin. The identification of new variants will improve the degree of HLA class II matching in organ transplantation, particularly when genetically unrelated donor-recipient pairs are considered. These new variants may also provide more precise disease susceptibility markers, and will increase the usefulness of the HLA system as a forensic tool.

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transfer to a nylon membrane. The membranes were hybridized with the Tenth Histocompatibility Workshop DRB probe, radiolabelled with [α-^{32}P]dCTP (Marcadet et al. 1989).

RESULTS

The DRw52 related haplotypes DR3, DR5, and DRw6, showed the expected DRB1 RFLPs. Table 1 demonstrates the TaqI restriction fragments associated with the DRB3 gene on the various DRw52 haplotypes. In addition to the well documented DRB3 fragments, 10.3 kb (DRw52a or c) and 12.2 kb (DRw52b), a DRB3 fragment of 11.0 kb, which identified a new allele provisionally called DRw52d, was seen to segregate in three families (Martell et al. 1989). This novel fragment was only seen in SA blacks and individuals of mixed ancestry and was always associated with DRw11(5).

Table 1. The TaqI restriction fragments seen with the various DRw52 haplotypes.

Allele	TaqI RFLPs
DRw52a or c	10.3 kb
DRw52b	12.2 kb
DRw52d	11.0 kb

Table 2. Restriction fragments associated with the DRw53 haplotypes, using the DRB probe and TaqI.

	TaqI RFLPs (kb)					
	DRw53		DRw53a		DRw53b	
HLA-DR	14.5	2.8	7.5	2.6	5.8	2.7
4	+	+	+	+	-	-
7	+	+	+	+	-	-
9	+	+	+	+	-	-
4	+	+	-	-	+	+
7	+	+	-	-	+	+
9	+	+	-	-	+	+

Table 2 demonstrates the pattern of TaqI DRB RFLPs seen with the 33 different DRw53 haplotypes. These haplotypes consisted of 19 DR4, 10 DR7 and 4 DR9 haplotypes. All the subjects studied were SA black or of mixed ancestry, with the exception of 4 SA white individuals. The usual DRB1 fragments correlating with DR4 (5.5 kb), DR7 (4.0 kb) and DR9 (4.1 kb)

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POLYMORPHISM OF HLA-DRB3 AND DRB4 GENES DETECTED BY RFLPs

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INTRODUCTION

All the characterized human HLA class II genes are encoded within the major histocompatibility complex (MHC) on the short arm of chromosome six. Among the five subregions of the class II region, HLA-DR is unique, in that the grouping of the beta chain genes is not the same for all haplotypes. The DRBI gene codes for the serologically detected DR1 to DRw18 antigens, while the DRB2 gene is probably a pseudogene in most haplotypes. The DRw52 and DRw53 supertypic antigens are coded for by two genes, DRB3 and DRB4, which are not allelic and are not present on all haplotypes. There is only one DRA gene which is not polymorphic.

The alleles of the DRB3 locus of the DRw52 family of haplotypes have been extensively analysed and are closely associated with the DRB1 alleles DRw11(5), DRw12(5), DRw13(6), DRw14(6), DRw17(3) and DRw18(3). Three DRB3 alleles have been recognized by a variety of reagents at the DNA, protein, and cellular level (Tiercy et al. 1988, Termijtelen et al. 1988). These alleles are referred to as DRw52a(Dw24), DRw52b(Dw25) and DRw52c(Dw26). The DRB4 gene, which is supertypic to the DR4, DR7 and DR9 antigens, has until now not shown any polymorphism.

In this study we have analysed further the polymorphism of the DRB3 and DRB4 genes using a full-length DRB cDNA probe and the restriction enzyme TaqI.

MATERIALS AND METHODS

Subjects

We studied 92 DRw52 and 33 DRw53 haplotypes in South African (SA) blacks, whites and individuals of mixed ancestry (Cape Coloureds) (du Toit et al. 1988). ALL subjects were HLA typed using standard serological techniques.

Southern Blot Analysis

The Southern Blot technique involved the digestion of genomic DNA with TaqI, electrophoresis in agarose gel followed by

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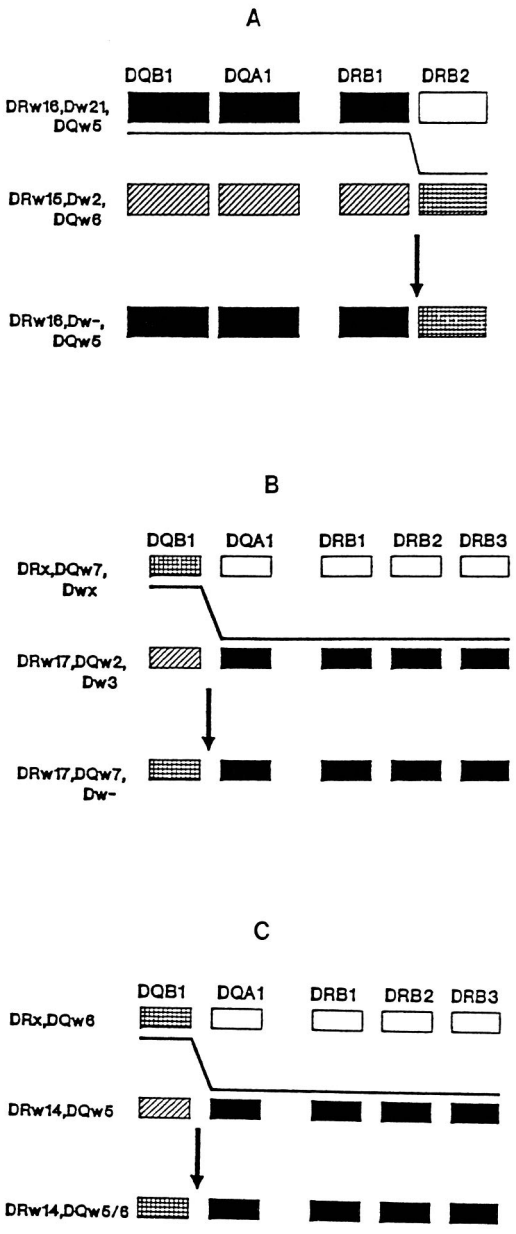


Fig. 1. Schematic diagram illustrating the probable site of recombination to generate (A) the DRw16(HER),DQw5,Dw"blank", (B) the DRw17,DQw7,Dw"blank" and (C) the DRw14,DQw1(MDI) haplotypes.

indicate the second expressed DRB gene in this paper) as shown in Fig. 1A. The results obtained for the DRw17,DQw7 haplotype indicate that this haplotype may have originated from a homologous cross-over between the DQB1 and DQA1 genes of a DRw17,DQw2 and a DQw7 haplotype (Fig. 1B) similar to that described for the DR4,DQw7 haplotype (Holbeck and Nepom 1988). The DQB RFLP pattern of the DRw17,DQw7 haplotype was identical to that of DQw7 haplotypes, whereas its DQA RFLP pattern was identical to that of DRw17,DQw2 haplotypes indicating that the serological DQw7 determinant resides on the DQB chain rather than the DQA chain. Fig. 1C shows the proposed recombination event for the generation of the DRw14,DQw1(MDI) haplotype indicating that this haplotype could also have originated from a cross-over between the DQA1 and DQB1 genes.

This study showed that recombinational events may have occurred between the DRB genes as well as the DQA and DQB genes during evolution and that they may be important elements in generating class II haplotype diversity. Of particular importance are the recombinational events between the DQA and DQB genes as they might result in new specificities, thus enhancing the degree of class II polymorphism.

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HLA-Dw typing was performed in duplicate by the method described previously (Oudshoorn et al. 1986). Fifty-six HTCs were used to define the specificities HLA-Dw1 to Dw11, Dw13 to Dw15, Dw17 to Dw19 as well as the specificity DKT2 and three locally defined specificities, Dw'RSH' (Oudshoorn et al. 1989), Dw'BME' and Dw'JOH' (Oudshoorn et al. 1986).

DNA was isolated from peripheral blood lymphocytes, digested with the restriction enzyme TaqI and used for Southern blotting by the method described for the 10WS (Marcadet et al. 1989).

RESULTS

The unusual haplotypes DRw17,DQw7; DRw16(HER),DQw5 and DRw14,DQw1(MDI) were analysed.

The serological patterns observed for the antigens constituting the unusual haplotypes did not differ from those of the "classic" haplotypes. HLA-Dw typing tests performed with the unusual haplotypes revealed no typing responses for any of the unusual haplotypes, indicating that these haplotypes carry unknown HLA-Dw specificities. The DRB and DQA RFLP results of the rare *DRw17,DQw7* haplotype were identical to common DRw17,DQw2,Dw3,Dw25(DRw52b) haplotypes, whereas the DQB RFLP pattern was identical to other DQw7 haplotypes. The *DRw14,DQw1(MDI)* haplotype showed DRB and DQA RFLPs identical to the "classic" DRw14,DQw5 haplotypes, but the DQB RFLP pattern was identical to DQw6 instead of DQw5, which is the allele commonly in linkage disequilibrium with DRw14. The DRB RFLP results of the *DRw16(HER),DQw5* haplotype showed fragments typical of both the normal DRw15 and DRw16 specificities. The results obtained using the DQB probe were identical to other DQw5 haplotypes and that of the DQA probe identical to the patterns observed for DR2 haplotypes such as DRw15,DQw6 and DRw16,DQw5.

CONCLUSION

Population differences in DR,DQ associations have been important in establishing the genetic organization of the HLA-D region (Oudshoorn et al. 1984; Hurley et al. 1989). These ethnically different DR,DQ haplotypes may also provide valuable insight into mechanisms such as homologous and non-homologous crossing-over, point mutation and gene conversion, by which the HLA region has evolved. Three unusual HLA-DR,DQ haplotypes were found in SA blacks and individuals of mixed ancestry. The origin of these haplotypes, DRw17,DQw7; DRw16(HER),DQw5 and DRw14,DQw1(MDI) could be explained by a single cross-over event.

The unusual DRw16(HER),DQw5,Dw"blank" haplotype which types serologically as DRw16 but shows a "hybrid" DRw15/DRw16 on DNA typing, may have arisen from a homologous cross-over between the two expressed DRB genes, DRB1 and "DRB2" (DRB2 was used to

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THE GENERATION OF CLASS II POLYMORPHISM BY RECOMBINATION

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INTRODUCTION

One of the most striking features of the HLA gene products is their high degree of polymorphism. The HLA class II polymorphism varies from locus to locus, e.g. at present there are 18 serological defined antigens at the DR locus and only half that number at the DQ locus (Bodmer et al. 1989). Within a polymorphic gene certain exons are more polymorphic than others, e.g.the gene segments encoding the first domains of DRB, DQA and DQB chains contain most of the sequence variation. Furthermore, even within the first domain, the polymorphism is concentrated in areas called allelic hypervariable regions (Bell et al. 1987). Various mechanisms have been implicated as being responsible for the generation of HLA polymorphism such as homologous and non-homologous crossing-over, point mutation and gene conversion.

Another striking characteristic of the class II region is the strong linkage disequilibrium between DQ and DR antigens and which varies from population to population. For example, Caucasoids who type serologically as HLA-DRw17 almost exclusively type as DQw2. In contrast, South African (SA) blacks with DRw17 can type as DQw2 or DQw7.

Several unusual HLA-DR,DQ haplotypes were found in SA blacks and individuals of mixed ancestry (Cape Coloureds) (du Toit et al. 1988) and studied by serological, cellular and DNA typing techniques to see if they may have arisen as a result of a recombination event.

MATERIALS AND METHODS

Three families with unusual HLA-DR,DQ haplotypes were studied, two of SA black origin and one of mixed ancestry (Cape Coloured). In addition, 25 homozygous typing cells (HTCs) were used in the RFLP analysis.

HLA-A, B and C typing was performed by the NIH complement-dependent microlymphocytotoxicity technique (Terasaki et al. 1974). HLA-DR and DQ typings were performed by the technique agreed upon for the Seventh International Histocompatibility Workshop. All families were typed with the Tenth International Histocompatibility Workshop (10WS) sera.

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Siberia, and North and South America. We have described that Mongoloids are divided into two groups, Southern and Northern group, and we Japanese belong to the Northern Mongoloids and its origin is around the Lake Baikal in Siberia. While the orign of Southern group is in south of China, Yunnan and Kuanhsi area, and people living in such area have over 95%, the highest rate of the Southern Mongoloids gene. About the group from Southèast Asia toward the islands in South Pacific Ocean they have over 70% Gm haplotype characteristic of Southern Mongoloids. So we were able to back up the hypothesis that their ancesters came from south of China. As shown in the table 1, the result of 293 islanders consisted of Rarotongan and Mangaian of Cook islands have 67% of Gm afb1b3 characteristic of Southern Mongoloids, on the contrary, are lacking in Gm ab3st characteristic of Northern Mongoloids.

The first migration of Mongoloids have taken place between 40,000 and 20,000 years ago crossing over the Behring strait from Siberia. This migrations probably lasted a long time in succession. American Indians are all classified as Northern Mongoloids on the basis of Gm pattern. South American Indians on the whole have only 0 - 3% of Gm afb1b3 characteristic of Southern Mongoloids. Accordingly, it became clear that they have little racial relationship with the islanders of Cook islands. Not only Cook islanders but also native inhabitants in Easter island who left giant stone statues Moai have their origin in the same Southeast Asia were also supported from the truth of existence of the giant stone statues, Haamogaa in Tonga and huge stone statues, Giki in Tahichi and also liguistic and archaeological evidences.

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statues called Moai are scattered here and there. Because of reduction of inhabitants owing to capture by slave trade and death from introduced diseases and additionally spread of racial admixture. It is almost impossible now to search their origin by investigating them.

In 1949 Thor Heyerdahl, an anthropologist in Norway, made an experiment on drifting with a raft named Kon-Tiki from Peruvian coast toward Polynesia from a foundation that the stone figures of Easter island were closely akin to those of Pre-Inca. As the result of his experiment, he reported that he proved the possibility of people in Easter island coming from South America and the stone statues orinating in also South America.

However, we judged that natives in Easter island have their origin in South of China from the result of research of distribution of Gm haplotype with Koichi Hiraiwa and others of Fukushima Medical College. So giant stone statues, Moai, were made by Southern Mongoloids who sailed across the sea over ten thousand kms away. The base is that we found islanders of Cook island situated at west of Easter island belong to the Southern Mongoloids by our research. People in islands spreading from Southeast Asia over Polynesia in South Pacific Ocean also belong to them, and it is quite possible that people in Easter island lying to the east of Cook islands belong to the Southern Mongoloids.

Table 1.

Frequencies of Gm phenotypes and haplotypes in Cook islanders.

Phenotype	Obs.No.	Exp.No.	Haplotype	Frequency
ag	15	19.8	ag	0.260
axg	14	13.0	axg	0.075
agfb1b3	110	101.3	ab3st	0.000
axgfb1b3	28	29.1	afb1b3	0.665
afb1b3	126	129.8		
Total	293	293.0		
χ^2= 2.127	d.f.= 1	P=0.146		

We have thus far investigated Mongoloids and neighboring populations of over 95 living over Southeast Asia, East Asia,

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Whose Heritage in the Giant Stone Culture in Easter Island ?

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INTRODUCTION

The genetic markers arise from mutation of existing genes and differences are due to the substitution of a single amino acid. These allotypes are inherited in fixed combinations termed Gm haplotypes. So far, it has been recognized that the Gm haplotypes common to Mongoloid populations are Gm ag, axg, ab3st, and afb1b3; among Caucasoids, Gm ag, axg, and fb1b3; and among Negroids, Gm ab1b3, ab1c, and ab3s. Thus, Mongoloids are characterized by the presence of Gm ab3st and afb1b3 haplotypes and Caucasoids, by Gm fb1b3. No Gm haplotype are found in common between Negloids and Caucasoids or Mongoloids. The Gm system thus provides genetic markers that are unparalleled in studies of human genetics, particularly in the characterization of different populations and in studies of genetic drift and gene flow determined by the presence of either a unique haplotype in a particular race or by differences in the frequencies of the same haplotypes in a given ethnic group. The authors investigated Gm haplotype distribution among the inhabitants on the Cook islands which is close to Easter island within southeasternmost Polynesia to make clear where the native people on the Easter island came from.

RESULT AND DISCUSSION

Easter island, a solitary island known with the giant stone statues, Moai, in the South Pacific Ocean, was discovered by the Dutch admiral Jacob Roggeveen on Easter Sundauy of 1722. In those days Polynesian of great voyage people had already lived on the islands of this area in the South Pacific Ocean. On the slope of this volcanic island about 900 huge stone

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Table Two

Gm3(g) Results

Stored Stains

	Positive	Negative	Inconclusive
App. 18 mo.			
NIB*	6	16	8
Serum sample by HAI**	10	19	1
4–12 years			
NIB*	7	9	0
Serum sample by HAI**	8	8	0

*Stain stored at room temperature
**Test on fresh sample

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NIB Procedure (continued)

8. Wash membrane in stain buffer (TBS, pH 9.1).
9. To membrane add substrate mixture of NBT/BCIP in ratio of 1:3 for 30 minutes. NBT (75 mg NBT, 0.7 ml dimethylformamide, 0.3 ml H_20) and BCIP (50 mg, 1 ml dimethylformamide).
10. Wash membrane briefly in deionized water, blot dry.
11. Dry completely before interpreting.

Bloodstains were extracted by placing six 4mm threads in 50 ul of deionized H_2O for 1 hour at room temperature. Doubling dilutions up to 1:64 were prepared. Serum/plasma samples were prepared by making dilutions of 1:250, 1:500, 1:1000, and 1:2000.

If staining occurred in any dilution, with the exception of the strongest, the test was interpreted as positive for the corresponding antigen. If staining occurred only in the strongest dilution the test was considered negative. If there was no staining in the strongest dilution the test was considered inconclusive.

Homozygous and heterozygous positive controls were used as well as a negative control.

RESULTS

Anti-G3m(b_o) polyclonal reagents were found unsuitable because of lack of specificity or sensitivity. Table One lists the results using anti-G3m(g) on frozen serum/stain samples. All stains show concordance between HAI and NIB for G3m(g) positive samples. On stains stored at room temperature (Table Two) no false positive were observed, however, on many samples results were inconclusive. This was not seen with stains stored for 4 - 12 years at -22°.

CONCLUSIONS

Polyclonal antisera for NIB must be carefully selected and are not as easy to use as monoclonal reagents. Use of this method required appropriate controls and antisera dilutions. Inconclusive results may occur if there is failure to elute the stain. Advantages of testing for GM by NIB with polyclonal reagents include the following: low set-up cost, permanent record of results, high sensitivity, minimal technician time and skills, no interfering antibodies, uncomplicated procedure, and small sample size.

Table One

G3m(g) Results

Serum/Stain Samples

	HAI	NIB	
	Serum	*Serum*	*Stain*
Positive	30	30	30
Negative	28	26	27
Inconclusive	2	4	3

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Allotyping By Immunoblot Using Polyclonal Antisera

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INTRODUCTION

Immunoglobulin allotyping can be valuable for discrimination purposes in the forensic laboratory. Current GM typing procedures include hemagglutination-inhibition (HAI) and enzyme linked immunosorbant assay (ELISA). HAI, the most commonly employed method, has some disadvantages including a high frequency of interfering antibodies as well as being a labor intensive test. The ELISA test method in microtiter plates eliminates some of the problems with HAI. A drawback of the ELISA system is technical complexity. To simplify the ELISA technique we have developed a nitrocellulose immunoblot (NIB) procedure. This procedure was initially developed for use with monoclonal antibodies (Weston 1988). Since limited reagents were available, we wanted to determine if polyclonal antibodies could be utilized.

MATERIALS AND METHODS

Samples used for this study included the following: two year old bloodstains and corresponding serum/plasma samples (33 Black, 2 Caucasian, 15 Amerindian, 10 Mexican-American); bloodstains stored at room temperature for 18 months, and bloodstains stored from 4-12 years. Unless otherwise noted all samples were stored at -22°C. All bloodstains were prepared on clean cotton cloth from samples drawn for the purpose of parentage testing. The blood samples from which all bloodstains were prepared had been allotyped by HAI during routine paternity testing.

For routine paternity testing HAI is performed in V-microtiter plates as follows:

1. 25 ul of appropriately diluted antiserum is added to test wells, 25 ul of 3% BSA is added to screen wells.
2. 25 ul of a 1:20 dilution of serum is added to test wells and screen wells.
3. 25 ul of 0.2% coated red cells are added to all wells.
4. Plates are incubated at 18°C for 60 minutes.
5. Plates are centrifuged.
6. Plates are set on a 60° slant board for 5-10 minutes.
7. Plates are read by streaming.

Three reagents were evaluated including anti-G3m(b_o) from Allotype (R-61), anti G3m(g) from Allotype (R-68), and anti-G3m(b_o) from the Netherlands Red Cross (3955). Evaluation included specificity and appropriate dilutions for samples and reagents.

The NIB procedure used for this study was as follows:

1. Apply 2 ul sample on nitrocellulose, dry overnight.
2. Block 1 hour in TBS (pH 7.5), 3% Liquid Hipure Gelatin, 5% Tween-20, .1% Hammarstein casein.
3. Wash membrane 10 minutes in wash buffer (1% block solution in TSB).
4. To membrane add 1:250 dilution polyclonal anti-Gm reagent in wash buffer. Rotate gently for 1 hr. at room temp.
5. Wash membrane 2 x 5 minutes in wash buffer.
6. To membrane add 1:5000 dilution of alk. phos. conjugated goat anti-rabbit (whole molecule IgG) in wash buffer. Rotate gently for 1 hour at room temp.
7. Wash membrane 3 x 5 minutes in TBS.

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Regrettably, the capture ELISA has a counterbalancing disadvantage in being complex and expensive: plates need to be coated with purified anti-allotype or anti-IgG subclass MAbs as capture antibodies; therefore, it should be set aside for special purposes.

To the best of our knowledge, only eight anti-GM MAbs have been produced in the past six years. At this pace it may take ten more years to produce MAbs against the rest of the known GM allotypes. For our present purposes, however, it is sufficient to have available one MAb each against allotypes representing each haplotype. In the Japanese, for instance, the four haplotypes *GM*AZG1U, GM*AZXG1U, GM*AFNBOB1B3B4B5U* , and *GM*AZBOB3B5ST* with frequencies of 0.4369, 0.1725, 0.1297, and 0.2609, respectively (Matsumoto and Takatsuki, 1968), determine the nine GM phenotypes; accordingly, anti-G1M X, anti-G1M F (or anti-G3M B1), anti-G3M T, and anti-G3M G1 are essential requirements for routine phenotyping. We have already produced the latter three MAbs, but anti-G1M X is yet to be produced. Finally, the quantitative analysis of GM allotypes requires 100%-pure allotype-specific mollecules of IgG to be used as a standard in a competitive or capture ELISA. They can be isolated from IgG by affinity chromatography only on an anti-GM MAb column. For that purpose it is necessary to obtain large quantities of ascitic fluids containing anti-GM MAb.

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(Kishida 1985; Kimura et al. 1989): IgG3 (G3M G1), for anti-G3M G1; IgG3 (G3M B3ST), for anti-G3M T; $F(ab')_2$ fragments (practically G1M F) of IgG1-enriched IgG (GM AFB1B3), for anti-G1M F. BALB/c mice were immunized by a subcutaneous injection into hind feet of 20 ug antigen in FCA. On day 10, their popliteal lymph node cells were fused with P3U1 myeloma cells. The hybridomas were cultured, selected, screened for antibody production (by an inhibition-ELISA in IgG-coated plates), and cloned, by the standard technique except that autoclavable serum-free culture medium ASF-103 (Ajinomoto Co., Inc., Tokyo, Japan) was used. Antibody-producing hybridomas were grown in mice for collection of ascitic fluids.

For GM typing by DIB, serial double dilutions of serum samples in tris-buffered saline (TBS), pH 7.5, were applied in the form of a dot (0.5 ul), and detected with the MAb and HRP-labeled anti-mouse IgG. The incubation time was 30 min each; the diluent was 0.5% BSA in 0.02 M TBS/0.1% Tween 20; the color development reagent contained 30 mg 4-chloro-1-naphthol, 10 ml methanol, 50 ml TBS, and 25 ul of 30% hydrogen peroxide.

RESULTS AND DISCUSSION

Mouse IgG1 MAbs against G1M F, G3M T, and G3M G1 were produced whose titers were up to 1 : 640 000 in the indirect ELISA. None of the MAbs reacted with GM-negative IgG. DIB allowed detection only of the GM-positive sera out of 100 GM control sera previously typed by HAI. The lower limit of detection was dilutions from 1 : 256 to 1 : 1024 (Fig. 1).

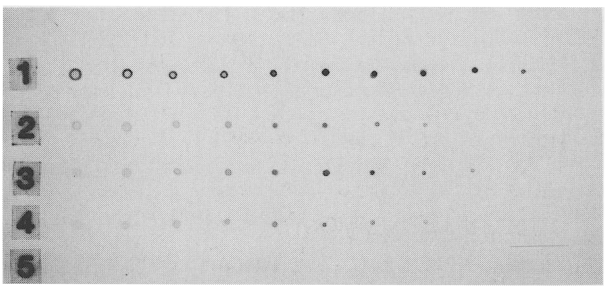


Fig. 1. G1M F typing by DIB of serial double dilutions (from left to right, 1/2 through 1/1024) of five serum samples of known GM phenotypes: 1, AFB1B3; 2, AG1FB1B3; 3, AXG1FB1B3; 4, AFB1B3; 5, AXG1.

Our purpose in producing anti-GM MAbs is not only to relieve the scarcity of human anti-GM sera but also to develop a new assay method to replace the cumbersome HAI method, thereby enabling the quantitation of allotype-specific IgG. To produce anti-GM MAbs, we persisted in using normal, and not myeloma, IgG subclass proteins as immunogens for their easy accessibility. The present study demonstrates that DIB is simple and practical in requiring only 2 h of bench work. In our experience, if more sensitivity is desired as in blodstain analysis, a direct-immobilization-ELISA is suitable in that serum dilutions of 1 : 10 000 to 1 : 40 000 can be readily GM-typed. A capture ELISA has an advantage over the above ELISAs in being far more sensitive. In fact, Francois-Gerard and Hoste (1987) and de Lange et al. (1989) detected GM allotypes in serum samples at dilutions of 1 : 320 000 to 1 : 2 000 000.

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Production of Monoclonal Antibodies Against IgG Allotypes and Their Use in Dot Immunobinding

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INTRODUCTION

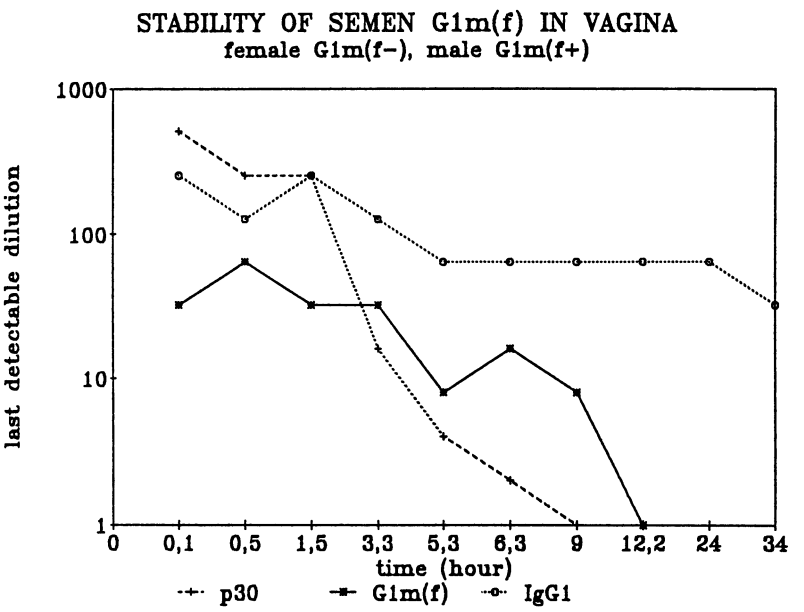
IgG heavy chain allotypes, GM, are extremely useful in population genetics and in cases of disputed paternity. Unfortunately, the longstanding shortage of typing reagents hampers the widespread use of GM allotyping. Moreover, the cumbersome hemagglutination inhibition (HAI) method of typing is still generally used: O, D(+) red cells coated with GM-positive anti-D antibodies are agglutinated by the anti-GM serum; prior addition of a serum sample to the anti-GM serum inhibits the hemagglutination if the test serum contains that particular allotype. Despite its ingenuity, HAI poses several problems: (1) The shelf life of coated red cells is relatively short; thus the cumbersome step of coating must be repeated at frequent intervals. (2) Since human anti-GM sera are scarce and of low titer, a large number of healty individuals need continually to be screened for anti-GM antibodies so that the stock of antisera may not be exhausted. The screening is laborious and presupposes the availability of GM allotyping control sera and anti-D antibodies of various GM specificities which are extremely difficult to obtain from certain racial groups. (3) The occaisional occurrence in serum samples of antibodies to IgG and/or red cell antigens invalidates the HAI test unless they are absorbed by a time-consuming procedure. (4) The HAI test can not be used for the quantitative analysis of GM allotypes. All of these problems are solved by the use of monoclonal antibodies (MAbs) in enzyme-linked immunosorbent assay (ELISA) or dot immunobinding (DIB) (Fletcher et al. 1983; Kishida and Tamaki 1984; Bird et al. 1984; Francois-Gerard and Hoste 1987; Kishida et al. 1988; Weston-Kirkegaard et al. 1988; de Lange et al. 1989). Therefore, we produced the first MAb against the G3M G1 allotype, using a protein A-purified normal IgG3 protein as an immunizing antigen (Tamaki et al. 1984). Shortly thereafter, Bird et al. (1984) described MAbs to G1M F. Subsequently, de Lange et al. (1986, 1989) reported the production of MAbs to G1M Z, G1M A, G1M F, G3M B1/U, and G3M G1. Recently, Kimura et al. (1989) have prepared an anti-G3M T MAb. In producing anti-GM MAbs, the European workers immunized mice with myeloma proteins (which are not easily available) and/or used splenocytes as fusion partners after multiple boosts. In contrast, we immunized mice with a single dose of normal IgG suclass protein and used their lymph node cells as fusion partners to expedite MAb production. The present paper provides a practical approach to the production of anti-GM MAbs, and describes their usefulness in DIB in relation to their ELISA applications.

MATERIALS AND METHODS

The immunogens used for MAb production were IgG subclass ptroteins or their fragments prepared from normal human serum by DEAE-cellulose and protein A or Ricinus communis lectin I column chromatography as previously described

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Fig 1 :



IN CONCLUSION,

This enzymeimmunoassay is a very sensitive and specific method, which can be applied to the typing of Gm antigens in semen and other body fluids.

The possible interference of Gm allotypes of vaginal origin and possibly from saliva or nasal mucus, has to be kept in mind when interpreting the results in sexual assault cases.

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One can see from table 1 that semen stains can be clearly typed using this method.

TABLE 2 : SENSITIVITY OF Gm SPECIFIC DETECTION IN VARIOUS BODY FLUIDS

		Range of Gm(a) or (f) detection (*)		
samples	N	liquid	N	fresh stains(**)
blood	20	1/64,000-1/1,000,000	20	1/1024
semen	55	1/800-1/25,000	10	1/16-1/1024
vag. sec			16	1/16-1/1024
saliva	5	1/256-1/1024	5	1/4 -1/16
nasal muc			10	1/2 -1/256
urine	12	0- (1/16, 1/32)	12	0
sweat			3	0 -1/2

(**) 5 mm x 5 mm extracted -2 to 3 ul

Table 2 shows the sensitivity range of Gm detection in various body fluids, liquid or dried, with the same amount of stain. To summarize, only the positive results for Gm (a) or Gm(f) are given; they were always in agreement with the serum Gm groups.The absence of blood or semen contamination was tested by using the benzidine and the p30 tests.

As for other semen polymorphisms, such as ABO and PGM, the main problem in forensic applications, is the presence of the same marker in vaginal secretions.

In two other body fluids, the Gm allotypes could also be clearly determined : saliva and nasal mucus. The Gm concentration in nasal mucus was extremely variable : in several samples it was as high as in semen stains.

All the liquid urines tested were negative, except one sample collected from a male donor and which was positive for p30 as well, and one urine from a female donor and which was negative for blood and for p30. This should be investigated further. Stains of all urine samples including these two, were negative or doubtful. Sweat stains did not drive to conclusive results.

Results of 63 stains ageing from 6 months up to 3 years showed the same excellent stability of the Gm(a) and (f) allotypes in dried semen and vaginal secretions as already known of those allotypes in dried blood.

The stability of semen Gm allotype in the vagina, after sexual intercourse could be studied in one case in which the woman was Gm(f-) and the man Gm(f+). They provided a semen sample, a semen free vaginal swab and a timed intervals set of post-coïtal swabs. For each swab, the same extract was tested for Gm(f), IgG1 and p30 content. Fig 1 shows that semen Gm(f+) could be detected in the vagina up to 12 hours after intercourse. The delay for p30 detection was identical.

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As for other EIA methods, important points are to select a capture antibody, to avoid cross-reactivity by using affinity purified reagents and to optimalize the dilution of each reagent.

The optical densities were measured by means of a spectrophotometer and the results expressed as the corrected optical density value (Δ OD = OD sample -OD blank). In cases of stains' extracts, they were alternatively expressed as the last sample dilution still giving a significant Δ OD value.

The amount of IgG1 in various body fluids was estimated by using a similar EIA procedure, with a monoclonal anti-γ1 (clone NL16 UNIPATH) 1:5000, in step 3.

Prostate specific antigen (PSA or p30) detected by a capture EIA (KAMENEV et al., 1989) was used as a control for the presence of semen.

RESULTS.

The monoclonal anti-Gm(a) and Gm(f) reagent were proved to be specific in this enzyme immunoassay, by testing a panel of 20 sera of various Gm phenotypes, including rare ones.

In order to determine a threshold of significant Δ OD values, the following negative controls were tested : various biological fluids from persons of Gm(a-) or Gm (f-) group : semen, semen stains, sera, blood stains, vaginal secretions, saliva, saliva stains, nasal mucus stains. The following samples of any Gm group were also tested : urine stains, sweat stains, faeces stains. The cutt-off was calculated as the mean + 2s of neat negative controls and found 0.097 (mean = 0.031, s = 0.033, n = 52) for Gm(a) and 0.260 (mean = 0.117, s = 0.076, n = 36) for Gm(f) respectively. .

A purified IgG1 standard allowed us to measure the IgG1 concentration of 55 liquid semen. The mean concentration is 32 $\pm$ 40 ug/ml, i.e.around 100 times less than in whole blood. As in blood, the individual variation is important. The mean IgG1 content of 5 liquid salivas was found to average 3 $\pm$ 6 ug/ml. IgG levels have been reported 1000 times lower in saliva than in blood (WAISSBLUTH and LANGMANN, 1971).

TABLE 1 : EXPERIMENTAL SEMEN STAINS.

Stains (**)	Age (months)	Serum Gm group	Semen Gm group (*) Gm(a)	Gm(f)
1	6.5	a-, f+	0	1/512
2	6.5	a-, f+	0	1/128
3	6.5	a+, f+	1/32	1/16
4	6.5	a-, f+	0	1/16
5	6.5	a-, f+	0	1/512
6	6.5	a+, f-	1/512	0
7	6.5	a-, f+	0	1/64
8	6.5	a-, f+	0	1/128
9	32	a-, f+	0	1/512
10	33	a-, f+	0	1/128
11	36	a+, f-	1/1024	0
12	36	a+, f+	1/256	1/128

(*) last dilution significantly detected by EIA.
(**) 5 mm x 10 mm extracted.

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ENZYME IMMUNO ASSAY FOR THE TYPING OF THE Gm(a) AND Gm(f) ALLOTYPES OF HUMAN IgG1 IN SEMEN, VAGINAL SECRETIONS AND OTHER BODY FLUIDS

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INTRODUCTION.

Gm allotypes of human immunoglobulins, which are highly polymorphic and stable, have always been of greatest interest for bloodstains' identification. However, the results obtained from semen stains' analysis by using the classical haemagglutination inhibition method, are often not reliable, due to the low immunoglobulin content of extracts.

Enzyme immuno assay (EIA) procedures known for their sensitivity might be better for Gm typing of semen, provided highly selected antibodies such as monoclonal reagents, can be used. We have previously applied a capture EIA to type Gm(f) in blood stains (FRANCOIS-GERARD and HOSTE, 1987). Its sensitivity was proved to be 1000 times higher than the haemagglutination inhibition method. Since the recent commercialization of several anti-Gm monoclonal reagents (DE LANGE et al., 1989), we now use anti-Gm(a) and anti-Gm(f) to type semen stains. These IgG1 markers are antithetical in Caucasian populations.

MATERIAL and METHODS.

MATERIALS.

-A panel of human sera phenotyped for Gm allotypic markers, obtained from G. De Lange.
-Purified IgG1 and IgG3 of known Gm phenotypes, obtained from Dr F. Skvaril from the WHO/IUIS Immunoglobulin Subcommittee, Berne, Switzerland;
-Semen samples provided by the Urology clinics;
-Vaginal secretions collected with plain sterile cotton wool swabs, obtained from the Gynaecology clinics and from volunteer donors;
-Saliva and nasal mucus samples provided by volunteers; blood and urine samples from blood donors. (Liquid salivas were not boiled, but stored at -20°C until analysis).
-Experimental stains on cotton cloth were air dried and stored at room temperature.

METHODS.

Extraction procedure : Stains (5 mm x 5 mm corresponding to 2 or 3 ul of body fluid) were extracted overnight at 20°C in 300 ul of phosphate buffered saline (PBS) containing 0,3 % (w/v) of bovine serum albumine (BSA) and 1 % (w/v) sodium azide. For older stains, a double amount was extracted for 36 h.

Enzyme immuno assay for Gm : a four steps test as previously described (1):
1.A capture antibody coated: rabbit Ig anti-human IgG (A090 DAKO) 1 : 4000.
2.The sample to be tested, in 11 two-fold dilutions.
3.The mouse monoclonal anti-Gm : anti-Gm(f) (clone TM14 from OXOID) 1:16.000.
anti-Gm(a) (clone 10H1 from G. De Lange) 1:20.000
4.Peroxidase conjugated rabbit anti-mouse Ig (P260 DAKO) 1:250.

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chain was localized on the surface membrane of centroacinar and intercalated duct cells. The type 1 chain cannot be fucosylated to the Le^a antigen because of the absence of the Le gene and instead, they are sialylated to be a criptic form in the Le(a-b-) individual.

Sialidase digestion also imparted the reactivity with MoAb against the type 1 precursor chain in islet cells of Langerhans from some individuals. Since sialidase digestion elicited the reactivity of islet cells with soybean agglutinin and peanut agglutinin which recognizes the type 2 and type 3 precursor chain, respectively(Ito et al. 1987), three types of precursor chains are produced and converted in their criptic forms by sialylation in these cells.

The results of the present study as well as previous ones demonstrated that the distribution pattern of blood group antigen is very complex in human pancreas; their expression is under the control of the ABO, H, Se, Le and X genes in acinar cells while in centroacinar cells and intercalated duct cells, only the Le and X genes are operating. In islet cells of Langerhans, these genes are not functioning although precursor chains may be produced and converted to their criptic forms by sialylation. These differences in the ability to express blood-group-related antigens are presumed to be related to the differences in function of these different cell types.

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whereas Le^a antigen was mainly localized on the surface membrane of centroacinar cells and intercalated duct cells. These results were obtained irrespective of the secretor status of the tissue donors. Expression of Le^b antigen in nonsecretors has been reported in some tissues and explained by the ability of the H gene-specified α-2-L-fucosyltransferase to use both type 1 and type 2 acceptor substance (Mollicone et al. 1988). In one Le(a-b-) individual, Le^a antigen was not detected in any cells while MoAb against Le^b antigen weakly reacted with acinar cells. The reason for such a discrepancy between the genotype and expression of antigens is not known. Such an unexpected reaction may be due to secondary to cross-reactions of the MoAb against Le^b antigen used. Since weak reactivity with the MoAb against type 1 precursor chain was occasionally detected in acinar cells from nonsecretor but not secretor individuals, fucosylation of the type 1 chain may be in part under the control of the Se gene. Previous studies suggested that fucosylation of O-glycosidically linked, type 3 precursor chain was also under the control of the Se gene(Ito et al. 1988b; 1989b). It may be presumed that both the H and Se gene-specified α-2-L-fucosyltransferases are expressed in pancreatic acinar cells and the enzyme coded by the H gene has lower affinity for the type 1 and type 3 precursor chains than that coded by the Se gene.

As reported previously(Ito et al. 1986), some clusters of acini elaborated A or B antigen, whereas others did not, and the distribution pattern of H antigen is exactly the reverse of that of A or B antigen. The results of the present study further demonstrated that the pattern of distribution of Le^b and Y antigen is likewise the reverse of that of A or B antigen. Thus, cell clusters producing the Le^b and Y antigen correspond well with those producing H antigen.

Although Le^b and Y antigens could not be detected in cells producing A or B antigen, prior α-N-acetylgalactosaminidase or α-galactosidase digestion revealed the reactivity with MoAbs against both Le^b and Y antigens as well as H antigen in cells producing A or B antigen. These results suggest that difucosyl A and B antigens(ALe^b, AY, BLe^b, BY) are produced in acinar cells along with the type 1, type 2 and type 3 based A and B antigens.

Since the Le^b structure, once formed, is not an acceptor for the A or B gene-specified transferase, it has been presumed that the ALe^b and BLe^b structure are constructed by the fucosylation of the C-4 position of GlcNAc of the type 1 based A or B antigen(Watkins, 1978). The process is catalized by α-4-L-fucosyltransferase coded by the Le gene. Similar mechanism may be operating in the synthesis of AY and BY structures(Lloyd, 1987). In this case, it is presumed that type 2 based A or B antigens are first produced and then they are fucosylated by α-3-L-fucosyltransferase coded by the X gene to give the AY and BY structures. Since the distribution of difucosyl A or B antigen is strictly confined to those producing A or B antigen and the reverse of that of Le^b and Y antigen, the mechanism proposed by Watkins(1978) and Lloyd(1987) may be operating in pancreatic acinar cells. Thus, the type 1 and type 2 based A or B antigen may be served as the precursor of difucosyl A or B antigen.

Sialidase digestion imparted the reactivity with MoAb against X antigen on the membrane of centroacinar and intercalated duct cells from some but not all the individuals examined. In some individuals, staining patterns with MoAb against the X antigen are quite similar to those with MoAb against Le^a antigen after the enzyme digestion. These results suggest that X antigen is criptic in its sialylated form in these tissue sites. In one Le(a-b-) individual, sialylated type 1

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Histochemical Analysis of the Chemical Structure of Blood-group-related Carbohydrate Chains in Human Pancreas

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INTRODUCTION

In a series of previous studies, we have developed the lectin staining method in combination with exo- and endoglycosidase digestion procedures to localize and analyze the definite chemical structures of blood-group-related carbohydrate chains in human pancreas and submandibular glands (Ito et al. 1987, 1988a, b, 1989a, b). The results of these previous studies suggested that type 1, type 2 and O-glycosidically linked, type 3 based ABH antigens are secreted in pancreatic acinar cells. In the present study, we developed immunostaining method using several monoclonal antibodies(MoAbs) against blood-group-related antigens combined with exoglycosidase digestion procedures to provide more definite information as to tissue distribution and regulation of blood group related antigens in human pancreas.

MATERIALS AND METHODS

Pancreatic tissues were obtained at autopsy cases. They were fixed in 10% or 10% neutralized formalin and embedded in paraffin and sectioned serially at 4 μm. The typing of donor blood group for ABO and Lewis was performed by routine hemagglutination testing of the donor's blood samples. MoAbs directed to A, B and H antigens were purchased from Dako(Santa Barbara CA, USA), MoAbs against Le^a and Le^b antigens were from Biotest Diagnostic(Frankfurt/Main, West Germany) and MoAbs against X, Y and precursor type 1 chain were from Cambridge Research Lab(Cambridge Mass, USA). They were used at dilution of x50-x10.

Tissue sections were de-waxed, hydrated and incubated in 0.1 M phosphate buffer pH 7.2, containing 0.2 M NaCl(PBS) three times(each for 3 min) at room temperature and then incubated in PBS containing 1mg/ml of bovine serum albumin for 20 min. After being blotted dry with filter paper, the tissue sections were incubated with MoAb at 0° C for 4 hr. Tissue sites reactive with MoAb were visualized with the streptavidinbiotin peroxidase complex method, using StreptAvidin Immunostaining Kit(BioGenex Lab., Dublin CA, USA). Finally, the sections were counter-stained with hematoxylin, dehydrated and mounted in balsam. Exoglycosidases used and digestion procedures of tissue sections were described in previous papers(Ito et al. 1987, 1988a, b, 1989a).

RESULTS AND DISCUSSION

Immunoperoxidase staining with MoAb against blood-group-related antigens showed that A, B, H, Le^b and Y antigens are secreted in acinar cells but not centroacinar cells, intercalated duct cells and islet cells of Langerhans. X antigen was often detected in acinar cells

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Seminal protein may contain two antigenic determinants: the peculiar proteins and ABO blood group. The ASPP reacts with seminal peculiar protein (SPP) and forms a reactive complex, in which the ABO blood group antigen determinant is present. After the NCF coated with ASPP is washed, only the SPP-ASPP reactive complex remains on the NCF. Hence the proteins in eluate contains ABO blood group antigenic determinant too. Then ABO blood typing could be determined by the egg white absorption-elution method.

The results indicate that the new method mentioned above is accurate, simple, time saving and sensitive.

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Isolation and Identification of ABO Blood Group of Seminal Stains Mixed with Vaginal Secretion or Blood

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The identification of seminal stains contaminated with body fluid is one of the most difficult problems in forensic medical biology. The methods such as electrophoretic separation, ABO blood typing of sperm in mixed stains and absorption-inhibition test etc, have been used for this purpose. This paper reports a new method for the ABO blood typing in seminal stains mixed with vaginal secretion or blood using nitro cellulose filter (NCF) coated with anti-semino peculiar proteins (ASPP).

Basic Procedures

1. Preparation of immunogen and immunization of rabbits

The semens obtained from a number of donors were mixed and the mixture was centrifuged. The supernatant was isolated by 1% agarose gel electrophoresis (pH 8.6, barbital buffer, 320V, 3 - 4 hrs, 10 C). The gel containing a large amount of seminoglycoproteins was cut off 5-6 cm far from origin to anode. Then the gel was frozen, thawed and extracted by centrifugation. The supernatant was concentrated into a solution of 1mg protein/ml and then used as immunogen. Crude antiserum was prepared by immunizing rabbits. Rabbits were intradermally injected with immunogens in the presence of Freund's adjuvant at intervals of 10, 15 and 30 days. When antibody titer reached 5000 fold, the blood was taken from rabbits and used as crude antiserum.

2. Purification of crude antiserum

The crude antiserum which had previously been absorbed with A,B,O type red cells was purified by affinity chromatography using CNBr - activated sepharose 4B. The human serum and vaginal secretion were used for coupling respectively. In coupling procedure, the human serum was diluted with 0.1 M NaH-CO3 (pH 8.4) into a solution of 10-15mg proteins/ml (pH 7.8). The vaginal secretion was extracted with saline and then centrifuged. The supernatant was dialyzed in saline for 48hrs and concentrated into a solution of 3mg protein/ml (pH 7).

3. ABO blood typing in seminal stains mixed with vaginal secretion or blood by NCF.

A piece of NCF (5 x 5mm) was put in the ASPP, absorbed in vacuum (-760mmHg) for 20 minutes at room temperature and washed in cold saline for removing the unabsorbed serum proteins.

The NCF coated with ASPP was put in the mixture of seminal-vaginal stains or seminal-blood stains, absorbed in vacuum (-760mmHg)for 10 minutes at room temperature and washed in cold saline for removing unreacted proteins.

Then the NCF was put in a well of the glass plate and one drop of saline was added to it. The substance specifically reacted with the antibody was eluted from the reactant on the NCF at 56C for 7 minutes. A stripe of cotton gauze (1cm in length) was immersed in the eluate and dried. Finally, ABO blood typing of seminal stains was determined by the egg white absorption-elution method.

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adsorption. To avoid the potential hazard of crossreaction, we produced anti-A2SGP MAbs of well-defined specificity. These MAbs worked well in the capture ELISA described here; however, for practical applications they need extensive testing with mixed body fluids and stains.

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dilution, the semen coats could be detected with anti-A2SGP at dilutions of 1 : 128 000 to 1 : 512 000. When plates were coated with dilutions of semen, the lower limit of detction with a 1 : 1000 dilution of anti-A2SGP was 1 : 10 240. When tested in a direct immobilization-ELISA, anti-A2SGP did not react with saliva or vaginal secretion coats, although the coats could be detected well with anti-A, anti-B, or anti-H irrespective of secretor status. The results indicate that the anti-A2SGP MAb does not react with ABO epitopes or the proteins of saliva or vaginal secretions; thus, we used the MAb in a capture ELISA to capture exclusively the ABO epitope-carrying protein of semen, A2SGP. Figure 1 shows the results of a capture-ELISA where a semen sample from a group B secretor was captured by anti-A2SGP for subsequent ABO grouping. Even a 1 : 12 800 dilution of semen could be detected and correctly ABO-grouped.

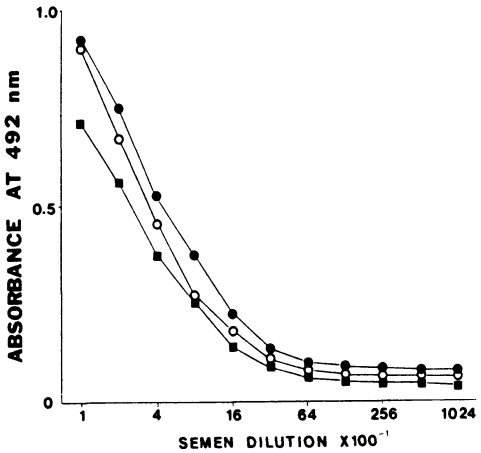


Fig. 1. Capture-ELISA for ABO-grouping of semen. Plates were coated with 1: 200(●), 1:1600(○), or 1:12 800 (■) dilution of anti-A2SGP, and incubated with serial double dilutions of group B secretor's semen. Captured A2SGP was detected with anti-B and HRP-anti-mouse IgM.

One of the major seminal proteins of forensic interest is γ-Sm (Koyanagi et al. 1972) or p30 (Sensabaugh 1978) and has long been used as a marker for semen identification. The present study demonstrates that A2SGP is another sensitive marker when detected by an indirect ELISA method.

Since the individualization of seminal fluids or stains in the investigation of rape cases is the final goal of semen examination in the forensic science laboratory, a number of forensic scientists attempted to ABO-group seminal fluids mixed with vaginal secretions after separation of seminal from vaginal components. Despite their claims of success, they failed to present convincing evidence of complete separation in the absence of information on the ABH substance of semen. Increasingly wide use of ELISA methods in recent years has stimulated a new approach to the ABO-grouping of semen. Using potato lectins or absorbed anti-semen serum in capture (or sandwich) ELISAs, Mukoyama et al. (1987) and Sagisaka et al. (1988) could ABO-group semen mixed with vaginal secretions in all ABO combinations. Tsuda et al. (1988) first isolated A2SGP and identified it as the ABH substance of semen. Immunizing rabbits with A2SGP, Iki et al. (1988) raised anti-A2SGP serum, but they had to absorb it with human red cells and serum before use. Although monospecific, absorbed anti-A2SGP serum may pose problems in ELISAs, such as crossreaction and nonspecifc

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Production of Monoclonal Antibody to ABH-Carrying α2-Seminoglycoprotein for ABO-Grouping of Semen by Enzyme-Linked Immunosorbent Assay (ELISA)

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INTRODUCTION

Recently, Tsuda et al. (1988) isolated a seminal glycoprotein, A2SGP, with a molecular weight of about 472 000 and identified it as the carrier of ABH epitopes; therefore, it is possible for antibodies to the protein backbone of A2SGP to capture A2SGP in a mixture of semen and vaginal secretions for ABO-grouping of the former without interference of the latter. For that purpose, we produced monoclonal antibodies to the protein backbone of A2SGP and used them in a capture-ELISA.

MATERIALS AND METHODS

A2SGP was isolated from seminal fluids by a sequence of chromatographic procedures as described elsewhere (Tsuda et al. 1988). BALB/c mice were immunized by a subcutaneous injection into hind feet of 20 ug A2SGP in Freund's complete adjuvant. Ten days later, popliteal lymph node cells were fused with P3U1 myeloma cells. Hybridomas were cultured, screened for antibody production by an indirect ELISA in A2SGP-coated plates, cloned, and grown in mice for collection of ascitic fluids, by standard tecniques. The ascitic fluid MAbs were precipitated with ammonium sulfate at 50% saturation and stored at 5 C until use. For ABO-grouping of semen by capture-ELISA, microtiter plates were coated with dilutions of anti-A2SGP MAb in coating buffer. The MAb coats were allowed to react with dilutions of semen. Afer washes, the captured A2SGP was allowed to react with a 1 : 20 dilution of monoclonal anti-A or anti-B or diluent alone. After washes, a 1 : 1000 dilution of horseradish (HRP)-labeled anti-mouse IgM or HRP-anti-H lectin was placed in each well. After incubation and washes, a HRP substrate was added to each well, the enzymic reaction was stopped with 12.5% sulfuric acid, and the plates were read on a microplate reader at 492 nm. All ELISAs were carried out under the following conditions: coating buffer, 0.05 M carbonate, pH 9.5; concentration of coating A2SGP, 10 ug/ml; coating time, 2 h at room temperature; diluent, 0.5% bovine serum albumin in 0.02 M tris-buffered saline conataining 0.1% Tween 20, pH 7.5; washing fluid, running tap water; reaction volume, 100 ul/well; incubation time, 30 min each at room temperature; HRP substrate; 0.005 M o -phenylenedamine and 0.001% hydrogen hperoxide in 0.05 M phosphate buffer, pH 5.0.

RESULTS AND DISCUSSION

Four IgG1 MAb-producing hybridoma clones were established. When plates were coated (in a direct immobilization type of ELISA) with semen at 1 : 200

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Table 1. Reaction of McAbs with human saliva and animal red cell

Samples	A antibodies			B antibodies			H antibodies			AB antibodies	
	RT3	SCA	AP	RT10	SCB	BP	HM1	HM2	HL	RT15	SCAB
Human saliva											
A type	-	+	+				-	+	+	-	+
B type				-	+	+	-	+	+	-	+
O type							-	+	+		
Animal red cell											
Pig	-	+								-	+
Rabbit				-	+					-	+

AP,BP: polyclonal antibodies. HL: lectin. + : reaction.
- : nonreaction.

Table 2. Epitopes in ABH antigens

	A epitopes		B epitopes		H epitopes	
	hrcS	hrcNS	hrcS	hrcNS	hrcS	hrcNS
Human red cell	+	+	+	+	+	+
Human saliva	-	+	-	+	-	+
Pig red cell	-	+				
Rabbit red cell			-	+		

hrcS: human red cell specific epitope.
hrcNS: human red cell nonspecific epitope.

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Different Epitopes of ABH Antigens Exist in Red Cell and Body Fluid

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INTRODUCTION

Our previous work have proved that anti-ABH monoclonal antibodies (McAbs) have a different affinity to animals' red cell. On the fact that one anti-A McAb reacted with pig red cell and the others did not, we surmise there are two epitopes in A antigen: pig sharing epitope and pig not sharing one. Two anti-B McAbs reacted with rabbit red cell and the others did not, so that we surmise there are two epitopes in B antigen: rabbit sharing epitope and rabbit not sharing one. In this work we used 8 McAbs to study ABH antigens in human saliva. We found that different epitopes exist in ABH antigens of human red cell and human saliva.

REACTION OF McAbs WITH SALIVA

The immunogen of McAbs we used was ABH antigen of human red cell. McAbs which react with human red cell in high affinity were used. The result shows some of McAbs reacted with ABH antigens in human saliva in high affinity and some of them did not reacted, while polyclonal antibodies and lectin reacted with those antigens. The McAb that reacted with A antigen in humen saliva also reacted with pig red cell and the McAb that reacted with B antigen in human saliva also reacted with rabbit red cell (Table 1).

DIFFERENT EPITOPES EXIST IN ABH ANTIGENS

This result clearly shows at least two epitopes exist in ABH antigens. One is human red cell specific epitope (hrcS) and another is human red cell nonspecific epitope (hrcNS) which coexists in human red cell, human saliva and some animals' red cell as seen in Table 2. Our previous works have proved that hrcS also has high species specificity; RT3, HM1 and RT15 only reacted with chimpanzee and orangutan red cell in very low affinity. RT10 did not react with all the 20 kinds of animals red cell including 7 kinds of primates. In this work we prove those McAbs did not react with human saliva, which means they reacted with hrcS. In forensic science those results supply us the possibility to use those McAbs which react with hrcS to discriminate human blood from human body fluids and animals' blood.

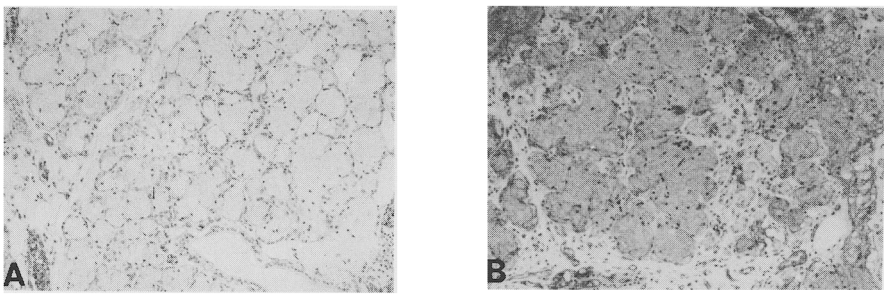
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Table 1. Blood groups of specimens tested in this study

German		cases	Japanese		cases
A_1	Le(a-b-)	2	A_1	Le(a-b-)	2
A_2	Le(a+b-)	1	A_1	Le(a+b-)	9
A_1	Le(a+b-)	2	A_1B	Le(a+b-)	5
A_1B	Le(a+b-)	1			
A_2B	Le(a-b-)	1			

Fig. 1 Immunocytochemical staining with monoclonal anti-A antibody of sublingual gland from a blood group A_1 German(A) and A_1 Japanese(B) nonsecretor. The antibody reacts well with blood vessels and red blood cells from both individuals whereas it reacts with mucous cells only from a Japanese nonsecretor.



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RESULTS

In all the cases of blood group A and AB Japanese nonsecretors examined, HPA and HAA moderetely or strongly reacted with the mucous cells of submandibular glands, sublingual glands, anteriol and posteriol lingual glands and the serous cells of von Ebner gland in tongues. On the other hand, other blood group A or AB specific lectins such as DBA, VVA, GSA-I and SJA did not bind to these cells. Monoclonal anti-A antibodies used in this study showed good reactivity with these secretory cells of salivary glands. No difference was recognized in the staining specificities among the monoclonal antibodies provided from four different sources.

In contrast, in the case of German nonsecretors, the mucous cells of salivary glands and the serous cells of the von Ebner glands showed no or feeble reactivity with HPA and HAA as well as other blood-group-specific lectins. The majority of these secretory cells likewise showed no reactivity with the monoclonal antibodies. Among the specimens examined in this study, the secretory cells from A_2 nonsecretor individuals showed lowest reactivity with these reagents. In fact, it was difficult to find the cells reactive with these reagents in the tissue sections from A_2 nonsecretor individuals.

Endothelial cells of the blood vessels and erythrocytes from individuals of both races showed good reactivity with HPA, HAA and the monoclonal antibodies whereas these cells hardly reacted with other blood-group-specific lectins.

As to the staining patterns of the secretory cells of salivary glands with conventional histochemical stainings, no observable difference was recognized between Japanese and German nonsecretor individuals. Hence, except the cases of HPA, HAA and the monoclonal anti-A antibody stainings, any histochemical difference of the secretory cells was not observed between Japanese and German nonsecretors.

Discussion

Recent development of biochemical methods for analyzing blood group substances has provided definite information as to the complexity and heterogeneity of back bone structures carring the blood group determinants(Hakomori 1984). Observed differences in the reactivity of lectins and antibodies with secretory cells and vascular endothelia may be explained in part by the differnces in the back bone structues carring A antigens. It may be assumed that the number of species of A antigens recognized by lectins other than HPA and HAA is restricted. Torres et al.(1988) reported that HPA could detect all A active glycolopids in human blood group A erythrocytes regardless of the type of precursor chains. Since HPA and HAA did not react with the secretory cells from blood group B and O secretor and nonsecretor individuals and these cells from blood group A Japanese nonsecretors reacted with monoclonal anti-A antibodies, it is reasonable to assume that these two snail lectins can recognize terminal GalNAc residues of certain kind of A antigen in these cells from Japanese nonsecretors. The results obtained in this study therefore suggest that certain kinds of A antigen which is recognized by HPA, HAA and monoclonal anti-A antibodies is secreted in the secretory cells of salivary glands from blood group A and AB Japanese nonsecretors, whereas such kind of A antigen is scarcely secreted in the cells from German nonsecretors.

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Different Expression of Blood Group A Antigen in the Secretory Cells of Salivary Glands from German and Japanese Nonsecretor Individuals

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INTRODUCTION

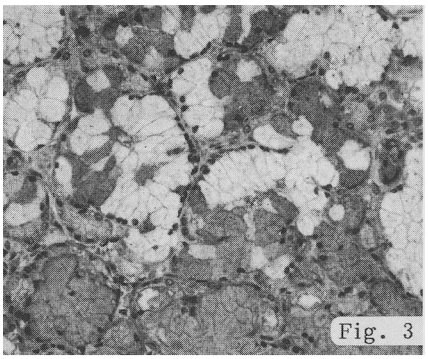
In a previous study(Ito et al. 1988), we compared the staining properties of several blood group A specific lectins in different human tissues and found that in submandibular glands from blood group A nonsecretors, Dolichos biflorus agglutinin(DBA), Bandeiraea simplicifolia agglutinin I(GSA-I), and Sophora japonica agglutinin(SJA) did not react with mucous cells, whereas Helix pomatia agglutinin(HPA) and Helix aspersa agglutinin(HAA) reacted with them. In a preliminary study, we found that these two snail lectins cannot react with mucous cells of salivary glands from German Blood group A nonsecretors, suggesting the existence of racial difference in the expression of blood group antigens. As to the racial difference in antigenicity of erythrocytes, Gibbs et al.(1961) reported that the reactivity of blood group B erythrocytes with anti B serum was strongest among Negroes, weakest among Mongoloids and intermediate among Whites. Wiener et al.(1972) also showed that the racial difference in the reactivity of the red blood cells with anti H lectin. These observation led us to suggest that it might be possible to exist the racial difference in the expression of ABH antigens in human secretory organs.

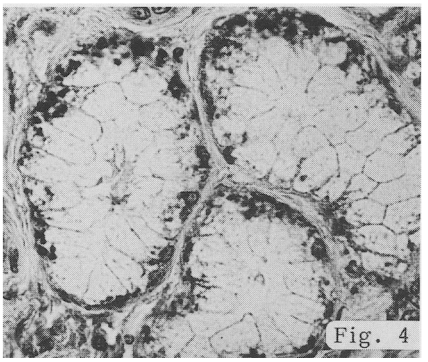
In the present study, we systematically compared the histochemical properties of secretory cells of various salivary glands obtained from blood group A and AB German nonsecretor individuals with those from Japanese nonsecretors.

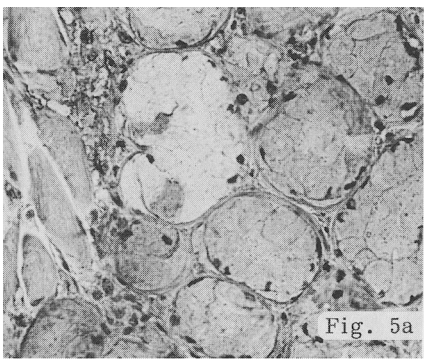
MATERIALS AND METHODS

Tissue specimens of the submandibular glands, sublingual glands and tongues were collected at autopsies. ABO and Lewis grouping of the donors were performed by routine heamaggulutination test. The secretor status of the donors was determined by the presence or absence of H antigen in the serous cells of submandibular glands. The distribution of the blood groups of the specimens examined in this study is listed in Table 1. Tissue specimens were fixed with 10% formalin and embedded in paraffin. Serial sections were used to detect blood group A antigen by the blood-group-specific lectins and monoclonal antibodies as well as the reactivity with PAS, alcian blue pH 2.5 and aldehyde pararosanilin stainings. Staining procedures with labeled lectins and monoclonal antibodies as well as conventional histochemical methods were described in our previous papers(Ito et al. 1986, Ito et al. 1987; Nishi et al. 1989). Monoclonal anti-A antibodies were purchased from four companies; Biotest(West Germany), Ortho(USA), Dakopatts (Denmark) and BioCarb(Sweden). Blood group A and AB specific lectins were purchased from Sigma Chemical Co(USA) or E.Y. Laboratories(USA).

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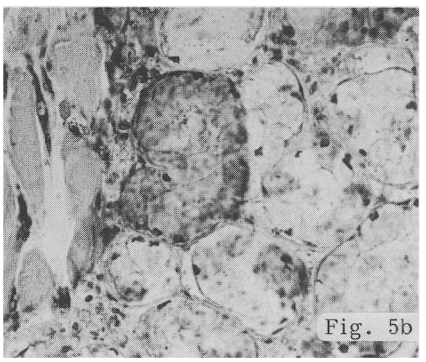


Fig. 3 Antigen A expression in an A-secretor sublingualis. Distinct mosaic phenomenon.

Fig. 4 Basal granular antigen expression in an A-secretor tongue gland. Primary antibody: anti-H.

Fig. 5a,b Vice-versa phenomenon in an AB-secretor tongue gland

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were stained in all areas of the gland. Le a could be detected well in non-secretors, but usually only with weak activity in secretors. Le b was always detected in secretors, but Le a only very weakly or not at all. In many cases the well-known phenomenon occurred, whereby in glands of A and B blood groups, either A or H, or B or H expression was observed; in AB glands this phenomenon occurred primarily between A and B (Fig. 5a and 5b). This staining behavior can be described as a vice-versa phenomenon.

In many sections a distinct granular antigen expression was seen at the base of the cells, whereas other cell parts did not react (Fig. 4). This phenomenon was first described by Pedal and Schmidt (1988) in tracheal glands of Le-negative individuals. It was particularly evident that from the 30 tongue glands studied, this granular antigen expression never occurred in the identification of A, B and Le a.

Discussion

The studies allow to conclude that the formation of glycosyltransferase is localized in the coarse endoplasmatic reticulum (Fig. 5). Depending on the blood group and the activity of the various glycosyltransferases, either the blood group substance formed first (e.g. H) remains predominant or a large amount of the blood group substance is further glycosylyzed. The mosaic phenomenon and the vice-versa phenomenon arise from a competition between the glycosyltransferases (Fig. 4a and 4b): in one part of the gland practically only A is formed, and in another only B.

The results of the ABH and Le reactivity in minor salivary glands appear to be significant to get further information on the synthesis of these antigens.

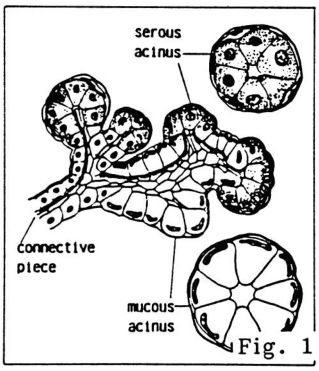


Fig. 1

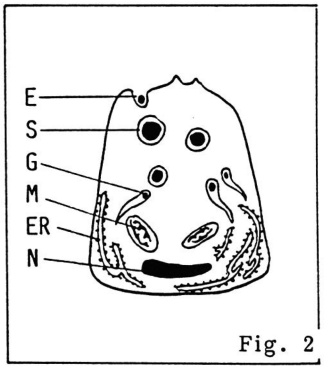


Fig. 2

Fig. 1 Light microscopic structure of a seromucous gland

Fig. 2 Electron microscopic structure of a mucous gland cell (N: cell nucleus, G: Golgi apparatus, ER: coarse endoplasmatic reticulum, M: mitochondrion, S: secretory granula, E: extrusion)

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Mosaic Phenomenon of ABH and Lewis Antigen Expression in Minor Salivary Glands - an Immunohistochemical Demonstration of the Transferase Activity

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Introduction

Immunohistochemical investigations of ABH and Lewis antigens in salivary glands, skin and hair, often showed a mosaic phenomenon (Takahasi and Kamijama, 1985; Lötterle and Heine, 1986, 1987; Nishi et al. 1989): in certain areas of the tissue, antigens exhibited by the individual could not be detected, whereas in other areas the antigens could be stained according to the blood group (Fig. 3). Furthermore, using serial sections, Takahasi and Kamijama (1985) and Lötterle and Heine (1986) demonstrated that in salivary glands of A-secretors certain areas exhibited either only A or only H but not both antigens at the same time.

The structure of the salivary glands, which can be either purely serous, serous and mucous or purely mucous, is given in Fig. 1. Fig. 2 shows a schematic representation of the electron microscopic structure of a mucous salivary gland cell (Schiebler et al. 1986). In the basal area of the cell around the nucleus the coarse endoplasmatic reticulum is located in which protein synthesis takes place. Further to the top is the Golgi apparatus, which is involved in the production of the secretory granulas. The mucoids produced in the cell are generally stored in granulas in the apical part of the cells and then transferred from there into the extrusion channel.

In order to obtain more information on the staining of the salivary glands, the identification of antigens in not yet investigated gland groups needed to be studied. The salivary glands of the tongue seemed appropriate for this study.

Material and Methods

Formalin-fixed and paraffin-embedded specimens taken from the minor glands of the tongue during dissection were serial sectioned and stained with a peroxidase-anti-peroxidase technique. A monoclonal anti-A (Dako, Code A 581, Lot 097, dilution 1:20), a monoclonal anti-B (Dako, Code A 582, Lot 018, dilution 1:20), a monoclonal anti-H (Dako, Code A 583, Lot 078, dilution 1:20), a monoclonal anti-Le a (Biotest, Ch 111055, dilution 1:15) and a monoclonal anti-Le b (Biotest, Ch 111055, dilution 1:15) served as primary antibodies. A peroxidase-conjugated rabbit antibody to mouse immunoglobulin (Dako, Code P 161, Lot 054, dilution 1:80) was taken as secondary antibody. The next reaction steps used a test kit from Ortho (Code 580000, Lot 56057: sheep antibody to rabbit immunoglobulin and PAP-complex of rabbit, stained with 3-9 AEC).

Results

The antigens A, B and H could be detected in secretors according to the blood group in all glands investigated, although in many cases not all the antigens

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These seven problem stains were tested by elution and ELISA in parallel tests. Elution was performed using the McAb selected for ELISA and by well tried polyclonal reagents.

In general elution appears to be slightly more sensitive than the ELISA and the selected polyclonal reagents used in elution produced slightly better results than the McAbs. In some instances the relevant ABH antigens could not be detected by any of the techniques used.

CONCLUSION

Modification of the ELISA developed by Fletcher and co-workers involving the omission of extraction and immobilisation and using direct attachment of bloodstained threads to acetate sheets appears to produce a simple sensitive and reliable test system.

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8. OD values greater than 0.1 considered positive provided blanks and negative controls below this value.

Controls: a. substrate only.
b. bloodstain, conjugate, substrate but no McAb.
c. bloodstain, McAb, substrate but no conjugate.
d. known positive and negative bloodstains.

Where the elution test was used this was as described elsewhere (Dodd and Lincoln, Antigen Antibody Reactions Revisited, American Association of Blood Banks 1982, pp223-239).

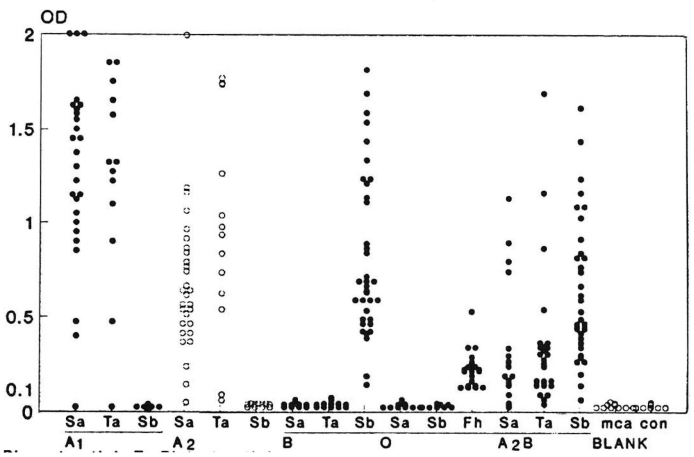
RESULTS

Various dilutions of McAb were used in preliminary experiments using 10 month old A2, B and O bloodstains. Dilutions of 1 in 2 for anti-A, 1 in 4 for anti-B and neat anti-H were selected for further investigations.

The results of ABO grouping of 213 bloodstains ranging in age from under 1 year (28 stains) to over 20 years (29 stains) are shown in Figure 1. All A, B and AB stains were tested with two anti-A reagents and one anti-B whereas the group O stains were tested with one each of anti-A, anti-B and anti-H.

206 stains were correctly typed. 7 produced results in disagreement with records of previous grouping of fresh blood samples. Four of these were 7 years old A_2B stains. (One failed to react with one of the anti-A reagents, two failed to react with either anti-A reagent used and one failed to react with the anti-B). The other three stains showing disagreements, one A_1, one A_2, one A_2B which were 20, 24 and 4 years old respectively all failed to show positive reactions with the relevant anti-A or anti-B reagents used.

Figure 1: The distribution of OD values of bloodstains tested by ELISA



Sa=Bioscot anti-A, Ta=Biotest anti-A
Sb=Bioscot anti-B, Fh=Fresenius anti-H
mca=McAb blank, con=conjugate blank

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A Modified ELISA for the Detection of ABH Antigens in Bloodstains

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Although solid phase ELISA using McAb has been reported for the determination of ABO groups of bloodstains there have been difficulties particularly relating to the stains of group A_2 and especially group AB.

Presented here is an ELISA technique which does not necessitate the extraction of antigens from the stain material or their immobilisation to the solid phase and does appear to overcome some of the previously reported problems.

MATERIALS

McAbs used include anti-A (blend of clones ES9 and LM103/107) and anti-B (clone ES4) purchased from Bioscot Ltd, anti-A (clone A003) donated by Biotest Folex Ltd and anti-H from Fresenius Diagnostics. Anti-mouse IgM alkaline phosphatase came from Sigma Chemical Co.

Over 200 bloodstains were prepared previously from laboratory staff and stored in the laboratory bloodstain file.

TECHNIQUES

1. 3-5mm bloodstain threads attached by one end to polycarbonate sheet using cellulose acetate glue.

2. 50ul of selected dilution of McAb added to each thread and sheets incubated overnight at $4^{\circ}C$ in sealed moist chamber.

3. Excess McAb removed with suction pump and threads washed with jet of ice cold PBS.

4. 50ul of anti mouse IgM alkaline phosphatase conjugate (1/400) added to each thread and sheets incubated at $4^{\circ}C$ for 2 hours in moist chamber.

5. Excess conjugate removed, threads irrigated with ice cold PBS and sheets immersed in tank of PBS for 2 hours at $4^{\circ}C$.

6. Blotted dry, threads cut off and transfered to wells of microplate.

7. Add to each well 100ul substrate (1gm/ml) P-nitrophenyl phosphate in diethanolamine buffer. Plates incubated at $37^{\circ}C$ on rotatest machine for 10 minutes and then 50ul of stopping solution (3M NaOH) added to each well. The threads are discarded, visual examination and OD readings taken.

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Therefore, when a value of over 300 mau was obtained for H with no significant reactions for A or B, then the semen stain could reliably be typed as O. In saliva, the H value was always low compared to A or B, and a value of 100 mau for H in the absence of A and B showed the stain to be group O.

The reason for this differential in semen and saliva H substance could be quantitative, qualitative or both. It is possible that more H substance remains unconverted to A or B in semen than in saliva. It is also possible that the reactions may be due to peripheral core variation (e.g. Type I and Type II) or the branched nature of the H substances forming antigenically distinct structures (Clausen and Hakomori, 1989) conferring differences to the H substance in the two body fluids. These differences could be recognised by the H monoclonal antibody we are using.

COMPARISON OF A AND B IN AB SECRETORS.

Body fluid stains from 35 group AB individuals have been studied. A few of these gave approximately the same amount of A and B, but approximately ¾ of the samples showed a reduction in one or other antigen. The loss was always related to the subtype of A. Extreme examples of these are shown in Fig. 4. and 5.

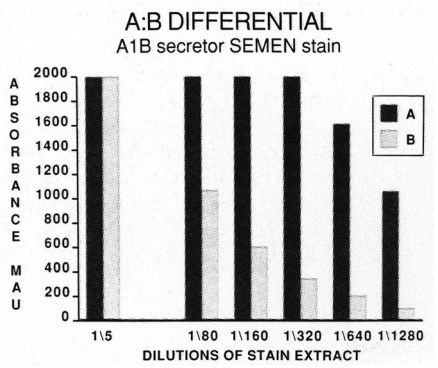


Fig. 4. Semen stain from a group A_1B secretor showing extreme A:B differential.

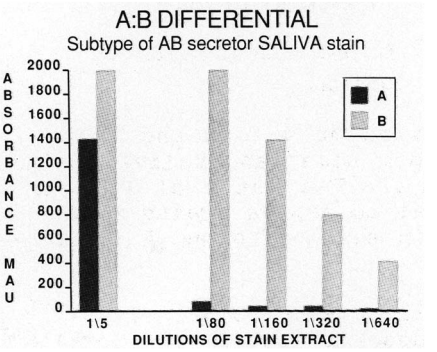


Fig. 5. Saliva stain from a group AB secretor showing extreme B:A differential.

The saliva stain result shown in Fig. 5. comes from a suspect in a rape case. A breast swab from the victim (group O) and seminal staining on her knickers gave reactions for group B only. A blood sample from the suspect was grouped as AB but with very weak reactions for A. This person was a group AB with either a weak A subtype or a super B gene (Yoshida 1983).

Besides these two examples there were many other group AB secretor saliva stains that gave differential values of over 500 mau. The two extreme examples (Figs. 4. and 5.) and the high differential values observed in others suggest that extreme caution is required when excluding group AB when either A or B only is detected. Therefore, until many more group AB samples are studied, a threshold value to exclude the possibility of an AB has been deferred in our laboratory.

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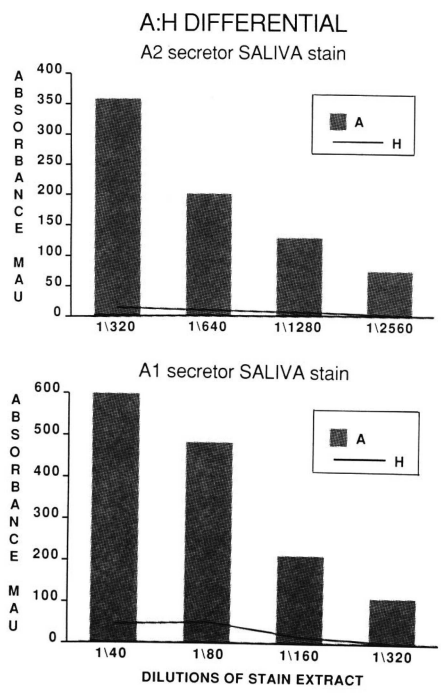


Fig. 1. A and H levels in A_1 and A_2 saliva.

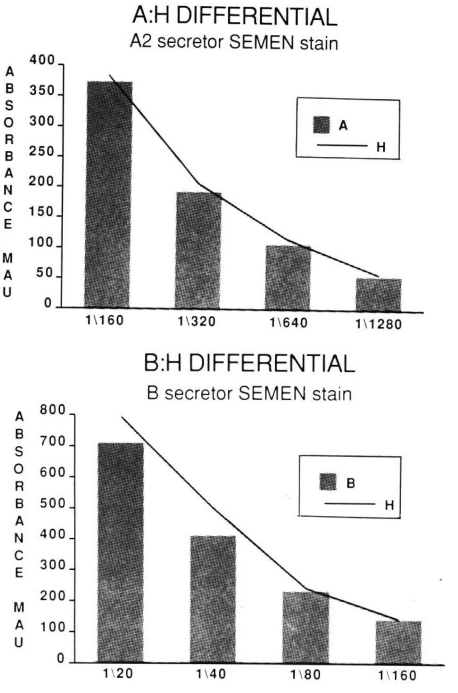


Fig. 2. A or B level compared to H in A_2 and B semen.

The ratios of A to H and B to H were calculated from a number of random semen and saliva stains, expected to contain more A_1 than A_2. The ratio of A to H was small in semen stains as compared to saliva stains (table 1). This indicates that the H level in semen of group A is not entirely related to subtype.

Table 1. A:H and B:H ratio. (The number of stains studied is in parenthesis)

	Semen	Saliva
A sec	1.8(38)	19.3(14)
B sec	1.7(16)	27.7(9)

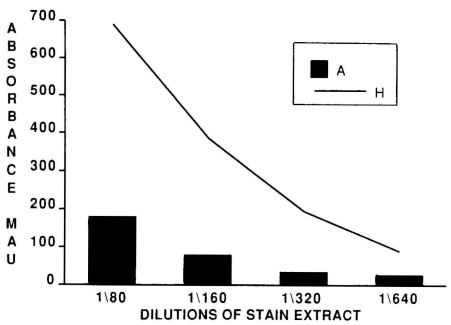


Fig. 3 Extreme A:H differential in a Group A semen.

These results alerted us to the possibility that in weak semen stains of group A or B the H may persist after A or B was lost. An extreme example of this is shown in Fig. 3. Observations of all these samples indicate that a semen stain of group A or B did not loose A or B and retain H at over 300 mau.

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DIFFERENTIAL DETECTION OF A, B AND H ANTIGENS IN SEMEN AND SALIVA STAINS DETECTED BY AN ELISA TECHNIQUE

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INTRODUCTION

In our laboratory we have modified an enzyme linked immunosorbent assay described by Fletcher (1989); and validated it for use in Forensic Samples. As part of the validation exercise a study was made of the differential levels of A, B and H antigens, in order to set a threshold value for the detection of one of the antigens at which the non detection of the others could be taken as their true absence. This is particularly important when grouping weak stains that are often encountered in Forensic work.

METHODS AND MATERIALS

Stains were made from semen and saliva samples from 1) laboratory donors, 2) Criminal cases and 3) a local fertility clinic. A 5mm. square piece of stained material was extracted and serial dilutions made such that the antigenic end point could be observed. The following studies were made:

1) Stains from group A_1, A_2 and B secretors, in respect to the level of H.

2) Comparisions of the levels of A and B in group AB stains.

A direct ELISA method was used where the antigen containing extract was incubated in microtitre plates. The A, B or H specific mouse monoclonal antisera pre-mixed with a conjugate of anti-mouse (μ chain specific) goat polyclonal alkaline phosphatase was added at appropriate concentrations. The enzyme concentration was measured by the addition of p-Nitrophenol phosphate. The colour change which represents the amount of antigen in the sample was meaasured in milli absorbance units (mau) using a Dynetech MR 700 microplate reader connected to an IBM computer.

RESULTS AND DISCUSSION

A TO H AND B TO H DIFFERENTIAL.

The ratio of A to H and B to H showed marked differences in the two body fluids investigated. In saliva the amount of H was low compared to A and B in groups A_1 and A_2 (Fig. 1.) and group B respectively. In semen however it appeared that the H level was high particularly in group A_2 and B (Fig. 2.) and in some A_1.

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2 Conventional Markers

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DNAs were amplified in reaction mixtures containing both the Y-specific primers and primers which amplify a highly polymorphic 242 bp region of the HLA DQ alpha gene. The Y-specific target sequence is present in roughly a thousand-fold excess compared to DQ alpha. In order to achieve approximately equal production of the Y-specific and DQ alpha PCR products, the amplifications were carried out in two steps: a ten-cycle amplification in the presence of DQ alpha primers alone followed by the addition of the Y-specific primers and an additional 25 cycles of amplification. When male DNA is amplified, both products are synthesized whereas only the DQ alpha target sequence is amplified from female DNA.

Using the above procedure, we have determined the sex of the donor in DNAs isolated from bloodstains, saliva samples (including buccal swabs, licked envelope flaps, cigarette butts, and simulated bitemarks), and single hairs. In the case of vaginal swabs subjected to the differential lysis procedure used to separate epithelial cell DNA from sperm DNA, male DNA was detectable in the epithelial cell fraction as well as in sperm fraction, presumably due to contamination of the epithelial cell fraction by sperm DNA. The DQ alpha type of both male and female donors was determined by a dot-blot assay using immobilized probes which distinguish six allelic variants at this locus. The presence of the Y-specific PCR product is also detectable by hybridizing with a Y-specific probe in a dot-blot format.

The concentration and ratio of the primers may be adjusted in the reaction mixture to obviate the need to open the reaction tubes once PCR is underway, thereby simplifying the procedure and minimizing the occurrence of product carry-over.

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Amplification of Y Chromosome-Specific Sequences in Biological Evidence

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INTRODUCTION

Determination of the sex of the donor of a biological evidence sample may be valuable in any investigation in which the identity of the donor is in question. The polymerase chain reaction (PCR) has been used to amplify a 149 bp segment of a 3.4kb repeat sequence which is specific to the Y chromosome and is present in as many as several thousand copies in male DNA. This simple and rapid method of sex determination was first developed by Kogan et al. (1987) for the prenatal diagnosis of fetuses at risk for X-linked genetic disorders. We demonstrate that this method can be applied to the analysis of a variety of samples, including bloodstains, vaginal swabs, saliva on various subrates, and single hairs.

MATERIALS AND METHODS

DNA was isolated from whole blood samples, single hair roots, saliva samples, and semen samples as described (von Beroldingen et al. 1989). A portion of each sample was amplified in 100 1 of reaction buffer containing 50 mM KCl, 10 mM Tris.HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 M each deoxynucleotide triphosphate, 2.5 units of Taq DNA polymerase (Perkin-Elmer/Cetus), 0.2 M each biotinylated DQ alpha primer (Saiki et al. 1989), and/or 0.2 M Y repeat primers (Kogan et al. 1987). PCR products were visualized by gel electrophoresis in a 3% Nusieve agarose gel. DQ alpha typing was according to the method of Saiki et al. (1989). Southern blot and dot/blot analysis of Y-specific PCR product was carried out using a horseradish peroxidase-labeled probe for the Y-specific target sequence.

RESULTS AND DISCUSSION

When DNA isolated from whole blood samples of male and female donors was amplified with the Y-specific primers, the 149 bp PCR product was only detected in those samples derived from males. The identity of this product was verified by Southern blot hybridization analysis using a probe specific for a sequence internal to the 149 bp Y-specific segment.

Inasmuch as the absence of the 149 bp Y-specific product could potentially be due to the failure of the amplification reaction or insufficient template DNA rather than the presence of female DNA, it was necessary to include a positive control that would be amplified in both male and female DNAs. Therefore, sample

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We will continue to use this method to look for additional Gc polymorphisms throughout the Gc gene and to look at the sequences of rare variants.

We acknowledge Dan Gregonis from the San Bernardino County (California) Sheriff's Crime Laboratory. He supplied us with most of the bloodstain material we have used and he retyped blood samples for us that had been erroneously typed by another lab. Our DNA typing system identified these errors and they were confirmed by protein typing.

This work was supported by grants from the Bureau of Forensic Services, State of California Department of Justice and from the National Institute of Justice (86-IJ-CX-0044).

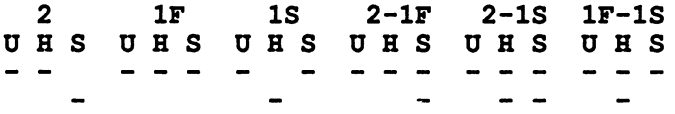
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If these restriction site combinations are characteristic of each of the three Gc alleles, then the digestion patterns of the 3 Gc heterozygotes can be predicted. The restriction patterns we observed for each of the six genotypes are shown below in a schematic diagram.



U: undigested PCR product
H: incubation with HaeIII
S: incubation with StyI

The patterns for the heterozygotes are those predicted by the restriction enzyme digestion data from the homozygotes. For example, for type 2-1F, neither the 2 allele nor the 1F allele contains a HaeIII site, and there is no digestion by HaeIII. In contrast, the 2 allele contains a StyI site but the 1F allele does not, giving rise to one undigested product and one StyI digested product in that lane of the gel. Each Gc genotype has a distinct restriction pattern, allowing us to type Gc unambiguously. We have analyzed 41 individuals by this method with no deviation from these restriction patterns.

The DNA polymorphisms of the 6 Gc genotypes were confirmed by direct DNA sequence analysis of the PCR products. The sequence differences between the 3 homozygotes are summarized below.

```
2       AATTGCCTGATGCCACACCCAAGGAACTGGCA
1F      ----------------------C----------
1S      ----------G----------C----------
```

These differences in sequence allowed us to identify the amino acid differences between the 3 Gc alleles in this region.

	aa 416	aa 420
2	*asp*	*lys*
1F	*asp*	*thr*
1S	*glu*	*thr*

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2-1F, 2-1S, 1F-1S). We found the sequences to be identical in every case; the sequences matched the published type 2 sequence. The apparent difference between the type 1 and type 2 sequences in this region is due most likely to an error in the published type 1 sequence. Another possibility is the type 1 sequence may have been derived from an individual carrying a rare Gc variant.

The polymorphisms at codons 416 and 420 involve only single base differences between the two types, but they have proved to be the most interesting. The continuous sequence through this region is shown below.

```
                    HaeIII
TYPE 1    ---CCTGAGGCCACACCCACGGAAC---

CODON              416          420

TYPE 2    ---CCTGATGCCACACCCAAGGAAC---
                              StyI
```

The generic type 1 sequence contains GGCC, which is the recognition sequence for the restriction enzyme HaeIII. However, this site is not present in the type 2 DNA because of the base change at codon 416. Similarly, the type 2 sequence contains CCAAGG, which is the recognition sequence for the restriction enzyme StyI. The base change at codon 420 results in the loss of this site in the type 1 sequence.

Because these sites are close together, we were able to make a PCR product containing both of these sites. If the PCR product contains the HaeIII site or the StyI site, then after digestion with the appropriate restriction enzyme there will be two fragments. The difference in size between the undigested PCR product and the digested fragments is easily distinguished on an agarose gel. We amplified DNA from 6 individuals representing the 6 Gc genotypes, incubated the PCR product with either HaeIII or StyI in separate reactions and analyzed the products on agarose gels. Our results of digestion of the 3 Gc homozygotes are summarized in the table below.

	HaeIII	StyI
2	-	+
1F	-	-
1S	+	-

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USE OF THE POLYMERASE CHAIN REACTION FOR TYPING Gc VARIANTS

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We have developed a polymerase chain reaction (PCR) system for typing group specific component (Gc). Our Gc DNA typing system has several advantages:

1. The 2, 1F and 1S Gc alleles can be typed unambiguously.
2. An individual's Gc type can be obtained from any biological sample from which DNA can be isolated and amplified (e.g. blood, hair, semen, saliva, bone).
3. The Gc typing can be combined with other genetic marker systems typed by DNA analysis (e.g. HLA DQα) to give a very high power of discrimination.

We made use of two published Gc cDNA sequences to determine which regions of the Gc gene to amplify and study. One Gc cDNA sequence is from a type 2 individual (Yang 1985) and the other cDNA sequence is from a type 1 individual (Cooke 1985). The subtype of the type 1 individual is not known. When these two cDNA sequences are compared, there are 4 regions containing base changes that correspond to amino acid changes. These sequence differences are summarized below.

CODON	152	310311	416	420
	gly	*valglu*	*asp*	*lys*
TYPE 2	---GGA----	GTAGAG----	GAT----	AAG
TYPE 1	---GAA----	GTGAGA----	GAG----	ACG
	glu	*valarg*	*glu*	*thr*

At position 152, there is a single base difference. We have not yet studied this region. Within the adjacent codons 310 and 311 there is a 4 base difference that results in a single amino acid change. We made PCR primers to amplify this region and have obtained sequence information from 6 individuals representing the 6 Gc genotypes (2, 1F, 1S,

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In order to specify the PCR products we looked for TaqI digestibility. The 1100 bp fragment could indeed be digested by TaqI into fragments of 300 and 800 bp. The fragment of 1100 bp was not completely digested by TaqI; about half of the amplification product possessed no TaqI digestion site. It seems that this band at 1100 bp represents a mixture of DNA sequences despite the specific PCR primers.

An unequivocal characterization of the PCR product was done by direct DNA sequencing of the 800 bp fragments using an internal start primer (Seq-PR) in the middle of exon 3, a variable region. The amplification with this primer and primer PR 2 resulted in the expected PCR product of 210 bp length. The sequence ladder can be read 29 bp apart from the 3'end of the start primer. Compared to the known sequence of A3 the homology was 98% (1 nt of a total of 58 nt) (Fig. 3, P1).

This should help to improve individual typing even by sequencing a short piece of 50 nucleotides in the variable HLA sequence.

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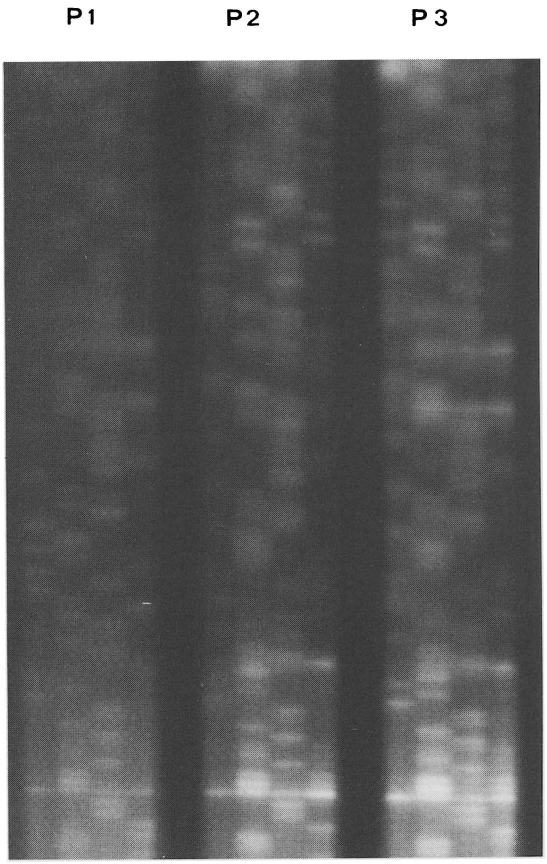
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fragment possessed a length of about 800 bp. The haplotypes defined by serologic HLA-ABC typing are genetically heterogenous. Irrespective of the serotype all PCR products had the same length. Differences of some nucleotides could not be detected.

The amplification products with primers PR 3 and PR 4 resulted in a uniform band of about 1100 bp, the expected length calculated with the help of the known sequences (including the known sequences of A2, 3 and w24).




```
A3    GCGCTCTTGGACCGCGGCGGACACGGCGGCTCAGATCACCAAGCGCAAGTGGGAGGCG
A2    ----------------------------A-------C--------A-------------
B7    ------C-----------------C---T--G---------C-----------------
Cw1   ------C--------C-------CC---------------C-----------------
P1    ----------------------------A------------------------------
P2    ------C--------C------?-----A-G---
P3    ---------------C--------?--A------
```

Figure 3 DNA sequences in exon 3 (nucleotide 120-178) of HLA A3 (Strachan et al. 1984), A2 (Koller et al. 1985), B7 (Davidson et al. 1985), Cw1 (Güssow et al. 1987) and the sequences we found. P1: A1,w24 B17,39 Cw6,7; P2: A2 B35 Cw4; P3: A1,3 B17,35 Cw4

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DNA amplification was performed according to Saiki et al.(1988) with 1 µg of genomic DNA, 300 ng of each primer and 1,5 mM $MgCl_2$ in 100 µl. Samples were heated to 94 C for 45 sec. (to denature the DNA), cooled to 60 C for 30 sec. (to anneal the primers) and heated to 72 C for 60 sec. (to extend the annealed primers). After 15 cycles the extension time was extended by 5 sec. a cycle for another 15 cycles, followed by a final extension step of 5 minutes.

PCR products were digested with restriction endonuclease TaqI (10 units/ug DNA) after precipitation with ethanol. Both digested and undigested samples were analyzed by agarose gel electrophoresis (3% NuSieve/1% SeaKem in TAE) and staining with ethidiumbromide (Fig. 2).

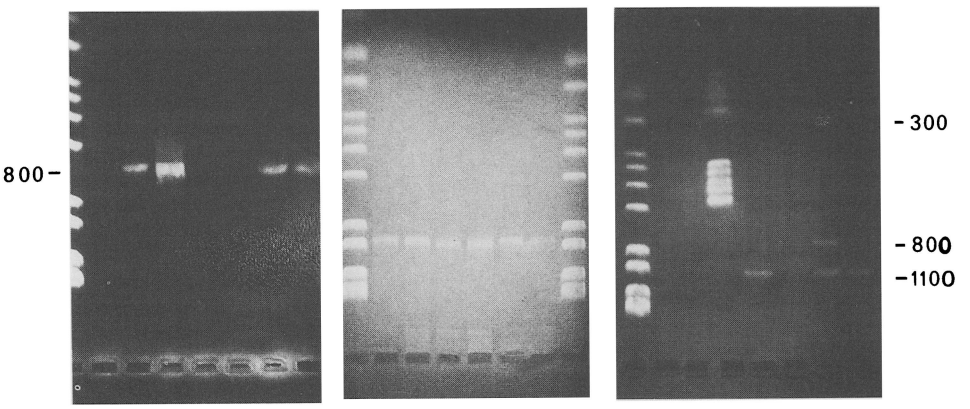


Figure 2 PCR-products with PR 1 and 2 (left), PR 3 and 4 (middle) and PR 3 and 4 digested with TaqI restriction enzyme (right). A panel of following serotypes was used: A28,w19 B5,35; A1,3 B35,57 Cw4; A2 B35 Cw4; A1,2 B16,44 Cw5; A1,24 B17,39 Cw6,7; A2,3 B15,35 Cw1,4; A2 B35 Cw4; A2,11 B17,44 Cw6,7.

Prior to DNA sequencing the PCR products were purified by Centricon 30 microconcentrators. Sequencing was carried out by the dideoxynucleotide chain-termination reaction (Sanger et al., 1977) using Sequenase kit from USB. The primer was radiolabeled using polynucleotide kinase and (γ-^{32}P)ATP. Samples were electrophoresed in a polyacrylamide/urea sequencing gel (Fig. 3).

Results:
The segments of genomic DNA chosen for amplification are shown in Fig. 1. Since the PCR primers are chosen to anneal to the HLA coding DNA regions with little or no variation , we succeeded in visualizing only one band in the ethidiumbromide stained agarose gels. With the primers PR 1 and 2 - annealing to known sequences of the serotypes A2, 3, w24, 11, B7 and 13 - the amplified DNA

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Typing by polymerase chain reaction mediated amplification of DNA fragments in the HLA class I region and direct DNA sequencing

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Until now, exploiting the polymorphism of the HLA class I genes for forensic purposes is limited to the surface (protein) antigens. The DNA polymorphism detected by restriction fragment length polymorphisms (RFLP) revealed a large number of bands varying between 15 and 25 (Mayr 1986). The DNA used for RFLP analysis has to be intact, a condition seldom met in forensic tasks. In addition, for RFLP analysis a certain microgram amount of DNA is necessary.

As assessed by Southern blotting, the HLA class I gene family contains 15-20 members, many more than can be accounted for the HLA-A, -B and -C genes (Geraghty et al., 1987). Until now, there are reports about the DNA amplification in the HLA class II region comprising a relatively low number of alleles (Higuchi et al. 1988).

Materials and Methods:
Genomic DNA was prepared from peripheral blood cells according to standard procedures. The primer sequences were chosen to span the variable alpha 1-domain, corresponding the exon 2, alpha 2-domain, corresponding exon 3, and the alpha 3-region, corresponding exon 4 (Fig. 1).

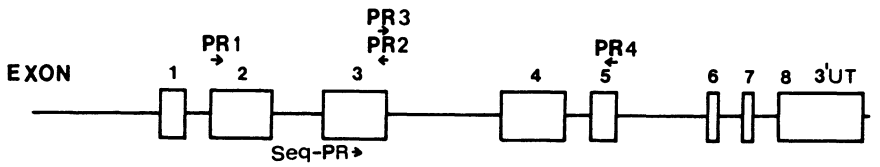


Figure 1 Amplification and sequencing strategy. The regions chosen for amplification and direct sequencing are shown along with the oligodeoxynucleotide amplification primers (PR) and the sequencing primer (Seq-PR). Arrows indicate the 5'→3' orientation of the primers.

PR 1 : 5'GCTCCCACTCCATGAGGTATT 3'
PR 2 : 3'CTATGGACCTCTTGCCCTTCC 5'
PR 3 : 5'AGATACCTGGAGAACGGGAAG 3'
PR 4 : 3'ACACCTCCTCCTTCTCGAGT 5'
Seq-PR: 5'GCGGGTACCGGCAGGACGCCTACGACGGCA 3'

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CONCLUSION

The DNA in evidence materials appears to be shielded from direct exposure to damaging UV radiation. It is thus unlikely that evidence DNA will contain significant DNA damage unless it is exposed to direct, unfiltered sunlight for extended periods. This work indicates that if evidence DNA is UV damaged, its amplification by PCR will be blocked.

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exposed to measured doses of short wave UV. The irradiated DNA was used as the template for a one cycle extension using a labeled primer and the reaction products were analyzed on a sequencing gel alongside sequence control lanes. The reaction products were fragments terminating at adjacent purines on the extending strand; this reflects termination at adjacent pyrimidines on the template strand. Doses up to 200J/M^2 resulted in fragments which were fairly evenly distributed along the length of the template sequence. Higher doses resulted in a shift of the fragment distribution toward shorter fragments; doses above 7200J/M^2 resulted in virtually no extension.

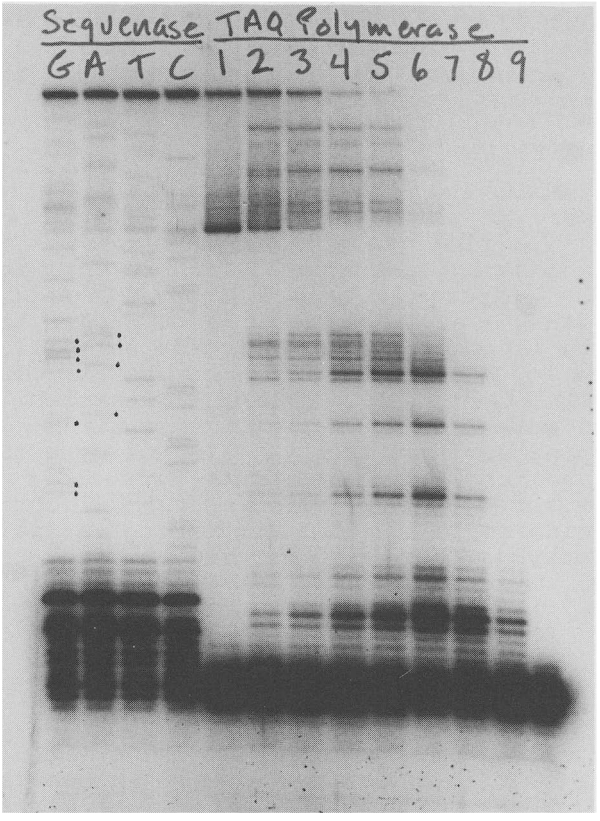


Fig. 1. Primer extensions by Taq polymerase on UV irradiated template DNA. Template strand exposures (in J/M^2) were 1, none; 2, 30; 3, 60; 4, 180; 5, 300; 6, 900; 7, 3600; 8, 7200; and 9, 14400. The sequencing reactions were run on un-irradiated DNA. Electrophoresis was on 7% acrylamide gels.

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EFFECTS OF UV DAMAGE ON DNA AMPLIFICATION BY THE POLYMERASE CHAIN REACTION

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INTRODUCTION

Exposure of DNA to ultraviolet radiation can result in DNA damage; prominent forms of damage are pyrimidine dimers, DNA strand cross-links, and DNA-protein cross-links (Friedberg, 1985). Many studies have demonstrated that the extent of DNA damage increases as the level of UV exposure increases. DNA in evidence samples may suffer some degree of UV damage and thus it is important to know what effect UV damage might have on DNA analysis. We have investigated the effects of UV damage, specifically pyrimidine dimer formation, on the polymerase chain reaction (PCR).

METHODS AND MATERIALS

Both "naked" DNA and DNA in whole cells were exposed to measured doses of short (254nm) and long (365nm) wave UV irradiation. Purified genomic DNA was exposed in solution and whole blood and semen were exposed in both the liquid and dried stain forms. Exposures ranged from 0 to 30,000 J/M^2 as measured by an UV exposure meter. DNA from the UV exposed samples was isolated by standard procedures (Gill, et al, 1985) and amplified by PCR for 30 cycles using the thermostable Taq polymerase (Saiki, et al., 1988); the target sequences for amplification were a 110bp sequence from the ß-globin gene and the 242bp typing sequence from the HLA DQã gene (Saiki, et al., 1986). The quantity of PCR product produced for each sample was then assessed using agarose gel electrophoresis and ethidium bromide staining. DNA sequencing was performed by the Sanger di-deoxy method using the protocol and materials supplied with the Sequenase™ ver. 2.0 kit (U.S. Biochemical Corp.)

RESULTS

Exposure of "naked" DNA in solution to short wave UV radiation at doses exceeding $200J/M^2$ resulted in reduced PCR product; above $2000J/M^2$, PCR product was lost. In contrast, there was little loss of PCR product from extensively irradiated liquid whole blood, liquid semen, or blood stain material. These results suggest that the DNA in these materials is shielded from UV exposure. Irradiation of dry semen stains with short wave UV resulted in greatly reduced DNA recoveries with corresponding PCR product reductions. Irradiation of DNA in solution or of body fluids with long wave UV had no detectable effect on PCR, regardless of dosage.

Analysis of primer extension products gives evidence that Taq polymerase is blocked at pyrimidine dimer sites (Fig. 1). Samples of purified ß-globin PCR fragment DNA in solution were

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Germany). Alternatively, the samples were absorbed onto LKB applicator tabs (2.5 × 5 mm), lightly blotted, and applied to the gel surface one cm from the cathode. The interelectrode wick distance was ten cm. Electrophoretic conditions were as previously described (Allen, et al 1989). Following electrophoresis, the gels were stained with silver (Allen, et al 1989).

Results and Discussion

Figure 1 shows that amplified fragment length polymorphism (AMP-FLP) typing of D1S80 can be performed using PCR and this simple polyacrylamide gel electrophoretic technique. Each band (or allele) is completely separated, and thus could be specifically designated without determining base pair size. The D1S80 alleles temporarily have been designated 1 through 13.

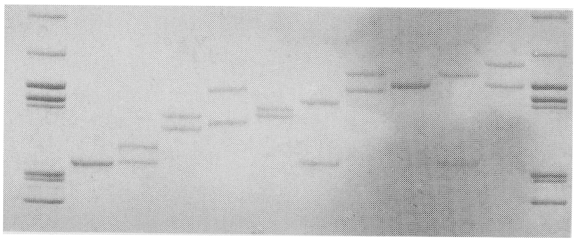


Figure 1. D1S80 profiles. The alleles have been temporarily named 1-13 (from smallest to largest in size). The genotypes of the samples from left to right are: 1-3-1, 7-5, 11-6, 8-7, 9-1, 12-10, 11-11, 12-1, and 13-11.

An advantage of analyzing D1S80 is that all alleles examined to date are less than 600 base pairs in length. The efficiency of amplification or yield of PCR products is related to the length of the target site between the primers; as observed for D17S30 (Horn, et al 1989), larger alleles could be less intense than smaller ones. Thus, unless proper quantities of amplified product were applied to the gel, incorrect genotype assignments could be made. However, with the D1S80 locus, there is no apparent difference in band intensity between the alleles of any heterozygote.

BIS is traditionally used as a cross-linker in polyacrylamide gels. However, PDA can be substituted on a gram-for-gram basis for BIS. It appears that polyacrylamide gels containing PDA have less background staining from silver than BIS gels. Thus, the sensitivity of detection of AMP-FLPs in PDA gels is enhanced and it is then possible to maintain silver-stained PDA gels permanently.

In conclusion, discontinuous gel electrophoresis can be used to type effective AMP-FLPs. AMP-FLP analysis offers these advantages over RFLP analysis of VNTRs: the ability to resolve all alleles at a particular locus, non-isotopic silver staining which will facilitate technology transfer, and relatively rapid assays. Compared with other PCR-related techniques the cost is relatively low, there are no requirements for probes, nylon or nitrocellulose membranes, and expensive assay chemicals. Additionally, the greater the number of samples analyzed at one time, the lower the cost of the assay will become. At present, we are generating population data on D1S80.

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Analysis of PCR Products (pMCT118) by Polyacrylamide Gel Electrophoresis

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Introduction

Identity tests rely on the genetic differences among individuals. The most informative genetic markers for the genetic characterization of people are variable number of tandem repeat (VNTR) loci (Nakamura, et al 1987). The detection of VNTRs is made possible by restriction fragment length polymorphism (RFLP) analysis via Southern Blotting (Southern, 1975). The RFLP procedure, although extremely effective for VNTR analyses, is time consuming and requires isotopic detection to type VNTRs in 10-50 ng of human genomic DNA samples (Budowle and Baechtel, 1989). Additionally, due to the inability of the technology to resolve discrete alleles of the VNTR loci that provide the greatest sensitivity of detection, more elaborate statistical analyses are required than for traditional genetic marker systems.

The polymerase chain reaction (PCR) (Saiki, et al 1985) offers a viable alternative to RFLP analysis of VNTRs, particularly for analyzing limited quantities of DNA, as it obviates the need for isotopic detection, and reduces assay time and costs. With appropriate VNTR loci and a high resolution electrophoretic system, amplification of specified DNA sequences by PCR could prove useful for identity testing, population genetics, and disease susceptibility studies. This paper describes a simple, high resolution discontinuous gel electrophoresis technique for the analysis of the VNTR locus pMCT 118 (D1S80) (Nakamura, et al 1988). The procedure resolves alleles of D1S80 into discrete entities, uses an inexpensive silver stain for detection, and provides a permanent record of the electrophoretically separated DNA fragments.

Materials and Methods

Whole blood was obtained in EDTA vacutainer tubes by venipuncture from donors at the FBI Academy. The DNA was extracted as previously described (Budowle and Baechtel, 1989).

Amplification of pMCT118 (D1S80) was achieved using the primers described by Kasai, et al (1989). The primers were 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3' and 5'GTCTTGTTGGAGATGCACGTGCCCCTTGC-3'. Each sample amplified contained 100ng DNA, 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 2.5 units of AmpliTaq™ DNA Polymerase (Perkin-Elmer-Cetus), 1uM of each primer, and 200 uM of each dNTP. The volume of each sample was 50 ul. The PCR was carried out in a Perkin-Elmer Thermocycler for 25 cycles. Each cycle was one minute at 95C for denaturation, one minute at 65C for primer annealing, and eight minutes at 70C for primer extension.

Ultrathin-layer polyacrylamide gels (5%T, 3%C; 400 um thick) were cast onto Gelbond (FMC, Rockland, Maine) using the flap technique (Allen, 1980). The gels were cross-linked with either BIS or piperazine di-acrylamide (PDA) (Hochstrasser, et al 1988). All gels contained 7.1% glycerol and 33 mM Tris-Sulfate buffer, pH 9.0. The electrode buffer contained 0.52 M Tris-Borate and 0.01% bromophenol blue. Electrophoresis was carried out horizontally on an ICE apparatus (EC Corporation,

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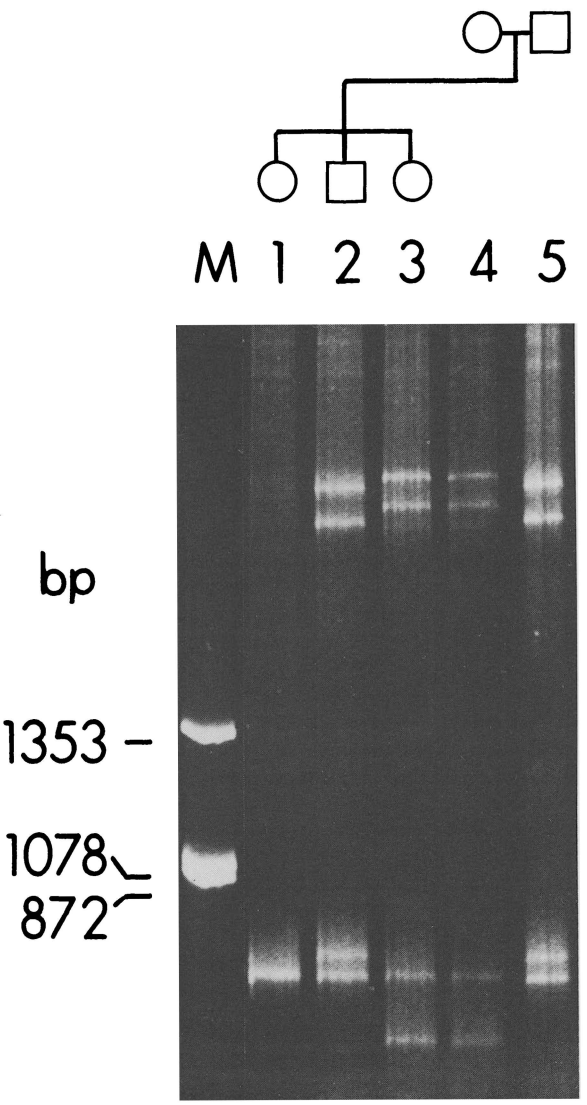


Fig. 4

subjected to polyacrylamide gel electrophoresis. Similar to what has been observed with the HLA-DQA locus, the polymorphic pattern is composed of 1-4 bands. The lower bands are believed to be homoduplexes and the upper bands are formed by interstrand combination of parental homoduplexes, i.e. heterduplexes. Similar results were obtained with the hypervariable region in the 3'-flanking region of the Type II collagen locus. The polymorphic pattern is composed of two to four bands.

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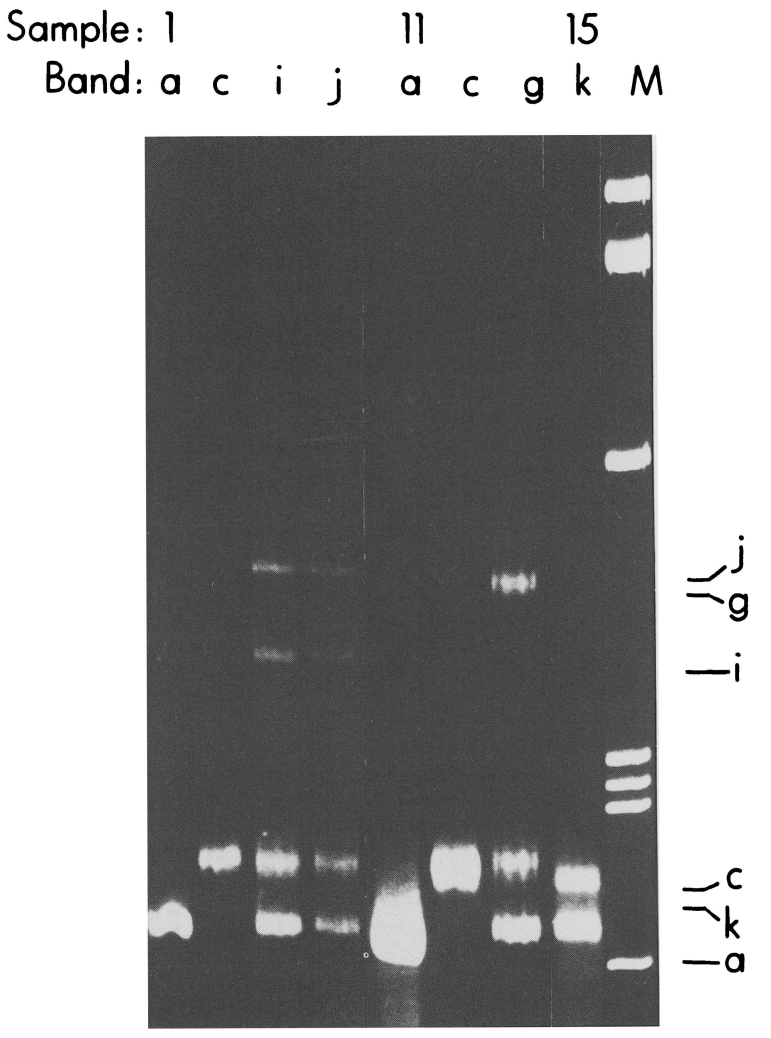


Fig. 3

In addition, sequencing of band b and c from sample 9 (Fig. 1B) confirmed the assignment of homoduplexes and heteroduplexes representing HLA-DQA allele 4 and 1.2 respectively.

APPLICATIONS IN GENOTYPING OF HYPERVARIABLE LOCI

Homoduplexes and heteroduplexes in hypervariable loci were examined. As shown in Fig. 4, polymorphic patterns were obtained when PCR products from the VNTR region associated with the locus 3' to the apolipoprotein B were

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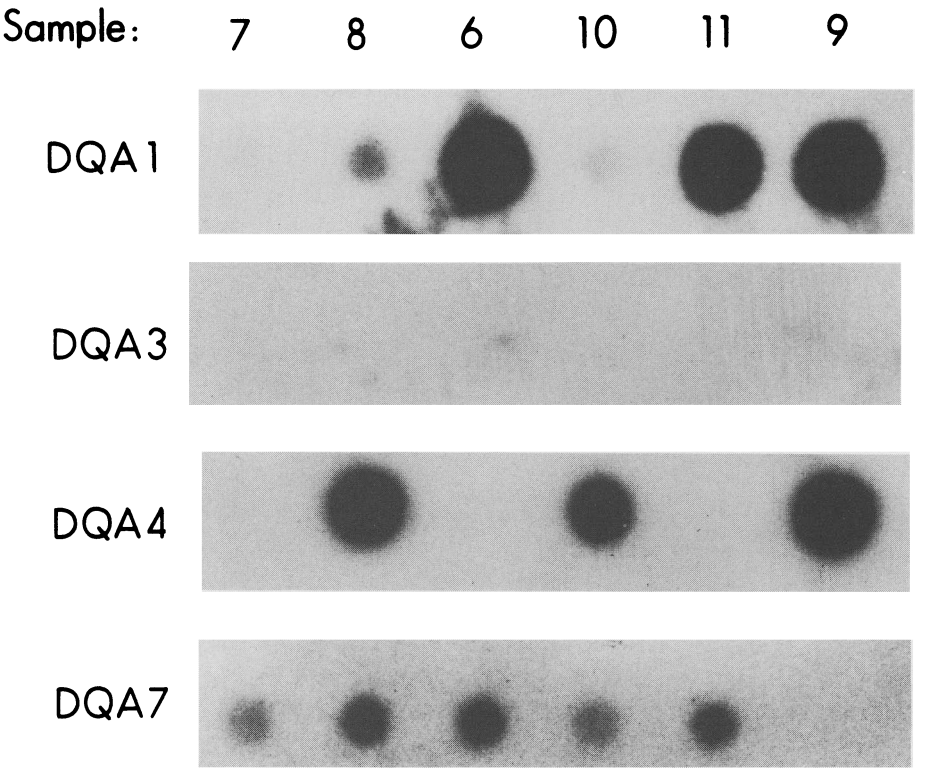


Fig. 2

Additional proof that the pattern resulted from homoduplex and heteroduplex formation is provided by the following experiments. As shown in Fig. 1B, the polymorphic pattern for sample 1 consists of bands a, c, i and j. When band a from sample 1 was reamplified, only band a was obtained (Fig. 3), Lane 1. When band c from sample 1 was reamplified, only band c was obtained, Lane 2. However, reamplification of either band i or band j from sample 1 resulted in all four bands (a, c, i and j,) Lane 3. These results show that bands a and c are homoduplexes while bands i and j are heteroduplexes. Similar results were obtained with sample 11 (Fig. 3).

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FORMATION AND DIFFERENTIATION OF HOMODUPLEXES AND HETERODUPLEXES

The validity of this procedure for genotyping was tested using the HLA-DQA polymorphic region. Amplified products from the HLA-DQA locus of different individuals electrophoresed through an agarose gel ran as a single fragment of approximately 240 base pairs, as shown in Fig. 1A. However, when the PCR products were run on a 15% polyacrylamide gel under non-denaturing conditions, several distinct polymorphic patterns were seen for different individuals (Fig. 1B).

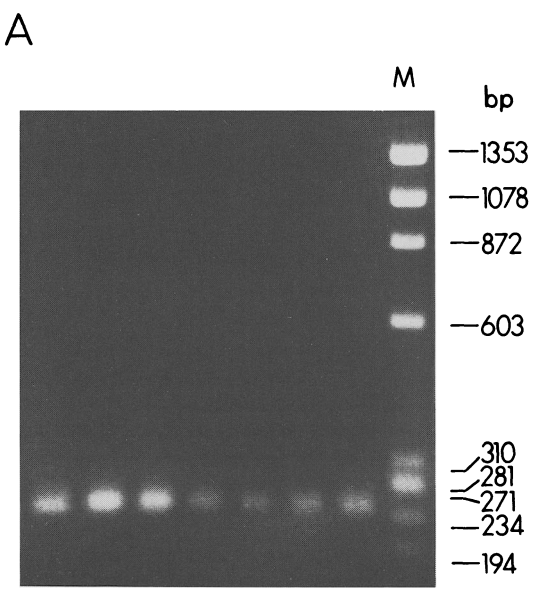


Fig. 1A

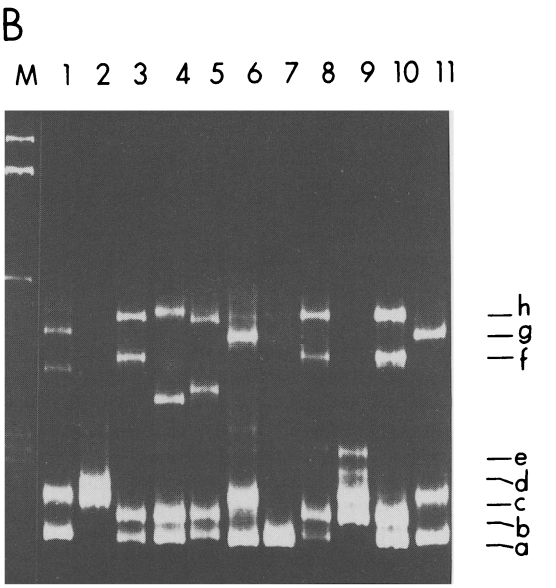


Fig. 1B

GENOTYPE ASSIGNMENT

The HLA-DQA genotype represented by each unique polymorphic pattern seen on the polyacrylamide gel were compared with the typing done by dot blot analysis using allele specific oligonucleotide probes. As shown in Fig. 2, amplified products from samples 6-11 of Fig. 1B were analyzed by the dot blot method. The polymorphic patterns associated with samples 9, 10 and 11 were typed as HLA-DQA (1,4), (4,7) and (1,7) respectively.

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A METHOD FOR THE ANALYSIS OF PCR AMPLIFICATION PRODUCTS TO IDENTIFY BASE-PAIR SUBSTITUTION AND VNTR POLYMORPHISMS

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INTRODUCTION

A Dot Blot format using allele specific oligonucleotides is commonly used to identify base pair substitution polymorphisms following the PCR amplification of target genomic DNA. A common application of this technique in forensic science has been to identify polymorphisms associated with the DQa region of the major histocompatibility complex (HLA DQa).

A method will be described which permits the identification of multiple base-pair substitution and VNTR polymorphisms using a single technique. The method relies on the simultaneous identification of homoduplexes and heteroduplexes resulting from the PCR amplification of target genomic DNA.

From Genomic DNA isolated from peripheral blood cells target sequences were amplified in a 100 ul reaction mixture containing approximately 300 ng genomic DNA, mixed nucleotides (dATP, dCTP, dGTP and TTP, 1.25 mM each), primers (100 pmol), Taq polymerase (2.5U, Perkin Elmer, Cetus, Emeryville, CA), and 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl at pH 8.3, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin). The primers used to amplify the HLA-DQA locus were SLI-243 (5'-GTGCTGCAGGTGTAAACTTGTACCAG) and SLI-244 (5'-CACGGATCCGGTAGCAGCGGTAGAGTTA) using standard amplification conditions.

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CONCLUSIONS

DNA profiling does provide an excellent technique for potentially individualizing forensic biological stains in criminal cases. DNA profiling can provide a clear and definite connection between a suspect and victim in many cases, and DNA profiling in some cases has also been used to eliminate a suspected individual. DNA profiling utilized within proper quality control and assurance guidelines and based on a scientifically sound and valid protocol provides a powerful tool for the forensic laboratory and the criminal justice system.

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Concurrent with implementation, the DNAU of the FBI Laboratory adopted a case acceptance policy which limits the examination of evidence to violent crimes with known suspects. DNA analysis is performed on probative evidence submitted by state or local crime laboratories lacking DNA typing capabilities following serological testing. Cases accepted without prior serological analysis are first processed in the Serology Unit of the FBI Laboratory for the identification of semen and/or human blood and blood grouping.

In the first ten months of operation, there have been approximately 1,000 cases accepted for examination by the DNAU. These cases represent submissions from Federal (approx. 10 percent), state (approx. 87 percent) law enforcement agencies and U.S. territories and possessions (approx. 3 percent). DNA analysis has been requested from nearly all 50 states.

A REVIEW OF THE FIRST 200 COMPLETED CASES SUBMITTED TO THE DNAU

Of the first 100 cases received in the DNAU, 71 percent were rapes, 27 percent homicides and the remaining 2 percent consisted of other violent crimes. Subsequent analyses of later 100 case groupings yielded nearly identical percentages. Serial rapes accounted for 16 of the first 71 rape cases. An examination of the RFLP results for the first 100 cases determined that 87 cases yielded successful DNA profile results. A successful result was defined as at least one probe result from a probative questioned body fluid stain which was compared to a known blood sample in a particular case. The remaining 13 cases yielded degraded or insufficient DNA. It is further noted that nearly all the first case samples were less than one year old and this likely contributed to the relatively high rate of successful DNA profile results. The variable number tandem repeat sequences recognized by the probes utilized in the DNAU exhibit a degree of polymorphism that approaches individualization of a sample which requires analysis with at least three or four probes (Budowle, 1988). The DNAU obtained results from four probings in 64 of the first 100 cases. For the second subsequent 100 case grouping, the addition of a more sensitive probe increased the overall number of four probe results to over 80 out of the second 100 cases. Our results suggest that a successful initial probing is likely to be followed by additional successful probings. For the first 100 cases, three successful probings were obtained in 14 cases, 2 probings in 8 cases and only 1 case yielded a single probe result.

One of the most significant contributions of DNA analysis to law enforcement is the ability to distinguish contributors of a mixed body fluid stain when compared to known blood samples. The differential DNA extraction procedure adopted by the FBI (Baechtel, 1988) allows for the separation of the male and female DNA components in mixed body fluid stains such as semen found on vaginal swabs. Of the first 71 rape cases submitted for DNA analysis, DNA profiles from 76 percent matched the suspects while 24 percent showed that the suspect was not the contributor to the male DNA profile. In most of these cases, conventional serological analyses did not eliminate the suspect as a possible source of the semen due to the inherent interpretational difficulties of body fluid mixtures.

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A Review of the Cases Submitted for DNA Analysis in the FBI Laboratory

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INTRODUCTION

The DNA Analysis Unit (DNAU) of the FBI Laboratory began accepting criminal cases for DNA analyses in December, 1988, and as of October, 1989, has accepted approximately 1,000 cases. The implementation of the restriction fragment length polymorphism (RFLP) protocol was the result of approximately three years of research at the FBI Laboratory Forensic Research and Training Center (FSRTC), Quantico, Virginia.

The RFLP protocol developed by the FBI Laboratory has been described in the literature (Baechtel, 1988, Budowle, 1988, Budowle et al. 1988). One of the most important determinations of the RFLP protocol development was that the DNA profile results were reliable, accurate and reproducible. The validation of the protocol currently used by the FBI Laboratory included studies involving outdoor daylight and dark exposure studies, time, temperature and substrate studies, chemical, biological and bacterial mixture studies, nonhuman cross-reactivity studies, and evidentiary stains from adjudicated criminal cases. The validation studies resulted in either accurate and reproducible profiles or no profiles at all. No false positives were obtained (Adams, 1988). Equally important, quality control (QC) and quality assurance (QA) programs were adopted within the DNAU to monitor the entire RFLP protocol and the interpretation of results (Mudd and Presley, 1988, Monson, 1988).

The most important quality control measure used in the DNA profiling protocol is the human DNA cell line. This internal control is treated in the same manner as known and questioned stains and serves to monitor the extraction, digestion, electrophoretic separation and ultimately the interpretation of the final autoradiographic results. Other quality control measures used by the DNAU have been set forth in the protocol.

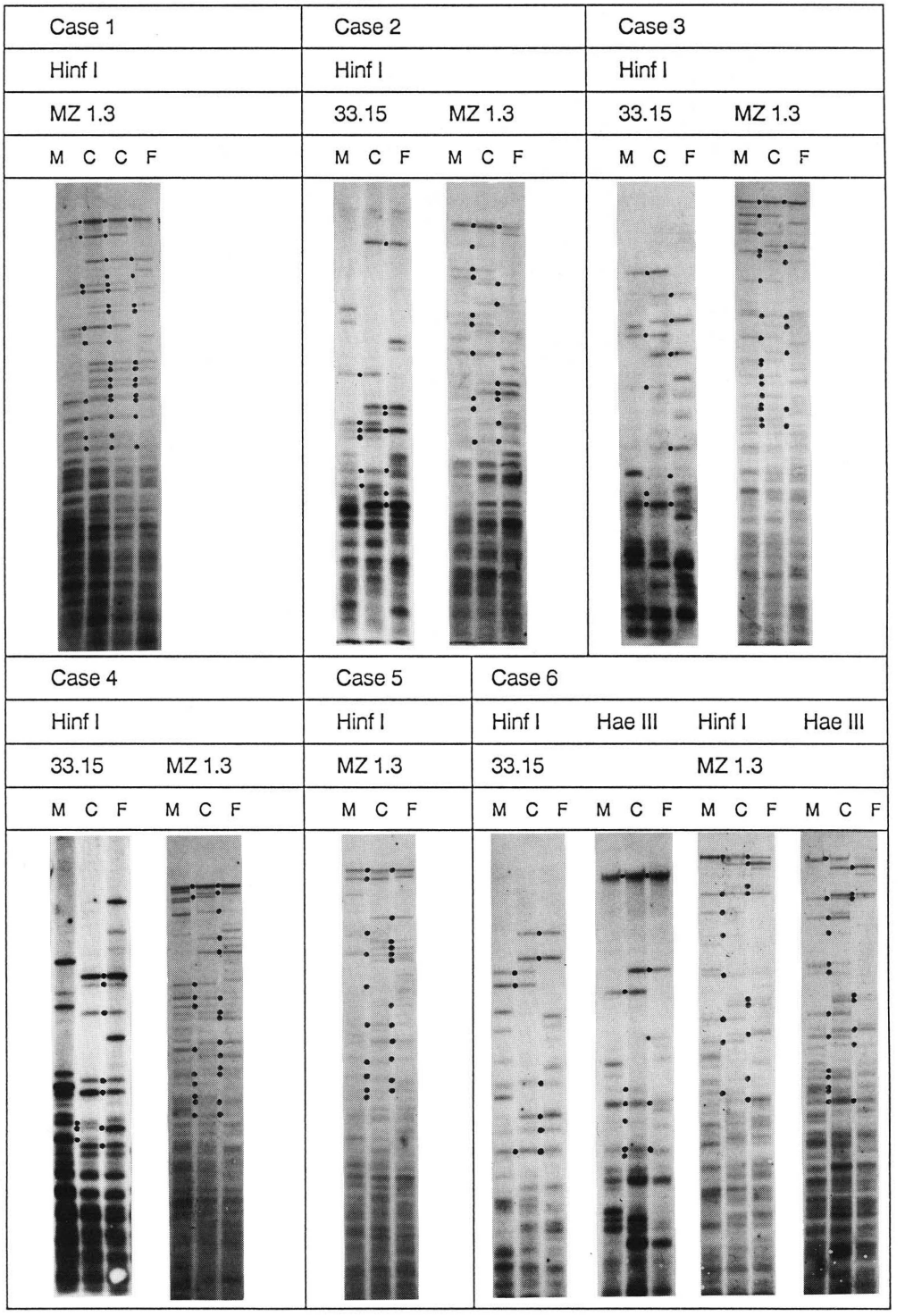
Quality assurance is regularly monitored in the DNAU by two kinds of proficiency tests. The first is an open proficiency in which samples are prepared by the FSRTC and submitted for analysis. These samples are known to the examiners as proficiency samples. The second kind of proficiency test is designated as a blind test. These samples, also prepared by FSRTC, are submitted by a cooperating law enforcement agency and simulate an actual case. The extent to which the blind sample cases are submitted is unknown to DNAU examiners.

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	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
ABO Rhesus MNSs, P_1 Kk,Fy,Jk	no exclusion	no exclusion	Fy(a-b+)/ Fy(a+b-)	no exclusion	no exclusion	no exclusion
Hp-S, Gc Gm(afxbg) Km(1), PLG Ag(x,y), C3 Bf, Tf, Pi	no exclusion	no exclusion	no exclusion	PLG 2/ PLG 1	no exclusion	no exclusion
ACP, PGM_1 ADA, Ak GPT, ESD Gt, GLO	no exclusion	no exclusion	no exclusion	GLO 1/ GLO 2	Gt Oron-1/ Gt 1 (6)	no exclusion
HLA-A,B,C	not done	not done	no exclusion	no exclusion	no exclusion	no exclusion
Additional tests	none	none	none	C6, α_2HS ORM_1, F XIIIB no exclusion	none	Lu^a, Co^b F XIIIB no exclusion
Bio-statistics	95,80%	91,32%	88,89%; with HLA 99,97%	with HLA 91,5%	>99,9999%	with HLA 99,993%; with add.tests 99,9998%
Conclusion after DNA-finger-printing	paternity confirmed	paternity confirmed	paternity confirmed; proband orignates from north Africa	paternity confirmed; silent alleles in PLG- and GLO-System	paternity confirmed; mutation to Oron-type	paternity confirmed; the accused man sub-mitted an expert evi-dence that he was infertile

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DNA FINGERPRINTING IN PATERNITY ANALYSES

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INTRODUCTION

Most cases of disputed paternity can be adequately resolved by testing 26 blood group, serum and enzyme systems, ev. completed by testing the HLA-System. In some cases however, the probability of paternity according to Essen-Möller is lower than 99,80%, which is the limit required by Swiss courts. In a few cases, exclusions, suggestive of a possible mutation or a null allele, make the analyses inconclusive.
A set of such cases was analysed by DNA fingerprinting (1, 2, 3, 4). In each case an unequivocal conclusion was reached.

MATERIAL AND METHODS

Leucocytes were collected from 10ml EDTA treated blood after centrifugation. Contaminating erythrocytes were lysed by addition of 10 volumes of 50mM KCl and incubation at 37°C for 15 minutes. After recovering the leucocytes by centrifugation, the lysis in 50mM KCl was repeated.
High molecular weight DNA was isolated according to Dykes (5) with some modifications: the leucocytes were resuspended in 9ml of 75mM NaCl, 25mM EDTA, 10mM Tris-HCl pH 7.6; then 0.9 ml 10% SDS and 100µl Proteinase K (10mg/ml in 10mM Tris-HCl pH 7.6, 1mM EDTA) were added.
After protein digestion, the peptides were precipitated by addition of 3ml of saturated NaCl solution and pelleted by centrifugation. The DNA was precipitated from the supernatant by addition of 2 volumes of absolute ethanol and was resuspended in 10ml Tris-HCl pH 7.6, 1mM EDTA.
DNA samples of 11µg were digested with restriction endonuclease Hinf I (Boehringer Mannheim, FRG) or Hae III (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.
After ethanol precipitation the DNA fragments were subjected to gel electrophoresis (0.8% agarose gel in Tris-borate buffer, at 40V for 40h). Then the DNA was transferred to a nylon membrane (Hybond N, Amersham, Buckinghamshire, UK) either by capillary transfer or by vacuum blotting.
Hybridization to the probe 33.15 (Cellmark Diagnostics, Abingdon, UK) labelled with ^{32}P by primer extension was carried out in 10% dextran sulfate, 0,5M NaCl, 1% SDS, 100µg/ml denatured salmon sperm DNA for 16h at 60°C. The membranes were washed twice in 2xSSC*, 0.1% SDS and twice in 1xSSC*, 0.1% SDS at 60°C and then autoradiographed at -70°C with AGFA-CURIX RP1L film (Agfa-Gevaert, Leverkusen, FRG).
Hybridization to the biotin-labelled probe MZ 1.3 (Biotest, Frankfurt, FRG) and subsequent colour development was carried out according to the manufacturer's instructions.
In some cases, the probe was removed from the filter by incubation in 0.4M NaOH at 42°C, followed by neutralization in 0.2M Tris-HCl pH 7.6, 0.1xSSC*, 0.1% SDS. Then the filter was hybridized again as described above.
*(20xSSC = 3M NaCl, 0.3M Na citrate)

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The increased sensitivity is due to a 20-fold amplified binding of MZ1.3 to its target

Hybridization with MZ1.3 was found to result in much higher band intensities than could be obtained with MZ7x1. This phenomenon could not be attributed to differences in labeling and thus was assumed to have structural reasons depending at least in part on the length of the probe. The binding of MZ1.3 to its target sequences was quantitated by the following experiment: pUC-MZ1.3 was cleaved with restriction enzymes PstI and BamHI to to release the repetitive insert. Serial dilutions of the restriction fragments resulting from the digest were separated by agarose gel electrophoresis and subsequently transfered to nylon membranes. The blot was probed with the complete radiolabeled plasmid pUC-MZ1.3 (Fig. 2).

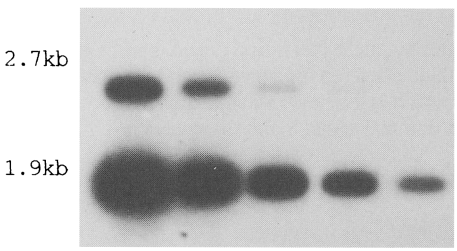


Fig. 2: Analysis of the amplification effect in MZ1.3 binding. Blot: pUC-MZ1.3, PstI/BamHI digest; 3 ng to 37 pg, dilut. steps 1 in 3; probe: pUC-MZ1.3, ^{32}P-labeled.

The 1.9kb-band representing the repetitive insert and the 2.7kb-band representing the vector fragment were excised and the radioactivity in the bands was determined. The results show that the radioactivity of the 1.9kb band is 20 times higher than that of the 2.7kb band indicating that the insert fragments bind about 20 times more probe molecules than the same number of vector fragments. Similar results were obtained when a mixture of insert and vector fragments instead of the complete plasmid were used as a probe. To analyse the influence of the length of the repetitive sequence on the sensitivity of detection, experiments similar to the one described for pUC-MZ1.3 were performed with pUC-MZ7x1 containing 7 repeats of the 27bp unit and plasmids containing 14, 28 or 56 repeats. As expected, amplification increased with the number of repeats in the probe and the target molecules. In addition an influence of the arrangement of the repeats on amplification was noted. Maximal binding required a non-interrupted repetitive sequence.

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RESULTS AND DISCUSSION

Minisatellite probe MZ1.3 allows non-isotopic fingerprint analysis

The minisatellite probe MZ1.3 was found to be a useful tool for genetic fingerprint analysis (Schneider et al.). Visualisation of radioactive RFLP-patterns requires only hours of exposure on X-ray films instead of days. This result indicated an extraordinary sensitivity of the probe that would be especially helpful for non-isotopic fingerprint analyses. A comparison of MZ1.3 fingerprint patterns obtained with isotopic and non-isotopic methods is shown in Fig.1. Except for some variations in relative band intensities the patterns on the duplicate filters obtained with either technique were identical. In addition to the obvious benefits in handling the non-isotopic detection advantage of better resolution of the RFLP pattern because dominant bands do not interfere with neighbouring faint bands. Non-isotopic finger prints could be generated from down to .5µg of restricted human DNA. Radioactive probes on the other hand proved to be at least one order of magnitude more sensitive.

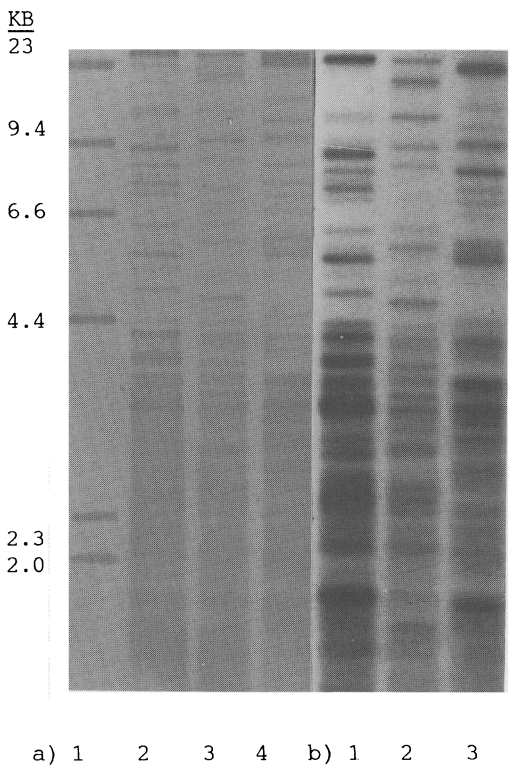


Fig. 1: Comparison of fingerprint patterns generated on duplicate nylon filters with Biotin- (a) and ^{32}P-labeled (b) MZ1.3 probes. a)1: Biotinylated size marker, Lambda HindIII dig.; a)2 and b)1 human DNA I; a)3 and b)2 human DNA II; a)4 and b)3 human DNA III; digest: HinfI.

The specificity of the minisatellite probe MZ1.3 is determined by a tandem repeat of a 27bp long sequence

In order to analyse the specificity and the sensitivity of MZ1.3 in more detail, Southern Blot experiments with MZ1.3 and subfragments were performed. MZ7x1 is a MspI fragment consisting of 7 tandem repeats of the 27 bp motif found in MZ1.3. It was cloned in the AccI site of pUC18. In a comparison of the MZ1.3 probe and the MZ7x1 probe in Southern Blot analyses with HinfI-restricted human DNA identical RFLP patterns were obtained. This result suggests that the binding specificity of MZ1.3 is determined by the 27bp repeat.

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Non-isotopic DNA fingerprint analyses with the minisatellite probe MZ1.3

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INTRODUCTION

RFLP analyses with minisatellite probes yield highly informative individual specific banding patterns (genetic fingerprints) (Jeffreys et al.). Because of the complexity of the pattern and marked differences in band intensities a good band resolution and high sensitivity of the probe are essential. By reason of the latter requirement to date most of the RFLP-studies are performed with radioactive probes. Although non-isotopic labels have been introduced into fingerprint analyses (Schäfer et al.; Medeiros et al.) ^{32}P-labeled probes are still superior with respect to sensitivity. Our approach to increase the specific signal intensity makes use of an amplification of the number of probe molecules bound per target molecule. The minisatellite probe MZ1.3 we describe here shows a 20-fold target-specific amplification in binding, facilitating the use of non-isotopic detection techniques.

MATERIAL AND METHODS

DNA-probes: MZ1.3 is a 1.9kb minisatellite probe isolated from a human genomic library by means of its crossreactivity to a repetitive sequence in bacteriophage M13 (Schacker et al.). It contains at least 40 repeats of the 27 bp motif 5'-CGGCGGWGGTGGTGGTATYGGTTGTGS-3', where W is A or T, S is G or C, and Y is T or C(4). The probe was cloned in the vector pUC18 (Pharmacia LKB, Freiburg, FRG). Radioactive labeling of probes: 100 ng of DNA was labeled with 50µCi deoxyadenosine triphosphate (NEG 012H,DuPont de Nemours, Dreieich, FRG) by random priming reactions (Random primed DNA Labeling Kit, Boehringer Mannheim GmbH, Mannheim, FRG) according to the manufacturers instructions. Non-isotopic probes: A biotinylated MZ1.3 probe (B.E.S.T.-Probe MZ1.3, Biotest AG, Dreieich, FRG) was used. Southern Blot analyses: Non-isotopic blots were prepared, hybridized and developed according to the instructions for the B.E.S.T.-Probe MZ1.3 Kit Radioactive hybridizations with ^{32}P-labeled probes followed the same protocol with the following modifications: The concentration of the radioactive probe was 1/10 of that of the non-isotopic probe. The prehybridization and hybridization solutions contained 2% skimmed milk powder. After stringency washes genomic Southern Blots were exposed to X-ray films (Kodak XAR-5) at -70°C with intensifying screens. Quantitation of radioactivity: Samples were mixed with scintillation cocktail (Quickszint I, Zinsser Analytik, Frankfurt, FRG) and counted in a Beckman LS3801 liquid scintillation counter.

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three or four offspring as shown in Fig. 1. For this analysis, paternal and maternal fragments of identical size due to band sharing were not included, as they are not informative. For each segregating fragment which was clearly inherited either from father or mother, we determined the distribution pattern within the offspring. Individual distribution patterns were taken as evidence for independent loci, whereas identical segregation patterns were assigned to the same or a closely linked locus. Using this approach in two unrelated families with 4 children, we found in both evidence for at least 9 independent loci which are recognized by MZ 1.3. In another family with three children (Fig. 1, Fam. II.), five independently segregating fragments were identified.

An example is given in Fig. 2, which represents a schematic interpretation of the informative fragments of family I.(Fig. 1). in Fig. 1. Twelve paternal and eight maternal fragments are informative in this family. Parental fragments of identical size are not shown. In the father, nine different segregation patterns are found and six in the mother. Patterns 2 and 8 into the father and 4 and 5 in the mother are represented at least twice suggesting that these are either linked or could be split in additional patterns by studying larger pedigrees. This method does not allow, however, to distinguish clearly between independent and allelic fragments. On the other hand, it provides a simple way to estimate the number of informative loci represented in a DNA fingerprint analysis.

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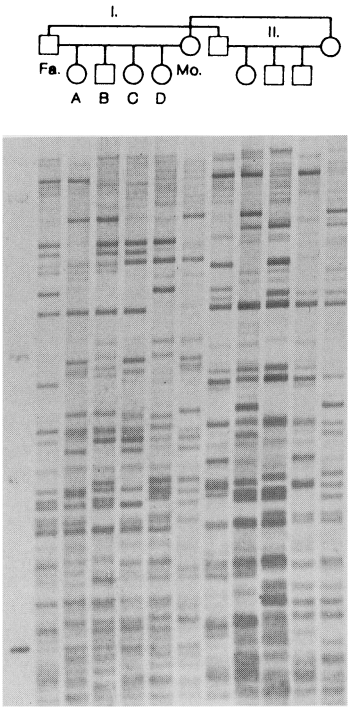
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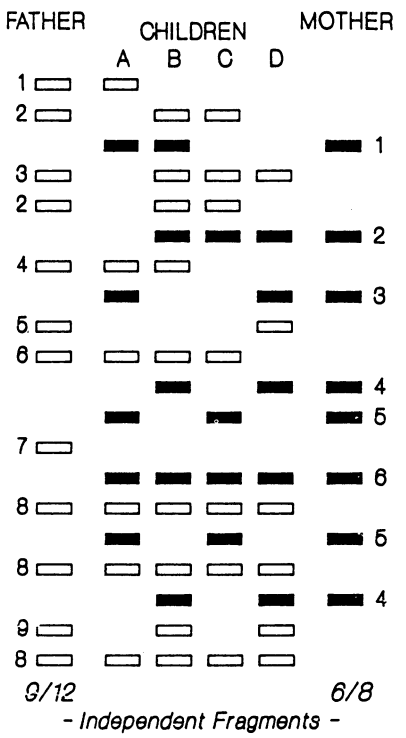


FIGURE 1 (left):
DNA fingerprint analysis of two related families (I. and II.) with the minisatellite probe MZ 1.3. Genomic DNA was digested with the enzyme Hinf I, subjected to electrophoresis in a 0.8 % agarose gel of 25 cm length for 40 h at 40 V, blotted onto nylon membrane and hybridized with the biotinylated probe MZ 1.3. Specific fragments were developed using enzymatic detection with streptavidin and horseradish peroxidase according to the manufacturer's instructions (Biotest AG, Dreieich, FRG). The family pedigrees are shown above. DNA size markers are in the left lane (top to bottom): 23.1 kb - 9.6 kb - 4.3 kb.

FIGURE 2 (right):
Schematic interpretation of unique parental DNA fragments of family I. (Fig. 1). Only informative fragments are shown, i.e. fragments which are clearly inherited either from the father or the mother. Parental fragments of identical size are omitted. The numbers of the both sides indicate unique segregation patterns for father and mother, e.g. pattern 1 (father) is found in child A, whereas pattern 2 from the father is found twice in children B and C. In the latter case, linkage has to be assumed due to cosegregation of the two paternal fragments. The total number of fragments as well as the number of independently segregating fragments is shown below for both parents.

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MINISATELLITE DNA PROBE MZ 1.3: APPLICATION IN PATERNITY TESTING AND ESTIMATE OF THE NUMBER OF GENETIC LOCI

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The use of hypervariable DNA minisatellite probes recognizing repetitive genomic DNA sequences has become a valuable and powerful tool in paternity testing as well as in forensic stain analysis (Jeffreys et al. 1985, 1987; Werrett et al. 1988). It has been shown that bacteriophage M13 DNA can also be used to obtain hypervariable DNA restriction fragment patterns in humans and other species (Vassart et al. 1987). To obtain more informative and specific fragment patterns for the DNA 'fingerprint' analysis in man, we have used M13 DNA as a probe to screen a human genomic library. Thus, we have isolated the minisatellite DNA probe MZ 1.3 (Schacker et al., in press). MZ 1.3 is a 1.9 kb fragment containing a 27 bp core sequence which is repeated 40 times. Alignment of the MZ 1.3 repeat with the 15 bp repeat of the M13 protein III gene has revealed identity at 8-11 positions, i.e. 53 - 73 %. No significant homology >70% was found by comparison of MZ 1.3 to the core repeat sequences of 33.6 and 33.15 described by Jeffreys et al. (1985).

With MZ 1.3, hypervariable restriction fragment patterns can be obtained by using the restriction enzymes Bst NI, Hae III, Hinf I, Mbo I, Pst I/Pvu II, and Rsa I. With Hinf I, we compared the fragment patterns of unrelated individuals as well as of parents and their offspring. The number of informative bands > 4.3 kb varied between 12 and 25 with an average of 18 bands. The band sharing frequency among unrelated individuals was 23.8 ± 7.2 % and 59.9 ± 7.8 % in parent-child comparisons. We were able to solve a number of special paternity cases with MZ 1.3, in which the conventional blood group analysis was either not informative or not applicable. These include a number of incest cases involving father, son and daughter/ sister within the same family, as well as paternity testing post mortem or after abortion (Schacker et al., in press; Rittner et al. 1989a, 1989b). Besides conventional labelling by radioisotopes, MZ 1.3 can also be used in a non-radioactive detection system (Fig. 1; B.E.S.T. Probe MZ 1.3, Biotest AG, Dreieich, FRG). It was found that a sequence-specific increase in binding enhances the sensitivity of the probe (Holtkamp et al., this volume).

Using minisatellite probes recognizing multiple loci throughout the genome, information on the number and linkage relationship of individual DNA fragments is difficult to obtain, as identical fragments may be derived from independent loci and allelic fragments are not recognizable. To get an idea about the number of genetic loci detected by MZ 1.3, we studied families with

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In forensic medecine, our probe represent a new system with wide application. The DNA fingerprint technique is routinely used in our laboratory for paternity testing. All the cases were resolved without ambiguity with probability of error as low as 1/10000 to $1/10^{7}$ for paternity confirmation and $1/10^{10}$ to $1/10^{18}$ for paternity exclusion. Out of 75 paternity confirmations (= 150 meiosis) no mutation band was detected with the M13 probe.

We have optimized the experimental conditions yielding high molecular weight DNA from different small blood and semen stain collected from different substrates and from different tissues collected from cadavers.

Tissue fragments are frozen in liquid nitrogen, powdered and incubated overnight with proteinase K at 50°C. Sperm cells (after separation of the vaginal cells) and dried blood samples are incubated with proteinase K and β-mercaptoethanol for 2 hours at 50°C. All samples were extracted with 1:1 phenol-chloroform solution (v:v).

All cases of rape we received were resolved following this technique; The different kinds of samples were: vaginal douches or swabs 4 to 24 hours after the rape, dried sperm on clothes and dried sperm mixed with dried blood on clothes.

Identification of suspects by comparison with fingerprint of dried blood samples depends of the nature of the support of the sample. Blood on cotton and nylon support permits extraction of high molecular weight DNA giving interpretable fingerprint.

To confirm the results, all tests were repeated with locus-specific probes as YNH24 . In some cases, we could not obtain a fingerprint with M13 because of problems such restricting the DNA (blood spots, on wood, paint or pavement) but hybridization with YNH24 gave weak but interpretable patterns. In vitro amplification of the DNA using the PCR technique would be helpful for these cases.

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DNA Fingerprinting with M13 Probe

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INTRODUCTION

The genome of vertebrates harbors, within the moderately repeated class of DNA, a large number of relatively short sequences repeated in tandem. These minisatellites show extreme variability in the number of motifs they contain at a given locus. Together with their dispersion throughout the genome, this characteristic has been exploited to generate individual specific DNA fingerprints (1).

In the course of a systematic search for Restriction Fragment Length Polymorphism (RFLP) with the thyroglobulin gene, we observed that the probe corresponding to DNA fragments subcloned in the bacteriophage M13 gave different results when used in the classical hybridization mixture containing Denhardt's solution and herring sperm DNA and the new blotto medium containing dried skimmed milk. In these conditions we observed a complicated and highly polymorphic pattern with the enzyme Hae III. It was evident that this pattern was unrelated to the nature of the insert and that wild-type M13 DNA gave the same results. The logical explanation for this finding was that a segment in M13 hybridized to a hypervariable minisatellite. This particular sequence could be competed for by fish DNA.

FINGERPRINTS IN FORENSIC MEDECINE - RESULTS

We have demonstrated that M13 constitutes an efficient probe to perform DNA fingerprints in man (2) and in animals (3) , provided no competitor DNA is used during hybridization. The patterns obtained are individual-specific and clearly different from those obtained with other probes.

We have determined the probability that two unrelated individuals show the same DNA fingerprint. The value of this probability, called Pc, is 0.27 for the M13 probe which is the same as for the C probe which was obtained by screening a bovine library with M13.

Our technique is derived from the classical Southern blot methodology. After purification, DNA is cut with the restriction enzyme Hinf I. The DNA fragments are separated in an 0.8% agarose gel and transferred to a nitrocellulose membrane.
Prehybridization in formamide 35%, SSCx6, dried skimmed milk 0.25% and EDTA 5 mM is carried out during 4 hours. The hybridization is continued overnight in the same medium containing the ^{32}P-labeled probe. After washing twice at room temperature in SSCx2-SDS 0.1% and twice at 65°C (15 min) in the same medium, the membrane is autoradiographied on X-Ray film with an intensifying screen at -80°C.

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et al. 1989) opens a new avenue to study the evolution of genomic and chromosomal organization. Due to the DNA stability and the information content the superiority of fingerprints over conventional marker serology has already been demonstrated in actual case work (Roewer et al. 1989; Sprecher et al. in press). Different oligonucleotides are available depending on the specific problem to be solved. Yet the whole subject of stain analyses by oligonucleotide fingerprinting has to be covered in more detail elsewhere (Roewer et al. to be submitted). Finally in addition to paternity and forensic questions many other problems in a variety of medical and veterenary disciplines can now be tackled with ease and unprecedented precision: zygosity determination in twins, genomic changes in tumours (Lagoda et al. 1989), chimaerism after bone marrow transplantation, semen identification, genetic relationship analysis, more effective animal and plant breeding programs. Nevertheless it should always be remembered, that fingerprints from simple repetitive sequences reflect only the length information of some 10-50 DNA fragments originating from "the barren stretch of desert" around the few "gene oases" in eukaryote genomes.

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ON THE METHODOLOGY OF NON-RADIOACTIVE OLIGONUCLEOTIDE FINGERPRINTING IN THE GEL



In order to continously simplify and speed up the routine fingerprinting pro-cedure, already initially we obstained from time-consuming Southern-blotting onto expensive membranes and hence hy-bridized the ^{32}P-labeled oligonucleotide probes directly in the gel. For man $(CAC)_5$ or the complementary $(GTG)_5$ ren-der identical, highly informative fin-gerprints. Meanwhile also the exact per-centage of mutated bands has been deter-mined in a large number of meioses (Nürnberg et al. 1989). Satisfying non-radioactive oligonucleotide fingerprint patterns, however, were then demon-strable only after DNA transfer onto PVDF membranes (Zischler et al. 1989a). Most recently both advantages could be combined (see preliminary report by Zischler et al. 1989b as well as the Figure on the left): Here about 10 ug of DNA from seven healthy, unrelated volunteer donors was digested with the restriction enzyme Hinf I and electro-phorezed in a 0.7% agarose gel in TBE buffer; the gel was dried and hybridized with the digoxigenated oligonucleotide $(CAC)_5$ as described (op. cit.). After extensive washing in 0.9 M NaCl at room temperature, 1 ml of a solution was spread onto the gel containing the mono-specific anti-digoxigenin antibody coupled to alkaline phosphatase (BOEH-RINGER-Mannheim, FRG) in a 1:500 dilu-tion for 3 h (37°C). The gel was laid overnight onto developing agar con-taining NBT and BCIP. Hybridization signals were recorded as sharp purple-blueish bands using AGFA-Ortho film. Note the individual-specific hybridi-zation patterns in each lane. Intensity differences between the samples result from the unequal amounts of DNA loaded. In a more carefully controled serial dilution experiment it was shown, that 4-5 ug of human DNA are absolutely sufficient for the non-radioactive fingerprint analysis (data not shown).

ON THE PRACTICAL APPLICATIONS

Because of the severely restricted space available, the whole range of applications and particularly the advantages of oligonucleotide fingerprinting cannot be addressed here adequately. Suffice it to say that in addition to fingerprinting purposes simple repeat oligonucle-otide hybridization in situ e.g. to metaphase chromosomes (Zischler

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Application of simple repeat oligonucleotides for DNA fingerprinting

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INTRODUCTION

Simple repetitive DNA sequences consist of short, tandemly repeated sequence motives, each motif consisting of up to ten bases, e.g. the dimeric ..CTCTCTCT.. or the quadruplet ..GATAGATAGATA.. The uninterrupted simple sequence stretches are in general some 50-200 (-500) nucleotides long and they are generally spread all over the chromosomal complement (Epplen 1988). Besides the "perfect" tandem repetitions, often there are also "degenerating" elements found in the genome and mixtures like $(GT)_{25}(GA)_{15}$. Residing in intergenic spacers and introns, certainly most of the simple elements do not exert any present-day sequence-dependent function und thus they do neither contribute to the phenotype nor even to the behaviour of a given organism. Because of their peculiar structure, simple sequences are prone to a number of mutational events, only two of which are mentioned here: i) Slipped strand mispairing probably generates variant numbers of individual sequence motives, while ii) unequal crossing over mutations result in large scale length variations. The former changes can be demonstrated in high resolution denaturing sequencing gels after molecular cloning (cave cloning artifacts of "poisonous" repetitive sequences in prokaryotes!) or better by direct genomic DNA amplification via the polymerase chain reaction. The latter long range mutations are more easily shown, e.g. by conventional agarose gel electrophoreses and subsequent probe hybridizations. Both methods proved the hy-pervariability of simple sequences (for review see Epplen et al. 1989). In summary their overall biological meaning may be best understood by regarding simple sequences as "the consequences of the properties of eukaryotic genomes". For the purpose of this discussion we shall restrict ourselves exclusively to the use of simple repetitive DNA as a fool-proof tool. Since all eukaryotes contain different sorts of simple repetitive DNAs interspersed in their genomes, it seemed reasonable to try to device a generally applicable method for the simultaneous multilocus detection, preferably by oligonucleotides. This allows to generate highly informative, individual-specific hybridization patterns in man (Nürnberg et al. 1989), in all investigated animal as well as plant species (Epplen et al. 1989).

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RESULTS

Using different biological materials the analyses of the $(GTG)_5$ fingerprints show identical patterns in all tissues from the same person, but different patterns between different persons. This was confirmed in more than 10 cases, one example of which is shown in figure 1. An example of paternity testing is demonstrated in figure 2. The DNA isolated from a blood clot of a child was compared to the oligonucleotide fingerprint of his parents. Clearly the child is the biological offspring of this couple since all its bands can either be referred to as maternal or paternal. In addition DNA fingerprints were generated from three pieces of dead bodies (all muscle tissues) which were found independently at different times and locations in a lake. The oligonucleotide probe $(GTG)_5$ proved that two of the pieces belonged to one body, while the third one (L 186/89) could be excluded (figure 3).

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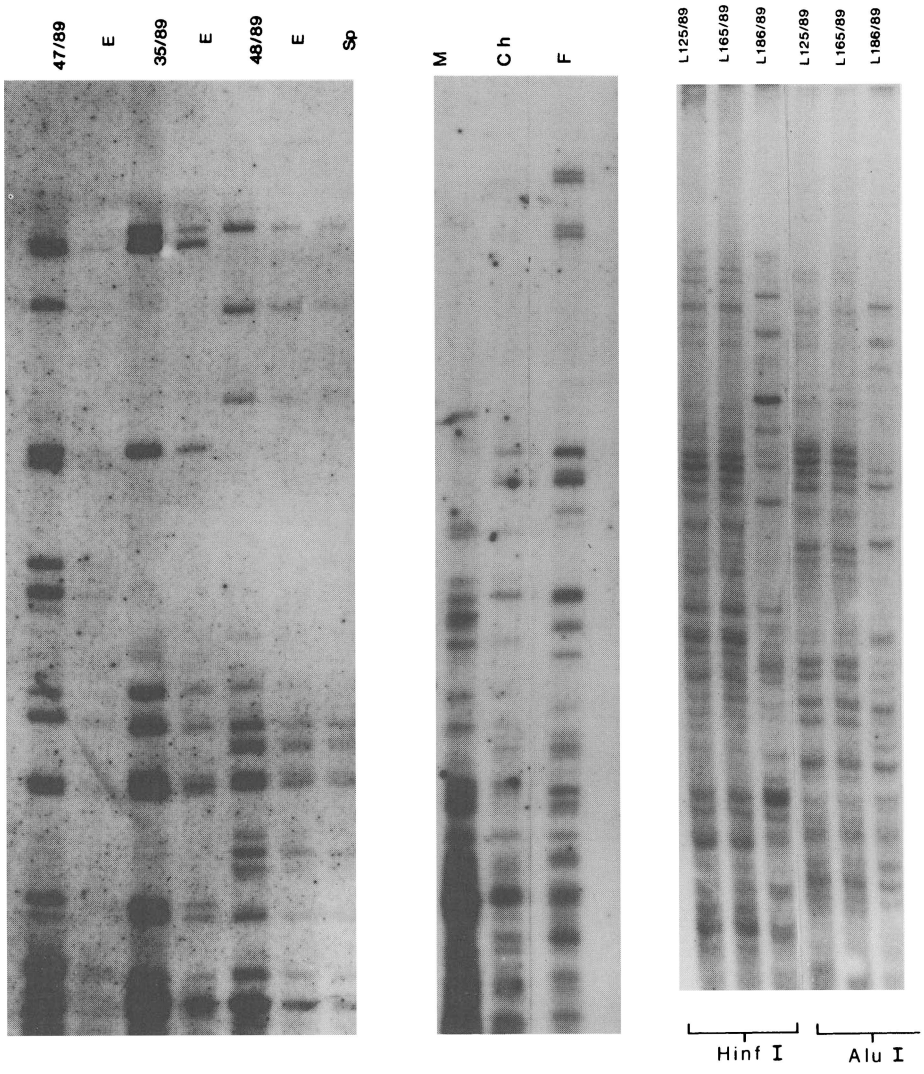


Figure 1: $(GTG)_5$ fingerprints of three persons. DNA obtained from different tissues (numbers=blood, E=epithelial cells from mucosa, Sp=sperm), digestion by Hinf I.

Figure 2: $(GTG)_5$ fingerprints of parents and their child. DNA obtained from blood, digestion by Hinf I. (M=mother, Ch=child, F=father).

Figure 3: $(GTG)_5$ fingerprints of 3 pieces of dead bodies. Digestion by Hinf I and Alu I. Piece L 125/89 and L 165/89 showing an identical pattern. L 186/89 does not belong to the same corpse.

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DNA fingerprinting with simple repetitive oligonucleotide probes in forensic medicine

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INTRODUCTION

DNA technology in forensic medicine (determination of the origin of biological materials and of consanguinity) has advanced dramatically over the last five years. Individual-specific fingerprints in man can be obtained using oligonucleotide probes specific for simple repetitive DNA sequences (Ali et al. 1986, Schäfer et al. 1988, Nürnberg et al. 1989). Simple repetitive DNA sequences consist of short, tandemly repeated sequence motives. They are spread all over the human chromosomes, i.e. in intergenic spacers and introns; they show extensive polymorphism (Epplen 1988, Epplen et al. 1989). The simple repetitive DNA elements do not exert any sequence dependent function. They do neither contribute to the phenotype nor to the behaviour in man. Therefore the only information obtained concerns the individual-specific hybridization pattern of an individual.

The most informative DNA fingerprints for paternity testing in man, including stains from blood clots, sperm traces and epithelial cells were obtained with the simple triplet repeat probe $(GTG)_5$ or its complement $(CAC)_5$. This is a highly discriminating probe which allows to differentiate all individuals in the 4 to 25 kilobase range of DNA fragments (Schäfer et al. 1988, Zischler et al. 1989). DNA fingerprints obtained by $(GACA)_4$ were also informative albeit showing less bands, allocated in the short fragment range (Roewer et al. 1989). This oligonucleotide probe can therefore be used even in partially degraded DNA.
In the following cases the $(GTG)_5$ probe was tested for its informativity by comparing the patterns from blood with those of sperm traces and epithelial tissues. Furthermore the method was also applied to identify parts of dead bodies of unknown origin.

METHOD

The DNA was isolated from peripheral blood, blood clots, muscle tissues, epithelial cells from mucosal smears and sperm, following modified protocols of Kunkel et al. (1977) and Bär (1988). The method for oligonucleotide fingerprinting has been described by Ali et al. (1986). DNA was digested by the restriction enzymes Hinf I and Alu I and electrophorezed in 0.7 % agarose gels in TBE buffer; the gels were dried and hybridized with the $(GTG)_5$ probe.

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Yield Gel

High Molecular Weight (HMW) DNA was estimated on a yield gel (0,9 % agarose, 2 hours at 80 volts) after staining with ethidium bromide ; 2 µg of HMW DNA being required to get successful result.

Digestion with Dde 1 or HAEIII

Samples of 2 µg of DNA were digested with 80 IU HAE III (Boehringer Ingelheim) or 80 IU Dde1(Boehringer Ingelheim) during 2 x 2 hours at 37°C.

Then DNA fragmentation was visualized by ethidium bromide staining after electrophoresis on 1.1 % agarose gel for 15-20 hours at 20 volts.

RESULTS AND DISCUSSION

Enough HMW DNA was extracted from 2 vaginal swabs, 5 blood stains and from all blood samples.
A 2.3 kb band was found to appear on the patterns of male DNA after digestion with HAE III. A similar band at 3.0 kb was identified with Dde 1. Confirmation was obtained by hybridization of X and Y probes on a nylon membrane (M.S.I.) after Southern transfer.
Appearance of the 2.3 kb or 3.0 kb bands among male patterns according to the selected restriction enzyme has been shown to be related to a human specific Y chromosome repeated sequence (Cooke et al 1982; Fowler et al 1987).
Thus male and female specimens can be clearly discriminated.
The method has been found to be sensitive enough to be successfully applied in forensic cases since no more than 2 µg of extracted DNA are necessary to perform the test.
In addition, this nonisotopic method does not require the use of a specifically designed laboratory or an expensive equipement.
Thus, it may be useful in forensic practice as for checking the presence of DNA of male origine in vaginal swabs in case of rape, in bloodstains in criminalistics or in blood samples in paternity testing.

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Rapid Sex Determination Using Restriction Enzymes Digestion

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In criminal investigation, the rapid determination of sex origin of bloodstains found at crime scenes or on clothing evidence provides an important clue to the identification of the suspect. Up to now, sex determination has been performed with morphological methods such as visualization of sex chromatin material (Zech 1969) or sex hormone analysis (Brown 1981 ; Yamamoto et al 1984).

Recently, several techniques dealing with molecular biology have been proposed by analyzing the Southern hybridization pattern of DNA to detect the presence of a Y chromosome specific DNA fragment (Vergnaud et al 1984; Fukushima et al 1988; Kobayashi et al 1988).
We report here a rapid method of sex identification using only a restriction enzyme digestion.

MATERIALS AND METHODS

The material consisted of :
- 6 vaginal swabs
- 6 bloodstains on coton and nylon fabric (150 µl)
-12 whole blood samples for paternity testing (0.5 ml).

DNA Isolation

Stains and swabs were cut into small pieces and incubated with proteinase K (150 µg/ml) and sodium dodecylsulfate (1 %) for two hours at 37°C.
Whole blood samples were treated with a lysis buffer (0.35 M sucrose, 10 mM Tris HCl pH 7.6, 1 % Triton X 100) before similar incubation with proteinase K.

DNA Extraction

DNA extraction was performed with phenol, chloroforme and isoamyl alcool following by dialysis against TE buffer for 2 x 4 hours.

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Maniatis et al (1982). The nylon filter blot was hybridized to YNH24 probe (Nakamura et al, 1987), and to a lambda MS1/MS31 probe mix (Wong et al, 1986), under stringent conditions. Both experiments of hybridizations provided unambiguous patterns of bands. No band sharing was observed between the suspect and the fetus.
This case showed us that numerous fetal tissues can be used for extraction of high amounts of high MW genomic DNA. Largest harvests are however to be expected from compact tissues, such as muscular tissues.

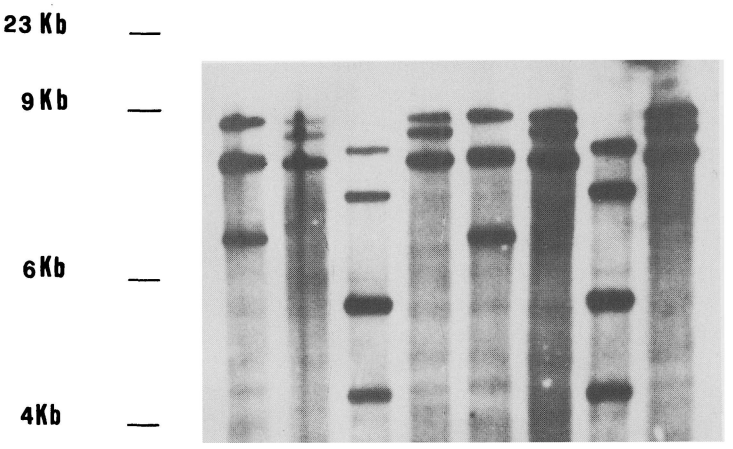


Fig.1. Allele specific profile pertaining to MS1/MS31 probe mix. From left: mother; fetal thymus; suspect; fetal spleen; mother; fetal lung; suspect; fetal heart muscle.

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Exclusion of an individual charged with rape by allele-specific DNA profiling on fetal tissues

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CASE HISTORY

A mentally handicapped young woman was raped by an unknown individual, who repeatedly abused of her. The crime was discovered only after she fell pregnant and aborted. By gestational age (16-18 weeks), the fetus was assumed to have been conceived when the woman was hospitalized. Hencefrom, a nurse was suspected to be her rapist, and was prosecuted. During the trial, our laboratory was asked to perform DNA profiles over some fetal tissues, the woman and the suspect. Following the analysis detailed here, evidence was given that the alleged rapist could not be the father.
This case allowed us to evaluate the relative amount of genomic DNA to be extracted from different fetal tissues.

DNA EXTRACTION

Triplicate specimens from different fetal tissues (thymus, lung, spleen, heart muscle, psoas muscle, kidney, chorionic villi) were made available to us. Twenty-five mg each were submitted to a standard SDS/DTT/Proteinase K lysis, with following standard phenol - chloroform treatment (Gill et al, 1985). Dosage of resuspended DNA samples was performed by spectrophotometry, fluorimetry and yield gel evaluation, to maximize informations on the quantity/quality of DNA harvests. Average quantitative results we obtained are shown in Table 1.

Table 1. Harvest of high molecular weight DNA from different fetal tissues (25 mg each)

Tissue	ug	MW	Tissue	ug	MW
thymus	3.3	high	psoas m.	10.5	high
lung	4.0	high	kidney	2.9	high
spleen	6.3	high	ch. villi	2.5	high
heart muscle	9.0	high			

Hinf I digests from the fetus/victim/alleged rapist DNAs were processed by a standard Southern blot analysis, according to

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The probability of occurrence of the combination of alleles produced by the three single locus probes used is also calculated in the three ethnic populations so far studied. When complete, the scanner will transfer the casework results to the laboratory main frame computer for addition to a case data index to enable searching outstanding cases against DNA profiles from nominated subjects and also to update ethnic frequency tables.

Using this DNA scanner data bases for λMS1, λMS31 and λMS43A have been derived from populations comprising 311 white caucasians, 154 Afro-Caribbeans and 60 Asians.

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give profiles even when microscopic examination suggests that the sperm concentration is very low.

Bloodstains are more problematic than semen stains. The number of nucleated cells is relatively small, and more material is necessary. Bloodstains also tend to be found on a wider spectrum of clothing and objects, which can cause problems with extraction and subsequent purification. Some textile dyes remain associated with DNA throughout purification, discolouring the DNA. In some cases this prevents restriction occurring.

Head hairs (about 10 anagen hairs) are sometimes used instead of body fluid controls. In case work, hairs found on weapons used in violent assaults can be successfully profiled, but those found on hats or masks (telogen hairs) give little or no success.

Cells derived by centrifugation from salvia can also be used for control purposes and often sufficient cellular material is found on cigarette ends for profiling. No success has yet been achieved from saliva on envelope flaps or stamps. DNA profiles have been obtained from nasal mucous found on handkerchiefs.

The small amounts of flesh from knives used for stabbing and flesh found on vehicles after hit-and-run accidents is particularly suitable for DNA profiling.

By use of single locus probes, mixtures of body fluids from more than one individual can be detected. If a sample from each relevant person is available as a control, we can say that a mixture of their DNA would be expected to produce a composite pattern such as that obtained. However the situation becomes more complex when one or more of the contributors does not provide a sample. This can occur in cases of multiple rape. Mixed profiles are sometimes seen when removal of victims' vaginal or buccal cells by preferential extraction is incomplete (Gill et al 1985).

DNA AUTORADIOGRAPH SCANNER

Due to the large number of measurements and calculations involved in reading results manually, a DNA autoradiograph scanner is being developed in this laboratory. This comprises a video camera and monitor coupled to an IBM AT computer via an Imaging Technology digital frame store. The mounted camera overhangs a light box on which the autoradiograph is placed. A printer is connected so that results, calculations and screen dumps can be printed out. Scanner software enables lanes to be scanned and allele sizes calculated with reference to calibration lanes on the autoradiograph. An error window, initially $\pm$ 1mm, is also calculated.

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Casework experiences with DNA profiling

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INTRODUCTION

This laboratory has been using DNA profiling in case work for 10 months and during this time has examined 400 cases. Of these, the majority have been sexual assaults, murders and violent assaults. DNA profiles have been obtained in approximately 70% of these cases.

METHOD

A modified southern blotting hybridisation method is used, where DNA is transferred to Hybond-N nylon membrane (Amersham) by vacuum blotting and hybridisation is carried out by the method of Church and Gilbert (1984) in a rotary hybridisation oven. Single locus probes λMS1, λMS31 and λMS43A are used sequentially although not in a specific order.

Allele sizes are calculated by reference to DNA size markers labelled with ^{35}S (Amersham). By use of population frequency tables (Cellmark Diagnostics) the probability of finding a second unrelated individual with the same DNA profile can be computed.

RESULTS

A high success rate of obtaining DNA profiles has been achieved with sexual assault cases.

In an examination of a rape victim, at least two vaginal swabs are routinely taken by a police surgeon. Combining the material extracted from these provides sufficient seminal DNA for profiles to be obtained, even when the swabs are taken up to 20 or 30 hours after the offence. It is our experience that this DNA is usually of good quality and profiles can be obtained from relatively small amounts. Semen stains on clothing are good sources of DNA, and, if large enough, will

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VNTR sequences in approximately 10 nanograms of DNA. D4S139 VNTR bands were detected in 93% of cases (n = 43), and 85% of all swabs (n = 118) contained DNA. Of these 73% (n = 89) had readable VNTR patterns. Thirty percent of the female case derived swabs (n = 33) had Y chromosome specific DNA present, indicating that male DNA was present. Eighty-nine per-cent of oral swabs (n = 28) contained DNA, suggesting that buccal swabs, saliva or dried saliva stains may be a useful source of DNA.

DNA SEQUENCING

DNA sequence data, obtained from a pH30 1.5 Kb spontaneous deletion plasmid, suggests that D4S139 may be from a new family of VNTR loci. The DNA sequence of ten pH30 repeat units has been obtained. The repeat units, rather than being in tandem arrays, fall in groups of two or three, each group being separated by an eight base pair spacer. Each 32 base pair repeat unit was approximately 60% G/C, with an eight base pair relatively invariant core. The consensus sequence was 78% purines. The regions flanking the repeats were also unusual. They were greater than 80% A/T, with many runs of A or T, some as long as eight base pairs. Based on this data, oligonucleotides have been made corresponding to a consensus sequence, and are being used in hybridization studies to determine if sensitivity can be further improved.

CONCLUSION

Observed data on the VNTR probe pH30, suggests that it is highly useful for DNA typing of bio-forensic evidence. It exhibits a high level of polymorphism and heterozygosity and may be the most sensitive probe so far described.Reference.

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Characterization of DNA Probe pH30 (D4S139) and Preparation of Related Synthetic Oligonucleotides

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INTRODUCTION

An extremely sensitive, highly polymorphic variable number of tandem repeat (VNTR) human DNA clone, pH30, has been isolated and found to be useful for the typing of bio-forensic evidence (Milner, E., et al 1989).

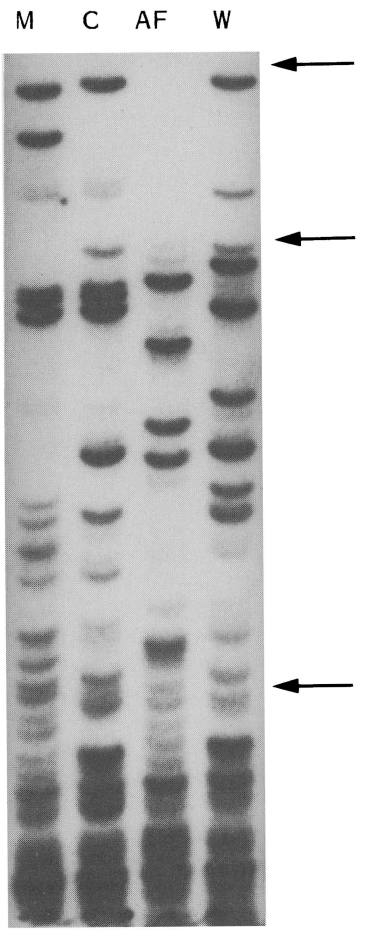
CHARACTERIZATION AND GENE MAPPING

Gene mapping studies with multiple mouse/human somatic cell hybrids were used to localize pH30 to chromosome 4. This locus has been assigned the Human Gene Mapping Workshop number D4S139. Mapping was accomplished by linkage analysis performed on 18 large three generation and 6 two generation families (CEPH families) using 53 chromosome 4 markers. Linkage was found at a distance of 16 cM, and a LOD score greater than three, to L231 and C82, markers previously mapped near the telomere of the chromosome 4 long arm. Heterozygosity was observed at 89% in Caucasoids. Over 50 bands have been detected by pH30, the most common occurring at a frequency of 13 to 15% depending on the ethnic/racial composition of the population group under study (unpublished). Clone pH 30 is complimentary to the VNTR sequence as it detects restriction fragment variants in human DNA with over 20 different restriction enzymes. There is also a rare variant that has a Hae III site within the VNTR locus. Plasmid pH30 detects relatively large restriction fragments on Southern blots of human DNA, (avg. 9 Kb) when compared to most other VNTR probes (avg. 3 to 4 Kb). This characteristic makes ph30 highly useful forensic DNA Typing analysis.

ANALYSIS OF SEXUAL ASSAULT EVIDENCE

The DNA probe pH30 has been used on Southern blots of a series of non-probative sexual assault evidence swabs, stored frozen at -20°C for periods of up to one year prior to analysis. Southern blots were first probed with pH30, and then a Y chromosome specific probe. pH30 demonstrates a high level of sensitivity, being able to detect single copy

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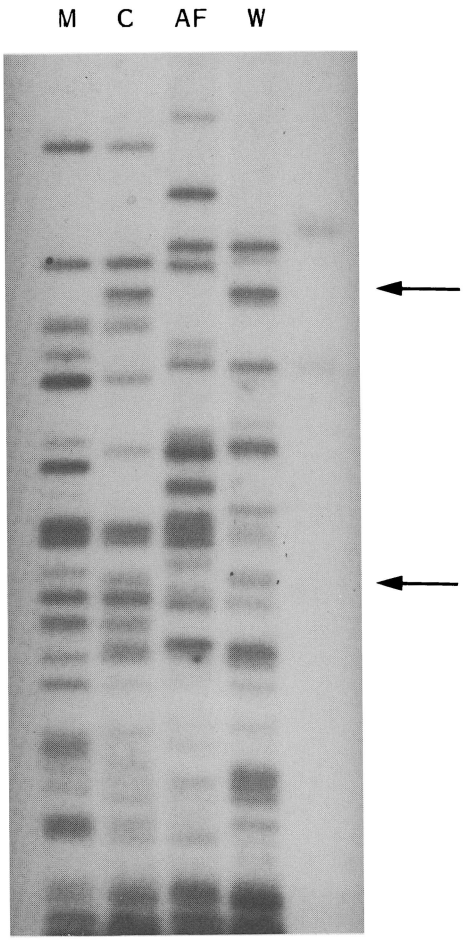


Fig. 5: DNA 'fingerprint' with minisatellite probe 33.15. Exclusions of the AF are indicated by arrows.

Fig. 6: DNA 'fingerprint' with minisatellite probe 33.6

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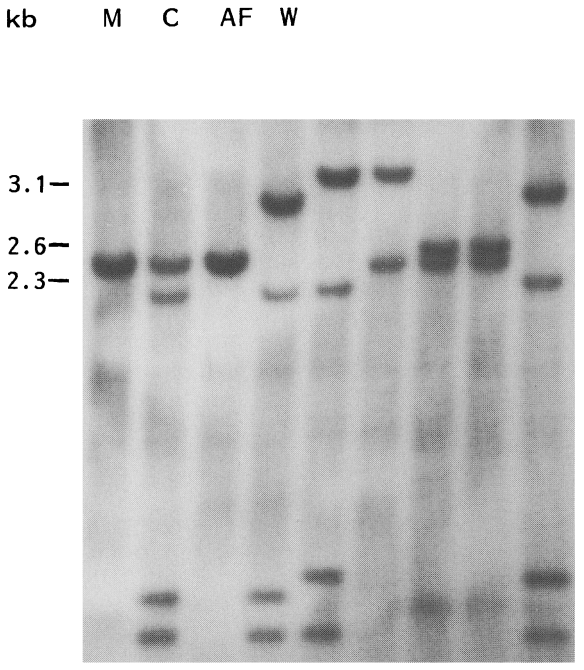


Fig. 2

TaqI polymorphism of Hras onkogen.

AF excluded
W included

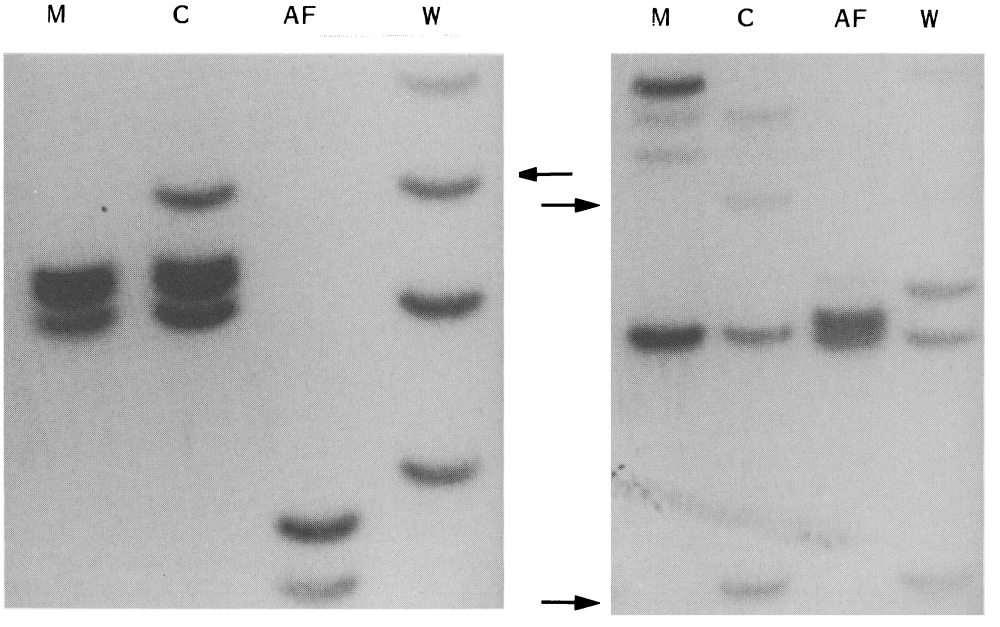


Fig.3: Mixture of single-locus probes MS31 and MS1

Fig.4: Mixture of single-locus probes MS8, MS43, g3

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CLARIFICATION OF A TWO-MAN CASE ONLY BY DNA MARKERS

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In a two-man case none of the men could be excluded from paternity in 36 blood group systems including HLA - A,B,DR. The men are not related.
On the base of mother-child combination the exclusion ratio of non-fathers came up to 99,96%. The calculation of the EM value for the accused man resulted lg Y/X + 10 = 6.3711 (W= 99,976%) PI= 4.255 and for the witness lg Y/X + 10 = 5.1862 (W= 99,998%) PI= 65.128 .
To resolve the matter we used the following combination of DNA probes:
1. EcoRI polymorphism of Hras onkogen (Wyman et al., 1980)
2. TaqI polymorphism of pAW101 (Capon et al., 1983)
3. Single-locus probes: MS1, MS31, MS43, g3, MS8
4. Two complementary multi-locus probes: 33.15 and 33.6 (Jeffreys et al 1988).
All nine DNA probes assued an exclusion of the accused man, but not of the witness.

MATERIALS AND METHODS

High molecular DNA was isolated from 5-10 ml EDTA blood by standard procedures. Individual DNA samples of 12 µg each were digested with 70 U of restriction endonuclease Hinf I (for 2 gels). Agarose gel electrophoresis was carried out in a 0,7% gel using a 134 mM Tris, 74,9 mM Boric acid, 2,55 mM Disodium ethylendiaminetatracetic acid pH 8,8 electrophoresis buffer. The gels (20x20 cm) were run at a constant voltage of 75 V for 24 hours. Treatment with 0,25 M HCl for 15 min., then with 1,5 M NaCl, 0,5 M NaOH for 30 min., and finally with 3,0 M NaCl, 0,5 M Tris ph 7,5 for 30 min. The gels were blotted overnight in 20xSSC auto nylon membranes (Amersham Hybond-N). Prehybridisation was carried out in 1 x Denhardt's solution for 30 min. at 62°C and in hybridisation solution for 60 min. at 62°C. Hybridisation was performed overnight at 62°C in the same buffer containing 20 mg 32P-labelled probe 33.15 and 33.6 or pooled inserts of MS1, MS31, g§ and MS43, MS8 (2 ng for each).

Hybridisation solution: 0,2 % Bovine serum albumin, 0,2 % Ficoll 400, 0,2 % Polyvinylpyralidone, 6,0 % Polyethylen glycol 6000, 50 µg/ml degraded Herring DNA 1 x SSC, Blots were washed 3 times in 1 x SSC, 0,1 % SDS at 62°C. Filters were exposed for 24-72 hours to X-ray film.

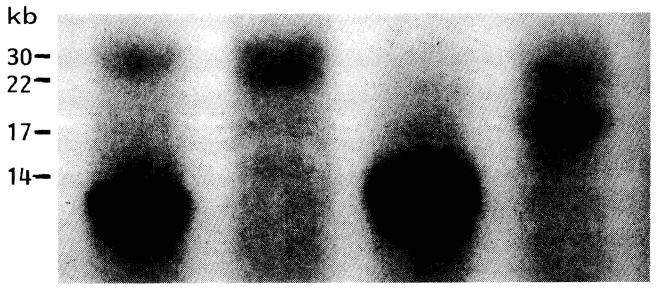


Fig. 1
EcoRI polymorphism of pAW 101 shows the exclusion from paternity of AF, but not of W.
(M=mother, C=child, AF= alleged father, W= witness)

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Table II Results from conventional tests and DNA testing using four single locus probes

In this Table the probability figures for cases where no exclusion was found using the conventional blood group polymorphisms are shown. The results using the four DNA probes on the same cases show whether or not evidence of an exclusion was found. In the cases where exclusions were found using the conventional systems they were also found using the DNA probes. In case No 38 involving a second order Duffy exclusion and an apparent discrepancy between the conventional and DNA polymorphisms, the investigation is incomplete.

	CONVENTIONAL		DNA			
	PI	%RCP	α globin 3'HVR	Mucin HVR	MR24/1 HVR	YNH24
1	175	99.4	+	+	+	+
2	160	99.4	+	+	+	+
3	3,800	99.97	+	+	+	+
4	6 million	99.9999	+	+	+	+
5	34,000	99.997	+	+	+	+
6	73,000	99.998	+	+	+	+
7	7 million	99.9999	+	+	+	+
8	1,470	99.93	+	+	+	+
9	24,000	99.996	+	+	+	+
10	80	98.8		+	+	+
11	22	95.7		+	+	+
12	690	99.85	+	+	+	+
13	1,000	99.9		+	+	+
14	1,700	99.4	+		+	+
15	1	50.0	+	+	+	+
16	90	98.9	+	+	+	+
17	32	96.9	+	+		+
18	21	95.5	+	+		+
19	5	83.3		+	+	
20	500	99.8	+	+	+	+
21	5,000	99.98	+	+	+	+
22	75	98.7	+	+	+	+
23	1,900	99.95		+		+
24	3,400	99.97		+		+
25	8,000	99.98		+		+
26	30,000	99.997				+
27	300,000	99.9997				+
28	61,000	99.998				+
29	186	99.5		+		+
30	50	98.0		+		
31	7,700	99.99		+		+
32	10,000	99.99		+		
33	Exclusion			Ex	Ex	Ex
34	Exclusion		Ex	Ex		Ex
35	Exclusion		Ex	Ex		Ex
36	Exclusion		Ex	Ex	+	Ex
37	Exclusion			+		Ex
38	2° Excl. PI = 106		+	+	+	+

KEY: + = no evidence of exclusion

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Table I Results from conventional blood group systems and DNA using L336

This Table compares the figures for the probability of the father's biological relatedness to the child (PI and RCP) derived from the conventional blood group polymorphisms with those derived from the L336 DNA polymorphism. The compound figures using both types of polymorphism together are also shown. Probability figures are also shown for L336 where it failed to produce evidence of exclusions in cases 35, 37 and 42.

	BLOOD GROUPS		L336		TOTAL	
	PI	RCP	PI (1%)	RCP	PI	RCP
1	95	99	19.9	95.2	1,891	99.947
2	745	99.87	10.5	91.3	7,823	99.987
3	380	99.7	2.3	69.7	874	99.886
4	6,186	99.98	17.5	94.6	108,255	99.9991
5	14,820	99.99	17.5	94.6	259,350	99.9996
6	1,166	99.91	2.3	69.7	2,682	99.963
7	1,687	99.94	9.9	90.8	16,701	99.994
8	200	99.5	13.5	93.1	2,700	99.963
9	5,000	99.98	3.1	75.6	15,500	99.994
10	9,000	99.99	105.0	99.1	945,000	99.9999
11	86	99	15.5	93.9	1,333	99.925
12	200	99.5	5.1	83.6	1,020	99.902
13	162	99.39	28.6	96.6	4,633	99.978
14	161	99.39	37.7	97.4	6,070	99.984
15	309	99.68	5.9	85.1	1,761	99.943
16	189	99.47	10.0	90.9	1,890	99.947
17	482	99.79	9.0	90.0	4,338	99.977
18	289	99.66	65.1	98.5	18,814	99.995
19	2,300	99.96	2.3	69.7	5,290	99.981
20	135,800	99.999	5.1	83.6	692,580	99.9998
21	3,000	99.97	14.1	93.4	42,300	99.998
22	13,000	99.99	10.5	91.3	136,500	99.9993
23	412	99.76	14.0	93.3	5,768	99.983
24	300	99.67	11.2	91.8	3,360	99.970
25	419	99.76	15.5	93.9	6,495	99.985
26	161	99.38	11.2	91.8	1,803	99.945
27	4,402	99.98	10.5	91.3	46,221	99.998
28	203	99.51	18.5	94.9	3,756	99.973
29	24,500	99.996	18.0	94.7	44,100	99.9998
30	104	99.05	8.7	89.7	905	99.8890
31	1,150	99.91	55.5	98.2	63,825	99.998
32	902	99.89	410.0	99.8	369,820	99.9997
33	5,840	99.98	189.0	99.5	1,103,760	99.9999
34	56	98.2	44.9	97.8	2,514	99.960
35	Excluded		9.5	90.5		
36	Excluded		Excluded			
37	Excluded		7.9	88.8		
38	Excluded		Excluded			
39	Excluded		Excluded			
40	412	99.76	Excluded			
41	Excluded		Excluded			
42	Excluded		7.6	88.4		

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Investigation of the use of DNA Probes L336 and YNH24 in Problems of Doubtful Parentage

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The application of the DNA probes L336 (from Collaborative Research Inc.), YNH 24 (Promega) and Mucin, MR24 and 3'α HVR (from Amersham International) was investigated in a series of families requesting parentage tests. The bands present in a putative parent's DNA may be used as part of the probability calculation of his biological relatedness to the child if allowance is made for the continuous variability of band size in the population and the inaccuracy of the methods for measuring band size. This was done by estimating the level of variation seen when repeated measurements were made of the band sizes in individuals on consecutive occasions and in parent/child pairs. The range of band sizes including the measured one was taken in order to determine the frequency of the band from the published tables for L336. The probability of relatedness derived from this frequency was then compared with the results obtained from the use of a wide range of conventional phenotypic polymorphisms (see Results Table I).

Using the four probes YNH 24, 3'α HVR, Mucin and MR24 the results whether or not an exclusion was found by visual examination of the autoradiographs was compared with the result of the conventional tests. These data are shown in Results Table II.

CONCLUSIONS

A high level of agreement was found between the results obtained using DNA probes and those from the conventional phenotypic polymorphisms in that (except in five cases) exclusions were not found using one method and not the other. Of these exceptional cases in three the conventional systems showed an exclusion and the single DNA probe used failed to do so. In one, the probe L336 showed an exclusion when the conventional systems had failed to do so, and in one the apparent discrepancy is probably the result of an unusual red cell antigen.

We consider that the use of a range of DNA probes in conjunction with the conventional polymorphisms will offer a very high compound chance of producing evidence of exclusions and will provide a powerful tool to investigate parentage problems.

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In case 8, with a measurement error of 1% the putative father is excluded the pattern of the plot was identical to the other excluded cases: a curve starting with W = 0.0 (standard measurement error is 0 kb) to W = 0.5 (standard error 3kb). Raising the error rate to 2% the 5 non fathers remained excluded, The putative father of case nr 5 was excluded by conventional systems, HLA and by the Hinf I RFLP detected by probe MS31, (W = 0.0), but the result of the Hae III RFLP with probe YNH24 was doubtful (I = 1.32). When in this case the measurement error increases, the W-value increases from 0.0 to 0.55 and in the end the W-value approaches 0.5 from below.
By plotting the W-value as a function of the measurement error seems to be a good method to avoid wrong conclusions and to discover cases in which further testing of other genetic systems is necessary.
In spite of the small number of paternity cases tested in this study the results suggest that the Hae III RFLP detected by probe YNH24 together with the Hinf I RFLP detected by probe MS31 can be an powerful tool for the analysis of paternity testing, comparable with HLA-A,B,C-testing.

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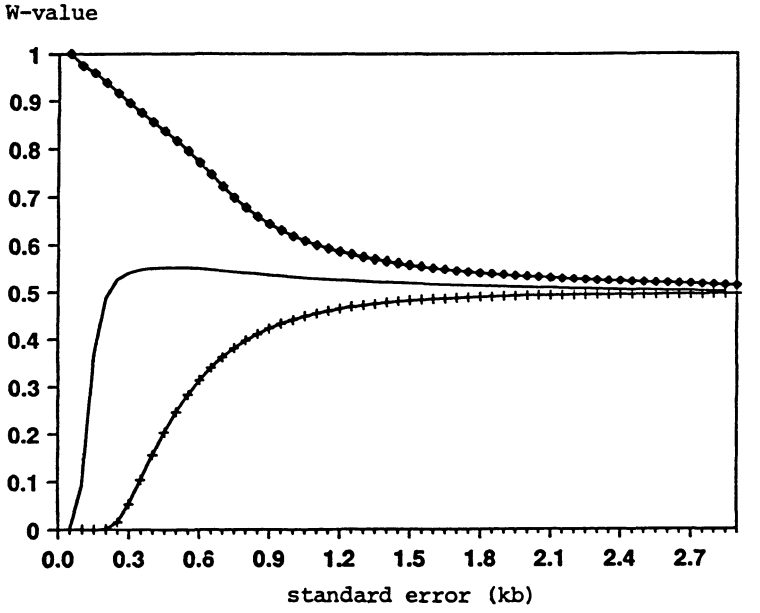
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Using the standard tests the putative father was excluded in 6 cases. In 17 cases the probability was greater than 99% (I > 99) when the conventional systems including HLA were tested. Analysis for HLA showed a probability greater than 99% in 7 cases. In 7 other cases the results were doubtful ($0 < I \leq 19$).
When the 24 paternity cases were analysed for the two RFLPs the putative father was excluded for both in 5 cases ($I = 0.0$). In two cases the results were doubtful and in 8 cases the paternity index was greater than 99. Combining the results of the conventional systems without HLA and the two RFLPs, one case was doubtful and in 18 cases the paternity index was greater than 99.
The method for the calculation of the paternity index is a function of the size measurement error. If for example in case 9, the measurement error is 0 kb the standard Essen-Möller method is used for the calculation of the W-value is 1.0. If the measurement error is increased the W-value approaches 0.5 which results in a loss of information (fig. 1).

fig. 1 W-value as a function of the error of measurement



—— case 5, Hae III/YNH24: Child (2.0,1.6)
Mother (2.0,2.8)
Father (2.6,1.8): doubtful

+++ case 8, Hinf I/MS31 : Child (6.3,5.9)
Mother (6.3,5.1)
Father (8.3,4.9): exclusion

●●● case 9, Hinf I/MS31 : Child (6.8,4.6)
Mother (9.0,6.8)
Father (7.1,4.6): indication

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Table 1. Paternity indices of 24 cases obtained with 15 conventional systems, HLA-A,B,C and RFLPs

case	conv.systems - HLA	HLA	conv.systems + HLA	RFLP Hae III YNH24	RFLP Hinf I MS31	conv. systems - HLA + RFLP
1	5.1	5.4	27.5	7.5	3.2	122.4
2	10.3	86.0	885.8	12.2	30.6	3845.2
3	132.0	5.4	712.8	8.6	2.9	3292.1
4	757.0	9.3	7040.1	5.6	12.3	52142.2
5	-	-	-	1.3	0.0	-
6	87.0	8.6	748.2	2.3	32.5	6503.3
7	59.0	539.0	31801.0	52.3	257.2	793642.0
8	-	-	-	0.0	0.0	-
9	512.0	21.0	10752.0	16.1	46.4	382484.5
10	26.0	283.0	7358.0	2.3	8.5	508.3
11	19.1	156.3	2985.3	4.6	42.9	3769.2
12	16.4	539.0	8839.6	10.2	36.4	6088.9
13	74.9	14.9	1116.0	7.3	17.0	9295.1
14	73.3	14.7	1077.5	24.8	10.8	19632.7
15	212.5	13.4	2847.5	5.9	6.4	8496.0
16	50.0	1000.0	50000.0	4.9	3.9	955.5
17	-	-	-	0.0	0.0	-
18	502.0	21.0	10542.0	2.9	7.0	10187.6
19	-	-	-	0.0	0.0	-
20	86.2	156.6	13498.9	3.4	271.7	79629.8
21	29.0	31.0	899.0	4.3	16.9	2107.4
22	-	-	-	0.0	0.0	-
23	1428.0	258.0	368424.0	3.5	2.9	14344.3
24	-	-	-	0.0	0.0	-

Table 2. Analysis of 24 Caucasian paternity cases with 15 conventional systems, HLA-A,B,C and RFLPs.

Category:	excluded	doubtful	probable	very probable
PI value:	0	0-19	20-99	$\geq$ 100
Conventional systems - HLA	6	4	8	6
Conventional systems + HLA	6	0	1	17
RFLPs:				
Hae III/YNH24 and Hinf I/MS31	6*	1	9	8
Conventional systems + RFLPs	6*	0	0	18

* In one case there was only an exclusion with Hinf I/MS31.

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restriction enzymes Hae III or Hinf I at a concentration of 7 U/ug DNA following the manufacturers specifications. Each sample was mixed with 5 ng digested lambda DNA.
Separation of DNA fragments was performed by electrophoresis in 0.8% agarose, followed by Southern blotting and hybridization with ^{32}P-labeled DNA probes, YNH24 (kindly given by Dr Y. Nakamura and Dr R. White, Salt Lake City, USA) and MS31 (kindly given by Dr P.G. Debenham, ICI, Cellmark diagnostics, Oxfordshire, UK), the radioactive bands were visualized by autoradiography. The filters were reprobed with ^{32}P-labeled lambda DNA and exposed to X-ray film. (Southern, 1975; Feinberg and Vogelstein, 1983).

Fragment size calculation

The fragments detected by lambda DNA probe were used as internal markers in each lane to calculate the size of the fragments detected by the probes YNH24 and MS31 with the use of a computer assisted digitizing system.

Standard paternity testing methods

Routine paternity testing methods were taken from standard protocols and consisted of the serological analysis of red cell antigen systems (ABO, MNS, P, Rh, Kell, Duffy, Kidd, Lutheran and Wright), serum proteins (Gc, Hp, Gm and Km), red cell enzymes (AcP and PGM1) and leucocyte antigens (HLA-A,B,C).

Fragment size frequencies and calculation of the paternity index

The frequencies of the fragments detected by the RFLPs as described here were determined by testing unrelated Caucasian individuals. The ratio of the total range in which the fragments were detected and the square root of the total number of detected fragments has been used as class width.
The paternity index or likelihood ratio (I) is calculated according to Gjertson et al.'s (1988) method of calculation of probability of paternity (w). If equal a priori probabilities are assumed than
$W = \frac{P1}{P1+P0}$ and $I = \frac{W}{1-W}$ in which P1 and P0 are the respective probabilities for paternity.

RESULTS AND DISCUSSION

One hundred fifteen individuals were analyzed for the Hae III RFLP detected by probe YNH24. The fragments found ranged from 0.96 kb to 6.21 kb. After sorting the fragments in class widths of $(6.21 - 0.96)/\sqrt{(2x115)}$ = 0.35 kb, the fragment size frequency was calculated.
Ninety-nine unrelated individuals were analyzed for the Hinf I RFLP detected by probe MS31. The fragment size frequency was calculated after sorting of the fragments in ranges of 0.50 kb.
The paternity indices of 24 paternity cases were calculated after analysis by conventional systems with and without HLA-A,B,C, and the Hae III/YNH24 and Hinf I/MS31 RFLPs (Table 1).
The paternity indices calculated by testing RFLPs is based on a error of measurement of 1% (data not shown). After classification of the results the data were summarized (Table 2).

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Analysis of Disputed Paternities Using Hae III/YNH24 and Hinf I/MS31 RFLPs

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ABSTRACT

The utility of the RFLPs detected by restriction enzyme Hae III and probe YNH24 and by restriction enzyme Hinf I and probe MS31 for the analysis of paternity cases was studied.
Because the differences in the size of the fragments detected by these highly polymorphic systems is much smaller than the variance due to errors of measurement, allele frequencies can not be obtained.
Based on the fragment size frequencies and the incorporation of the measurement error into the Essen-Möller version of the probability of paternity, paternity indices were calculated, and the results were compared with those of conventional bloodgroup testing. The results showed that the RFLPs Hae III/YNH24 and Hinf I/MS31 can make a major contribution to the analysis of paternity.

INTRODUCTION

Typing for 16 conventional polymorphic systems which include bloodgroup antigens, red cell enzymes, serum proteins and HLA antigens, it is possible to exclude in average 99% of falsely accused men in Caucasian paternity cases. To improve the analysis of disputed parentages in general it has been demonstrated that restriction fragment length polymorphisms (RFLPs) can be very useful (Baird et al., 1986; Kanter et al., 1986; Balasz et al., 1989).
Because the differences in the size of the fragments detected by highly polymorphic probes is much smaller than the variance due to the errors of measurement, allele frequencies can not be obtained and therefore fragment size frequencies has to be calculated. For the calculation of paternity indices the Essen-Möller version of the probability of paternity was used as a function of the measurement error of the fragment size (Essen-Möller, 1938; Gjertson et al., 1988).
In this paper the results of the analysis of 24 paternity cases, performed on two highly polymorphic RFLPs, enzyme Hae III with probe YNH24 and enzyme Hinf I with probe MS31, were compared with those of 16 conventional bloodgroup systems applied in the same cases.

MATERIALS AND METHODS

DNA preparation and Southern blotting

Genomic DNA was isolated from peripheral blood from unrelated Caucasian individuals and from 24 paternity cases according to standard procedures (Maniatis et al. 1982). DNA samples (3 ug) were digested with the

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considerably more expensive. By comparison, as shown here with RFLP analysis using a proper choice of only one particular probe, the efficiency of the two procedures were about equal ; moreover, in a single case, even after the inclusion of more than 20 blood, serum and enzyme polymorphisms (including HLA - A, B), we were unable to distinguish between to potential fathers in a particular paternity analysis ; using the 3' HVR probe, it was then possible to eliminate one of the father. We conclude that RFLP analysis, if using a proper choice of probes, is extremely promising for use in future paternity diagnostics in some special situations.

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MATERIAL AND METHODS

In our experiments, blood was obtained from French families and from unrelated healthy controls. DNA was obtained from the nuclear pellet of each blood sample by phenol-chloroform extraction. DNA (10 µg) was digested to completion with 20-30 units of *Pvu* II under the conditions recommended by the manufacturer (appligène). The DNA fragments were electrophoresed in a 1 % agarose gel and transferred onto nitrocellulose filters by Southern blotting. The 4 kilobases (kb) *Hinf* I insert from the recombinant pSEA1 was used as a probe, labelled with 32 P by nick-translation. High-stringency conditions were obtained by hybridization with 50 % formamide at 42° C and post-hybridization wasking in 0.1 x SSC at 65° C. Autoradiography with Amersham Hyperfilm was carried out at -70° C with an intensifying screen (Dupont Lighting Plus) for 1 - 4 days.

RESULTS

To determine the variability parameters, DNA samples from 120 unrelated French individuals were examined. Alleles detected by the probe were grouped into 100 bp size classes ; a total of 49 different alleles have been found far ranging in size from 0.7 kb to 9 kb and more. The most common allele detected in French was 2.3 kb (gene frequency = 0.147). Observed individual heterozygosity is 0.916, and the average *a priori* probability of exclusion is about 86 %.

The paternity results are summarized for the 3' HVR probe obtained in a two-years pilot study at the INTS Institute of Paris. Before DNA analysis, all cases were tested with a full-test battery of red blood cell antigens, blood cell enzymes and serum proteins (and with HLA - A and B markers in some cases) which had a combined probability of exclusion of about 0.99. In our results on 24 families tested we found 14 cases of exclusions with the 3' HVR marker alone, 13 of them being also demonstrated with the battery of classical markers ; only family number 21 demonstrated a 3' HVR exclusion, case not excluded unambiguously by other marker systems.

CONCLUSIONS

In conclusions, we have been involved in studies aimed at evaluating the feasibility of applying RFLP analysis to routine paternity testing. DNA technology is at present clearly second to the classical serological methods concerning practicability, since DNA analysis requires more hands-on time and is

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Paternity testing using the hypervariable 3' HVR DNA probe

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INTRODUCTION

The human genome contains many polymorphic loci known as hypervariable regions (HVR) of DNA, which result from a variable number of tandem repeats of a short core sequence. There are very informative for genetic analysis since there are many alleles at each locus. These regions may occur dispersed throughout the genome, or they may be clustered on a single chromosome. Several HVRs associated with the alpha-globin gene cluster on the short arm of chromosome 16 have been characterized (Higgs et al., 1981). The alpha-globin 3' HVR (Jarman et al., 1986) is a tandem repeat array of 17 base pairs (pb), the number of which varies considerably between different alleles. Under hybridization conditions of high stringency, the 3' HVR probe reveals as highly polymorphic locus with heterozygosity approaching 1 (Higgs et al.,1986). The 3' HVR is a hypervariable region located approximately 8 kb downstream of the α1-globin gene ; the repeat segment is related to three previously identified HVRs by the sequence 5'- GNGGGG (N) ACAG - 3' ; a probe to the 3' HVR detects multiple, mendelianly inherited DNA segments, suggesting that it too may represent one member of a dispersed minisatellite family (Fowler et al., 1986).

Several possible applications of DNA fingerprinting using such probes have been explored ; however, the analysis of DNA fingerprints produced by minisatellite probes is complicated by several difficulties assessing which bands are allelic, and also in genetic linkage studies by the lack of chromosomal localisation of the bands. We described here a very usefull approach to genetic analysis which overcomes some of these problems : the method involves digestion of DNA with an enzyme and hybridization with the conventionally labelled 3' HVR probe, which detects a *Pvu* II restriction fragment length polymorphism (RFLP), whose alleles are conveniently sited on the gel ; the resulting band pattern is a simple constituent allelic band system of known chromosomal localisation. This approach to genetic characterisation would be of particular value in paternity testing (Wainscoat et al., 1987) and should prove be simple in pratice, and complementary to other methods of analysis.

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Table Three

CASES WITH POSSIBLE MUTATION IN pL336 (Rsa I)

	Child	*Mother*	*Tested Man*
Case 1	7.41	7.85	13.0 *
	4.12	4.84	4.12
Case 2	6.24	6.03	4.96
	4.12	4.27	4.12
Case 3	6.53	5.07	6.31 +
	4.17	4.17	4.17

*Direct exclusion of paternity in ACP

+Residual index using 10 systems is 2,247,726

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Table One

RESULTS OBSERVED WITH TWO SINGLE LOCUS PROBES

	Number	%
Excluded by DNA	23	14.6
Two probes	*15*	*65*
pL336 (Rsa I) only	*7*	*30*
pS194 (Pst I) only	*1*	*5*
No exclusion by DNA	135	85.4
Total	158	

Table Two

CASES WITH PROBABLE SILENT ALLELES

	System	*Child*	*Mother*	*Alleged Father*	*Residual PI All systems*	*Residual PI 2 Probes*
Case 1	Rh	R2	R1R2	R1	859	145.0
Case 2	PGM1	2+	2+	1+	1,635	7.6
Case 3	PLG	2	12	1	289	52.0
Case 4*	FY	a	a	b	265,417	8325.0
Case 5*	FY	a	a	b	2,454	71.0
Case 6	GLO	1	12	2	34,724	51.0

*Hispanic, 2 children, same alleged father

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Results on Selected Trios Tested with Two Single Locus DNA Probes

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The purpose of this paper is to report on the results obtained from selected paternity cases in which two single locus DNA probes were used. Many of the trios in this study were referred from other laboratories because of a previously low likelihood of paternity or single exclusion using conventional methods.

MATERIALS AND METHODS

DNA was extracted for all samples using a non organic solvent procedure (Miller 1988). Samples were quantitated and adjusted to a concentration of 0.35ug/ul with Tris-EDTA buffer. Each sample was tested with pS194 (locus D7S104) after restriction with Pst I and pL336 (locus D1S47) after restriction with Rsa I. The probes were obtained from Collaborative Research, Inc. (Bedford, MA) and biotin labeled with a nick translation kit obtained from ONCOR, Inc. (Gaithersburg, MD). All gels contained a visible lambda Hind III marker, a human control, and size markers in every ninth lane. The trios were run on the same gel and included a sample mix of the alleged father and child. The gels were run approximately 46 hours and Southern blotted as described by Dykes (1988). The nylon membranes were hybridized and stained using a kit from ONCOR. A high resolution video camera was used to obtain a computer image of each membrane. Analysis of this image to determine band position and kilobase size was done by a program developed in house.

RESULTS

Table one lists the results of 158 cases. Exclusions were found in 23 cases. In 85 cases, where only the probes were used, the PI ranged from 12.3 to 16,667 (<50 = 16, $50 - 100$ = 18, >100 = 51).

In 73 cases data from other systems were available. Table two shows results in six of these cases in which an apparent indirect exclusion was observed in another system. Based on the high residual index, we concluded that a null allele was responsible for the apparent exclusion (Polesky 1988). In two of the 73 cases where the probes failed to provide evidence of exclusion, direct exclusions were observed in other systems (BF & F13B; HLA).

Table three shows the results in three additional cases. In each of these there appears to be a mutation detected by pL336.

In three cases of the 158, the initial results using pL336 and Rsa I showed a single band of different sizes for the alleged father and child. The use of another restriction enzyme (Pst I) showed an additional band shared by both the alleged father and child.

SUMMARY

Both of the probes described have been helpful in establishing an exclusion for some additional men, but alone will not exclude all non-fathers. The use of these probes has resolved cases with low paternity indexes and helped establish paternity in cases with null alleles. Based on our observations, pL336 many have an mutation rate in excess of 0.1.

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Table Two

Effect of DNA Testing on the Paternity Index (PI)

	Observed Value			
	Excluding DNA		Including DNA	
Index	No. of Cases	%	No. of Cases	%
< 100	418	51	94*	11.4
> 100	404	49	728	88.6

* In 34 cases the PI < 50.

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give evidence of exclusion in as many of the non fathers (cases with an exclusion in another system) as expected; however, pS194 did provide the only evidence of non paternity in 3.5% of the exclusion cases and confirmed a single exclusion with another system in 16.6% of these cases. The finding of an exclusion with the DNA system significantly reduced the number of cases in which we would have requested additional samples for further testing.

The addition of a highly polymorphic DNA RFLP system to a group of less polymorphic systems, as expected, increased the PI. In 88.6% of the cases without an exclusion the value reported was over 100. Though this increase in observed index values is probably not significant in determining paternity, it is helpful in obtaining parentage estimates that are mandated by statutory requirements of several US states. The higher PI values found by using pS194 also decreased the number of cases in which we would have tested additional systems or requested additional samples.

Several problems have been encountered in routinely using a DNA marker system. In some trios it has been difficult to recover adequate amounts of DNA from one or more of the individuals to be tested. Usually this has been related to the age or amount of sample available. On several occasions reagent related problems have occurred, most frequently this has been failure of the commercially obtained restriction enzyme to perform as expected. Careful attention to technical details including handling of gels, temperature of the stringency wash, and preparation of reagents is essential.

SUMMARY

Routine use of a single locus DNA probe enhances the results found with other marker systems. As additional probes are used or combined to resolve cases of disputed parentage, it is important that the outcome of the testing be validated by comparison with results obtained by well established marker systems.

Table One

Results in 1189 Cases Tested with pS194 and other Systems

	Number of Trios	% of Total	% of Excl Trios
Total with an exclusion	**367**	**30.9**	**---**
Excl by multiple systems	**335**	**28.2**	**91.3**
Excl by pS194	**260**	**21.8**	**70.7**
Excl by pS194 & one other system	**61**	**5.1**	**16.6**
Excl only by pS194	**13**	**1.1**	**3.5**
No exclusion observed	**822**	**69.1**	**---**

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Routine Use of a Single Locus DNA Probe

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INTRODUCTION

This report evaluates the results of using a single locus DNA probe on routine parentage testing cases in addition to a battery of tests expected to exclude at least 95% of falsely accused men.

METHODS

Samples from 1189 trios were tested for markers in the following systems: ABO, Rh, MNSs, Kell, Duffy, ACP, PGM1, GC, BF, F13B, PLG, and TF. DNA was extracted from each sample following the procedure described by Miller (1988). A standardized quantity of DNA was restricted with Pst I. Samples from each member of the trio and a mixture of DNA from the alleged father and child were run in adjacent lanes on the same gel. After electrophoresis and transblotting samples were probed with pS194 (locus: D7S104) obtained from Collaborative Research (Bedford, MA). The detection method (Dykes 1988) utilized a nonisotopic label. The position of each band was determined from a digitised video picture using an image analysis routine developed inhouse. Calculation of the paternity index (PI) was based on population frequency values that include bands located within +/- 1% of the measured location of the bands detected.

RESULTS

Table one shows that 30.9% of the tested trios were excluded by combining the single locus DNA probe with the other test systems. Of trios excluded, approximately 30% had an exclusion in one or more of the other systems but were not excluded by pS194. In 19 of these 107 cases a single exclusion was observed [13 direct, 2 indirect (JK, PLG), and 4 (3 PLG , 1 F13B) with a very high residual index and a probable null allele]. In 13 trios (5.0% of cases with a DNA exclusion) pS194 provided the only evidence of non-paternity. In 61 trios (23.5% of cases with a DNA exclusion) one other system in addition to pS194 provided evidence of non paternity. In 14 of these trios (23%) the other exclusion was indirect (4 MNSs, 3 GC, 2 PGM1, 1 Kell, 1 PLG, 1 F13B) and would have required additional tests or new samples to confirm non-paternity.

Combining the results of pS194 with the other test systems increased the PI to greater than 100 in 324 (77.5%) of 418 cases where it fell below 100 when only the results of red cell antigen, red cell enzyme and serum protein systems were used for the calculation (Table Two). In 60 additional cases the PI was increased to a value between 50 & 100. Thus of the 822 trios without an exclusion, a W $\geq$ 98% (prior probability = 0.5) was obtained in 95.9% of the cases.

DISCUSSION

The 1189 parentage cases reported here illustrate the usefulness of a single locus DNA probe as part of the battery of tests used in resolving disputed paternity. The addition of an RFLP marker system (pS194) with an expected exclusion probability of greater than 0.8, made it possible for us to reduce the number of systems tested, while increasing the cumulative probability of exclusion (CPE) of our routine test battery. The RFLP system did not

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Figure 3

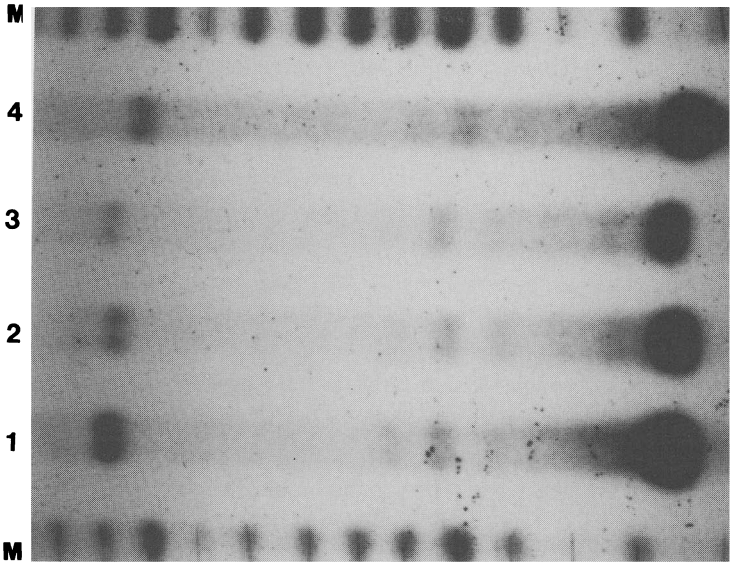


Figure 3. Hybridization with probe which detects the monomorphic DXZ1 locus.

of the fragment size) in a Gaussian distribution. When results of constant bands are included to correct for interlane mobility differences, two fragments are considered to match if they are within 1.8% of each other.

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Figure 1

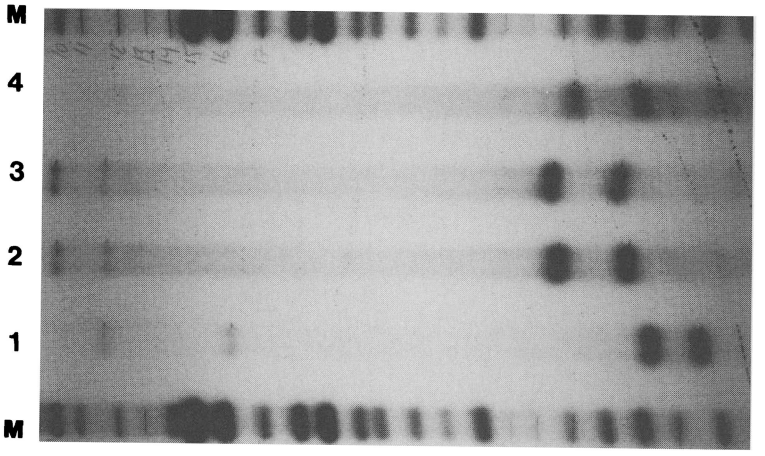
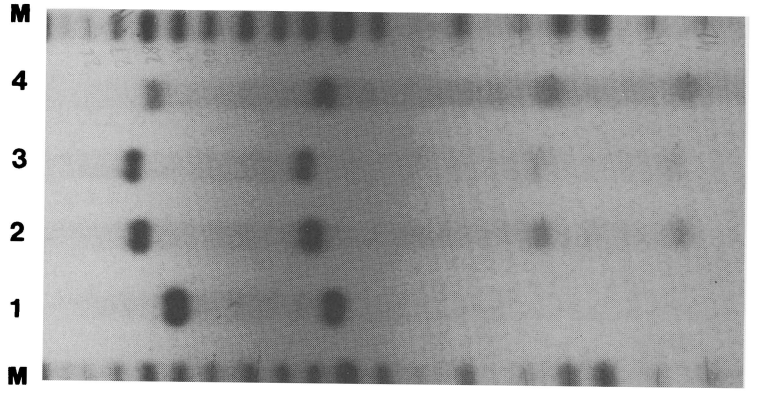


Figure 2



DNA was isolated from the following samples:
20951 - blood from rape victim
20952 - vaginal swab from victim
20953 - semen stain from victim's clothing
20954 - blood from suspect

Figures 1 - 3. Hybridization of the nylon membrane from Gel 1121. Lanes M contain size markers. Lane 1 contains victim. Lane 2 contains vaginal swab. Lane 3 contains suspect. Lane 4 contains semen stain.

Figure 1. Hybridization with probes which detect the highly polymorphic D2S44 and D17S79 loci.

Figure 2. Hybridization with probe which detects the highly polymorphic DXYS14 locus.

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concentration. The entire digest from each sample is loaded and lanes are skipped between samples. From a typical rape case the gel is loaded with DNA isolated from victim, evidence, suspect, and whenever possible a single lane containing DNA from evidence and suspect. A piece of numbered nylon mesh is embedded in the gel for identification. The gel is stained with ethidium bromide, photographed, and transferred to a nylon membrane.

Hybridization and Autoradiography:

DNA probes are labelled with 32P and applied sequentially to the membrane to generate the DNA Print. The following polymorphic loci are routinely examined: D2S44, D14S13, D17S79, DXYS14, D18S27, and D14S1. In addition probes which recognize constant regions within the DNA DXZ1 and DNF24, are used to document any interlane mobility differences and the extent of degradation. Hybridizations are performed using standard conditions at 68oC aqueous using oligonucleotide probes. Membranes are washed to 0.1XSSC at 68oC, dried briefly, wrapped in plastic and placed against x-ray film in a cassette for 1 to 5 days to generate autoradiographs. The x-ray film is developed to produce the autoradiograph or autorad (Figure 1). Membranes are stripped of signal with 50% formamide at 68oC and rehybridized (Figure 2).

ANALYSIS

All autoradiographs are reviewed to determine if any overall DNA Prints match visually. If a sample is partially degraded, larger fragments may not be present, for example the D2S44 locus. It has been shown that the DNA Print pattern does not change in degraded samples due to environmental conditions or substrate (McNally et al., 1989A and 1989B). Mobility differences may occur, especially in degraded samples and are documented by the use of probes which recognize monomorphic loci (Figure 3).

POPULATION GENETICS

The frequency of occurrence of the pattern at each locus, except for DXYS14 is calculated by multiplying the allele frequencies of the DNA fragment(s) according to the Hardy-Weinberg equation. All frequencies within plus and minus 1.8% (3 average deviations) of the size of the fragment are added together to determine the allele frequency. For the DXYS14 locus, because haplotypes exist to produce patterns with one to eight bands per person, the database is queried to determine how frequently a particular pattern has been observed. Each of the loci appear to meet the assumptions of Hardy-Weinberg equilibrium because the number of observed and expected combinations appear in agreement. Each locus is on a separate chromosome which is good evidence that there is no linkage disequilibrium. The final frequency of occurrence is calculated by multiplying the individual frequencies of occurrence at each locus. Running the same DNA sample multiple times indicates that sizes fall within 3 average deviations (1 average deviation equals 0.6%

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Analysis of Case Work Samples by Single Locus VNTR Polymorphisms

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INTRODUCTION

For the past several years, restriction fragment length polymorphism (RFLP) analysis of DNA isolated from forensic samples has been used to provide evidence in forensic cases. To date, Lifecodes Corporation has analyzed over 2000 cases and has presented testimony in over 100 US court cases in 21 states. Examples of casework samples are presented.

EXTRACTION

DNA from semen stains and vaginal swabs is obtained by a differential lysis procedure (Giusti et al. 1986) to enrich for male DNA and the supernatant (female DNA) can be used to establish the victim's DNA pattern. Liquid blood and dried blood stains are processed for DNA as previously reported (Kanter et al. 1986). The quality and quantity of DNA is evaluated from the intensity of ethidium bromide staining after electrophoresis of about 1% of undigested DNA on a 15 cm Yield Gel which includes concentration and size controls.

DIGESTION

DNA is digested with the restriction endonuclease Pst I which recognizes the 6 nucleotide sequence CTGCAG. The initial digestion is performed with a 20 fold excess of enzyme for 1 hour at conditions recommended by the manufacturer. Although 50 nanograms of high molecular weight DNA can yield RFLP results, more evidentiary DNA (about 1 microgram) is usually required because the DNA is often partially degraded. After ethanol precipitation, the cut DNA is resuspended in a small volume and redigested with Pst I. About 10% of this digestion is evaluated on a 15 cm Test Gel including male and female controls. After electrophoresis and staining by ethidium bromide, digested DNA appears as a smear from the top of the gel to below 2 kilobase pairs (kb), similar to the control samples. If the sample is partially degraded, there is less florescence at the top of the lane. The amount of DNA is estimated from the staining intensity relative to the controls.

Electrophoresis and Transfer

The digested DNA is size separated by electrophoresis for three days on a 30 cm Analytical Gel. Size markers composed of bacterial viral DNAs digested with a variety of restriction endonucleases flank the samples and act as a sizing ladder to determine fragment sizes from samples. A control human DNA from cell line K562 is included on each gel at a 100 ng

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range. Thus, a single blot generated from a standard 0.8% agarose gel run overnight can be sequentially hybridized to the probe collection with the general assurance that alleles at each locus will be adequately resolved for subsequent analysis.

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in size from about 1-10 kb (Table 1). The size of alleles produced at each locus with Pvu II is such that an overnight electrophoretic run of the digest in a 0.8% agarose gel of standard format (ie 15 X 20 cm) provides good separation of restriction fragments. Alleles at each locus exhibit an apparent continuous distribution of fragment sizes over the size range and frequencies of alleles generated with Pvu II are similar to those observed when Pst 1 or Hae III are used to digest the DNA as would be anticipated (results not shown).

The discriminatory capabilities of the four probes in parentage testing have been examined in a large number of paternity trios for which results of standard testing methods were known. The results (Table 2) indicate that this collection of probes used in conjunction with Pvu II will exclude approximately 99.90% of falsely accused men in paternity suits.

Table 2. Discriminatory capabilities of four HVR probes used with Pvu II for parentage testing.

Probe	PEX[1]
3'HVR	0.90
YNH24	0.88
pHRAS	0.60
pS194	0.80
Cumulative PEX= 0.9990	

1. PEX refers to the ability of a given probe (or collection of probes) to exclude a falsely accused man in a paternity dispute.

DISCUSSION

The results presented here indicate that a collection of four probes, commonly used for identity testing, are compatible with Pvu II restriction enzyme. Pvu II has several advantages over either Pst 1 or Hae III when used with this collection of probes. One advantage is the higher relative activity of Pvu II compared to the other enzymes which results in a lower number of chromosomal DNA samples that need further processing before complete digestion is obtained. The second advantage relates to the size range of alleles produced at each locus. Pvu II produces alleles at each locus that fall into a 1-10 kb

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digested approximately 98%. The least active enzyme was Pst 1 which digested only 92% of the DNA samples.
A comparison of allele sizes produced with the different enzymes at each locus is shown in Table 1. The data indicate that Hae III can produce small alleles at the D16S85, D2S44, and HRAS1 loci. Small restriction fragments, which represent the most common alleles in the population at the D16S85 and HRAS1 loci (unpublished observations), can electrophorese out of the gel during extended electrophoresis, or, can diffuse within the gel during blotting, making accurate sizing of fragments difficult. Thus, Hae III is not the enzyme of choice for studying polymorphisms at the D16S85, D2S44, or HRAS1 loci.

Table 1. Characteristics of alleles produced at four HVR loci with different restriction enzymes.

Probe/Locus	Size Range of Alleles		
	Pvu II	Hae III	Pst 1
3'HVR/D16S85	1.5-10 kb	0.5-8.0 kb	0.6-8.0 kb
YNH24/D2S44	2.0-10.0 kb	<1-7.0 kb[1]	6.0-17 kb[2]
pHRAS/HRAS1	2.5-6.5 kb	0.3-4.0 kb	1.5-5.0 kb[3]
pS194/D7S107	1.3-8.0 kb	N.P.[4]	5.0-12.5 kb[5]

1. Michael Baird, Lifecodes, personal communication.
2. Balazs et al 1989.
3. Baird et al 1985.
4. Not polymorphic, Tim Keith, Collaborative Research, personal communication.
5. Dykes 1988.

Digestion of chromosomal DNA with Pst 1 produces relatively small alleles at the D16S85 locus, moderately sized alleles at the HRAS1 locus (Baird et al 1988 and unpublished observations), and large alleles at both the D2S44 and D7S107 loci (Table 1) (Balazs et al 1989, Dykes 1988). Adequate separation of alleles at the D2S44 and D7S107 loci therefore requires an extended period of electrophoresis (Balazs et al 1989, Baird et al 1985,1988, Dykes 1988) which is generally incompatible with visualization of small alleles at both the D16S85 and HRAS1 loci. Thus, Pst 1 is not the enzyme of choice for use with these four probes.

In contrast to Hae III and Pst 1, Pvu II digestion of chromosomal DNA produces alleles at all four loci that range

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locus (Barker et al 1987). Standard conditions used for RFLP mapping included an overnight digestion of chromosomal DNA with a fivefold excess of restriction enzyme. Each sample of chromosomal DNA (5-10 ug) was mixed with 0.5-1.0 ug of lambda DNA prior to adding enzyme, and the extent of digestion was evaluated by electrophoresing 10% of the digest on an analytical agarose gel that was subsequently stained with ethidium bromide. Restriction fragments in samples digested to completion were recovered by ethanol precipitation and centrifugation and electrophoresed in 0.8% agarose gels in 89 mM Tris-borate pH 8.3 + 1 mM EDTA. Gels were blotted onto Zetabind membranes (AMF Cuno, Meriden, CT) in 20 mM NaOH (Fowler et al 1988) over a 4-6 hour period. Blots were hybridized to probes labeled with ^{32}P-dCTP by oligonucleotide primer mediated labeling (Feinberg and Vogelstein 1984). Filters were hybridized and washed under conditions of high stringency.

RESULTS

The purpose of this study was to evaluate RFLP mapping data generated with a collection of four HVR probes used in conjunction with different restriction enzymes. The probes used in this study have been shown to be informative for identity testing and are widely used in parentage testing and forensics. Although these probes are common to a number of laboratories, they are used in conjunction with different restriction enzymes; most notably Pst 1 and Hae III.

The activities of Pvu II, Hae III, and Pst 1 were compared using chromosomal DNA isolated from random blood donors. 1-2 ug aliquots of chromosomal DNA were mixed with 200-400 ng of lambda DNA and digested with a 5 fold excess of restriction enzyme overnight at 37^{o}C. The samples were then electrophoresed in an agarose gel which was subsequently stained with ethidium bromide to visualize the pattern of restriction fragments produced. Restriction enzymes were obtained from several commercial suppliers (BRL, New England Biolabs, Boehringer Mannheim, U.S. Biochemicals). If the expected pattern of restriction fragments from lambda DNA was observed in the gel along with a hazy smear of chromosomal fragments, the digestion was scored as complete. If the expected pattern of lambda fragments was not observed, a second aliquot of DNA was digested and analysed under the same conditions. If the second aliquot digested to completion, it was scored as complete. If neither the first nor the second digestion was complete, the sample was scored as incomplete. The results of this study indicated that Pvu II digested approximately 99% of the DNA samples followed by Hae III which

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Compatibility of Several HVR-Type Probes with Pvu II Restriction Endonuclease and Advantages of Pvu II Over Other Commonly Used Enzymes

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INTRODUCTION

One characteristic of chromosomal DNA loci exhibiting VNTR polymorphisms is the ability to study such polymorphisms using RFLP mapping with multiple restriction endonucleases. Three restriction enzymes, Pst 1, Hae III, and Hinf I, have been widely used in RFLP mapping for identity testing with a number of commonly used DNA probes.

There are several considerations in choosing a restriction enzyme for use with a collection of HVR probes for identity testing. One consideration is the activity of a particular enzyme and its susceptibility to inactivation by contaminants present in some samples of chromosomal DNA. A second consideration in choosing an enzyme is the size of restriction fragments produced at each locus being analysed. Ideally, one wants an enzyme that is highly active in digesting chromosomal DNA and produces allele sizes at each locus that are compatible with analysis on a single Southern blot.

We describe here the characteristics of a collection of commonly used, and readily available probes that can be used for identity testing in conjunction with Pvu II restriction enzyme. Pvu II has advantages over other restriction enzymes commonly used for identity testing in that Pvu II is highly active in digesting chromosomal DNA. In addition, Pvu II produces alleles at each of the loci that range in size from about 1-10 kb; a range of allele sizes easily separated during an overnight electrophoresis in a standard gel system employing 0.8% agarose.

MATERIALS AND METHODS.

The probes used in this study include: 3'HVR (Higgs et al 1981) detecting the D16S85 locus (kindly provided by Dr. David Werrett, Home Office Forensics Unit, England), pHRAS, a oligonucleotide probe detecting the HRAS1 locus (Krontiris et al 1985, Thein et al 1986), YNH24, an oligonucleotide probe detecting the D2S44 locus (Nakamura et al 1987), and pS194 (Collaborative Research, Bedford, MA) detecting the D7S107

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These observed values are lower than those calculated using the Ito formula, which uses allele frequencies to derive the Power of Exclusion. Nevertheless, these probes have a high combined Power of Exclusion, and thus provide a very powerful tool for paternity determination.

In addition to increasing the Power of Exclusion of our battery of tests, DNA analysis provided very useful evidence in support of paternity in those cases where no exclusion was found. The median Paternity Index observed in our laboratory was 145 for Caucasians and 404 for Blacks, when all three probes were used. These values are 10 to 12 fold lower than those previously reported (Balazs et al., op cit) reflecting the fact that we have used a more conservative means of calculating the PI than that used by these authors. Even so, our data indicate that DNA typing using these three probes can provide strong evidence in support of paternity when no exclusion is found.

CONCLUSION

We have examined the effect of using DNA analysis in addition to the standard battery of genetic tests currently used in our laboratory for paternity testing. We find that the data obtained from DNA typing are generally in agreement with the results of traditional testing, and that all exclusions found with our standard tests were confirmed by DNA analysis. In addition, 9.9% of men included by red cell and HLA testing with PIs of less than 50 were excluded by DNA analysis, reflecting the high combined power of exclusion of the three probes used in this study. In those cases where no exclusion was found with any of the tests used, DNA typing provided strong additional genetic evidence in support of paternity. Thus, we conclude that the use of these three probes in addition to standard testing provided a powerful means of separating "true" fathers from falsely accused men.

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RESULTS AND DISCUSSION

Table 1 presents the results obtained when DNA analysis was used in addition to our standard battery of tests. All direct exclusions obtained by standard testing were confirmed by DNA analysis (exclusion by at least 2 probes). Out of the 133 cases in which the alleged father was not excluded by traditional testing, 8 exclusions were found by DNA testing. Four of these DNA exclusions had one or more indirect red cell exclusions (Table 2). Of 33 cases involving non-excluded men with an initial PI of less than 20, 15% were excluded by DNA analysis. DNA exclusions were also found in 3 of the 48 cases with an initial PI of 20-49. No exclusions were observed in any case with an initial PI of greater than 50, supporting the contention that most alleged fathers having Paternity Indices of 50 or more are probably true fathers.

TABLE 1: A COMPARISON OF TRADITIONAL TESTING AND DNA TESTING IN 145 PATERNITY CASES

INITIAL PI	INITIAL W	# CASES	# EXCLUSIONS WITH DNA	% EXCLUSION WITH DNA
0	0	12	12	100%
0-1	0-50%	6	3	50%
1-9	50-90%	11	2	18%
10-19	90-95%	16	0	0%
20-49	95-98%	48	3	6%
50-99	98-99%	37	0	0%
99 or >	99% or >	15	0	0%
		145	20	13.8%

TABLE 2: EIGHT CASES IN WHICH ALLEGED FATHERS WERE EXCLUDED BY SUBSEQUENT DNA TESTING

RACE	INITIAL PI	INITIAL W	INDIRECT RBC EX.	# OF PROBES EX. THE AF
C	<0.01		KIDD & MNSs	2
C	0.07		Rh	2
B	0.08		Rh	3
C	1.7	62.96%		3
C	6	85.71%	Duffy	2
C	26	96.30%		2
B	28	96.55%		3
B	48	97.96%		2

The Power of Exclusion (PE) for the three probes used in this study have been calculated using the formula described by Ito et al. (1985) and reported elsewhere as 99.8% for Caucasians and Blacks (Balazs et al., 1989). We have empirically determined the Power of Exclusion of these probes by creating novel "trios" composed of the mother and child from one paternity case and an alleged father from a different, unrelated case. Since we assume that the new "alleged father" is not the biological father of the child, this analysis provides a measure of the frequency with which each probe would exclude a falsely accused man. We considered that the "alleged father" was excluded if his allele(s) differed from the obligate band by more than 2%. Using this approach, D14S13, D2S44, and D17S79 were found to have a combined Power of Exclusion of approximately 98% in Caucasians and 99% in Blacks.

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A Comparative Paternity Study: DNA vs Traditional Testing

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INTRODUCTION

We have analyzed 145 paternity trios using both traditional serology (six red cell systems and the HLA system), and RFLP analysis with three single-locus VNTR probes -- D14S13, D2S44, and D17S79 (Lifecodes Corporation). This study was done to evaluate the benefit of using DNA testing in association with our traditional battery of tests. Specifically, we wished to (1) determine if DNA testing corroborated exclusions found by traditional testing (2) determine whether additional exclusions would be found by DNA analysis (3) evaluate the strength of the genetic evidence provided by DNA testing in cases where no exclusion was found. The results of this study indicate that DNA analysis is a very useful additional system for distinguishing non-excluded non-fathers from "true" fathers, and for providing convincing evidence in favor of paternity in cases of non-exclusion.

MATERIALS AND METHODS

Blood samples from paternity trios were typed for the following blood group systems: ABO (A,B,O); Rh (D,C,c,E,e); MNSs (M,N,S,s); Kell (K,k); Duffy (Fy^a, Fy^b); Kidd (Jk^a,Jk^b) and HLA (16 A-locus, 31 B-locus, and 8 C-locus antigens). All serological assays were performed in duplicate, according to standard protocols.

DNA analysis was performed using the LifePrint^{TM} system (Lifecodes Corporation, Valhalla, NY). DNA isolated from peripheral blood was digested with the restriction enzyme PstI. Approximately one microgram of the restricted DNA was electrophoresed through a 0.9% agarose gel and transferred to nylon membranes. Following overnight hybridization with ^{32}P-labelled probes (D2S44, D14S13, and/or D17S79) the membranes were washed and exposed to X-ray film. The resulting autoradiograms were analyzed using a digitizing pad (Scriptel Corporation, Columbus, Ohio) and software developed by Lifecodes Corp. to determine allele sizes.

The Paternity Index (PI) and Probability of Paternity (W) were calculated according to standard methods (Walker, 1983). A conservative method of determining the Y value was used, in which all allele frequencies within 3 standard deviations (± 1.8%) from the measured size of the obligate gene were summed.

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However, 9 white and 6 black putative fathers were excluded only by YNH24. In comparing the paternity index (PI) obtained by use of HLA and YNH24 in 28 white and 42 black non-excluded cases, respectively, the PI was higher with HLA in 15 white and 25 black cases. In contrast the PI obtained by use of YNH24 was higher in 13 white and 17 black cases. Although, we do not have as much case work experience with use of PHINS 310 as compared to YNH24, based on the population frequencies of alleles detected by these two VNTR probes the cumulative exclusion probability would be approximately 98.5% in whites and 95.4% in blacks.

SUMMARY

The VNTR probes evaluated provide a powerful system for resolving disputed cases of paternity. However we feel that the population sampled is not adequate to detect all the alleles that may exist in the population. We are presently attempting to increase our population database to include at least 500 white and 500 black individuals. In addition, it will be important to compare the allele frequencies detected by these probes in whites and blacks from various geographic regions. It is known that the degree of white admixture in the black population varies from one region of the USA to another (Reed 1969). Thus, it will be essential that regional databases of allelic frequencies be established for paternity case work. When adequate databases of allelic frequencies for the various racial and ethnic groups represented in the USA becomes available, DNA typing should be a single technique with sufficient power to resolve most disputed cases of paternity.

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from the putative father the lane containing the DNA mixture was very informative in determining if indeed there was an exclusion.

Since VNTR probes detect highly polymorphic regions of the genome with the alleles often varying in size from one to a few hundred copies of the tandemly repeated sequence, it is important to establish the size range within which one can reproducibly measure a given fragment in the population. Based on blot to blot comparisons of the allelic control fragments in the 0.3 kb to 4 kb size range, fragments can be reproducibly measured within plus or minus 50 base pairs. However, due to the low frequencies of fragments greater than 4 kb we have arbitrarily made the operating assumption that one can only measure within plus or minus 400 base pairs which corresponds to pooling over several identifiable fragments. Based on these considerations we have established mathematically derived ranges of fragment sizes called bins within which the sample alleles can be grouped in order to calculate the allele frequencies. It should be noted that these bins may change in future as we obtain greater accuracy and reliability in measuring fragments over the entire size spectrum.

RESULTS

Utilizing YNH24 and Hae III restriction endonuclease we have observed fragments that range in size from 0.2 kb to 6.4 kb in the black population and 0.8 kb to 8.8 kb in the white population from Alabama. Using size bins which span either 100 base pairs for fragments in the range of 0.2 kb to 4 kb or 400 base pairs for fragments greater than 4 kb, 44 alleled can be assigned within a sample of 153 blacks and 41 alleles within a sample of 156 whites for the YNH24 probe (Table 1). The size range of fragments detected by PHINS 310 and PVU II restriction endonucleases range from 0.6 kb to 4.7 kb in the black population and 0.5 kb to 4.6 kb in the white population. As can be seen in Table 1 the polymorphic information content (PIC) is higher for the loci detected by the YNH24 probe in the white population (Botstein 1980). The PIC for each of the two loci is less in the black population than for the white population. The YNH24 probe has the greatest exclusion probability. This probe has the power to exclude approximately 94% and 85% wrongly identified black and white men, respectively.

Table 1. Power of VNTR probes to exclude falsely accused individuals

Probe	Enzyme	Race	Sample N	Alleles N	PIC %	Exclusion Probability %
PHINS 310	PVU II	W	262	31	92.2	74.9
		B	100	30	89.4	69.0
YNH24	Hae III	W	156	41	94.7	93.9
		B	153	44	86.7	85.0

Using the YNH24 allelic frequencies that have been estimated for the Alabama population we have compared the power of DNA polymorphism testing to HLA antigen testing in resolving 58 white and 81 black cases of paternity. We were able to exclude with both HLA and YNH24, 21 white and 33 black putative fathers.

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1.3 Practical Use

Comparison of HLA Antigens and DNA Polymorphisms in Parentage Testing

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The power to exclude wrongly identified men involved in paternity disputes has been increased by recombinant DNA technology. A variety of human DNA probes have been cloned which can be used in Southern blot analysis to detect highly polymorphic loci within the genome of individuals. The probes that detect loci consisting of variable numbers of tandem repeats (VNTRs)appear to be especially useful in identity testing (Nakamura 1987). Of the 7 immunologic genetic tests that the joint American Medical Association - American Bar Association Committee recommended to establish non-paternity in routine investigations, the HLA system provides the greatest probability of excluding a wrongly identified man (Abbott et al. 1976). This article will describe the strategy we have taken for estimating the alleles in the population detected by use of VNTR probes as well as evalualting the power of HLA as opposed to DNA polymorphism testing for excluding wrongly identified men in paternity disputes.

The population investigated consisted of black and white paternity cases from Alabama who presented to the University of Alabama Health Services Foundation, Immunogenetics/DNA Diagnostic Laboratory for testing. All cases were assessed utilizing HLA-A and B typing and the VNTR probes YNH24 and PHINS 310 representing loci D2S44 and INS (CH. 11), respectively. The Promega GenePrint™ DNA typing protocol that was largely developed at the FBI Academy in Quantico, Virginia was followed for Southern blot analyses. The standards for DNA polymorphism testing of the American Society for Histocompatibility and Immunogenetics and The American Association of Blood Banks also were followed. Each electrophoretic gel run contained an internal allelic control consisting of DNA from an individual whose restriction fragment alleles were known as well as standard DNA fragment size markers that spanned the entire range of VNTR fragment sizes detected in the two populations. Fragment sizes were estimated by use of a digitizing pad connected to a PC programmed for autoradiograph reading and line fit. For a case consisting of a trio the DNA samples from the mother, child and putative father were loaded into the gel as well as a mixture of DNA from the child and putative father. In the event that the child's paternal fragment was similar in size to a fragment

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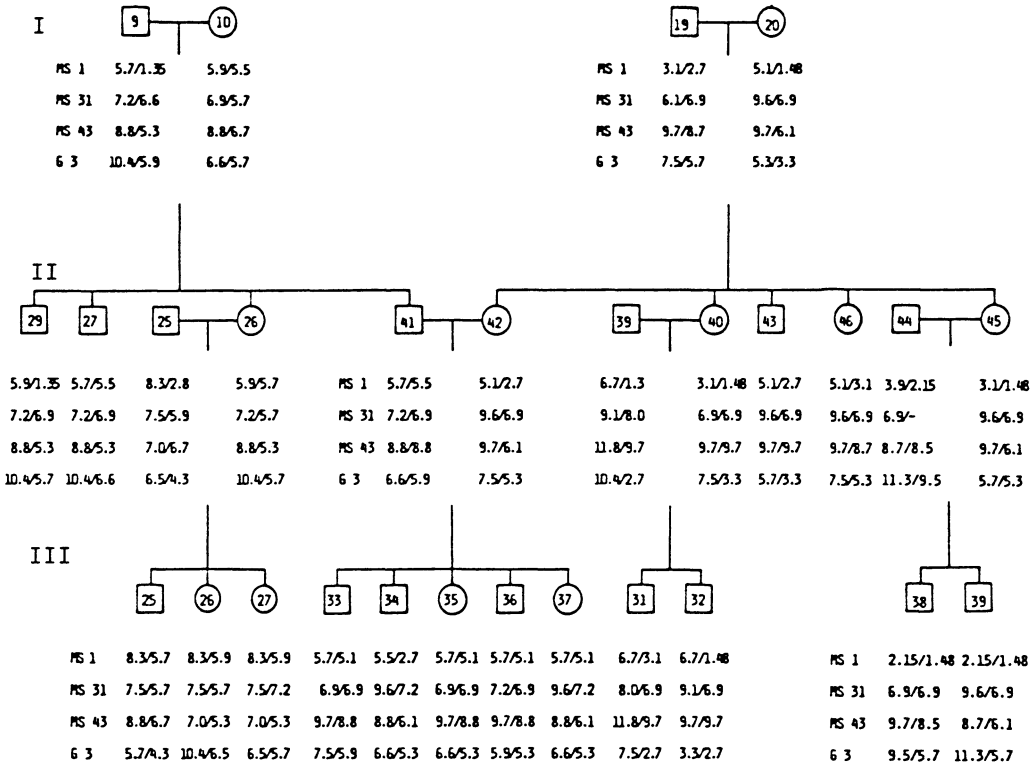


Fig. 2
Exerpt from the pedigree. Given are the respective kb sizes of restriction fragments as revealed by probing with MS1, MS31, MS 43 and G3, resprectively.

93 members of a 3 generation family could be included in the study. The fragments obtained with the probes MS 1 (D1S7), MS 31 (D7S21), MS 43 (D12S11) and G 3 (D7S22) showed a clear segregation. In two children fragments were detected with probe MS 1 which were not attributable to either parent. The differences between the fragments were small (4.8-5.1 kb, 5.8-5.9 kb). Thus we believed that the differences were due to mutational events. No deviations from the expected mode of inheritance were observed, when probes MS 31, MS 43 and G 3 were used. Offspring II/41 is homozygous with respect to his Hinf I 8.8 kb fragment at locus D12S11, whereas offsprings III/33 and III/35 are homozygous for their Hinf I 6.9 kb fragment at locus D7S21. The mentioned homozygous fragments are quite common (frequency ~ 3.5 %) in the Caucasian population.

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Segregation of Single-Locus DNA-Fragments in a Large Family

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Material and Methods

The family tested is of Caucasian origin and lives in all parts of West Germany. DNA was extracted from EDTA-blood by either phenol-choroform extraction or the salting out method (1). After quantification and control of high molecular weight 5 µg DNA digested with Hinf I was loaded onto a 0.7 % agarose gel. After Southern transfer on Hybond N (Amersham) the hybridisation was performed in a hybridisation oven.
Prehybridisation was carried out for 1 h in 10x Denhardt's and 3 h in hybridisation solution (10x Denhardt's, 6 % PEG 6000, 0.1 % SDS, 1x SSC, 50 µg/ml herring sperm DNA) followed by the hybridisation in respective solution and the probe labelled with ^{32}P by oligo-labelling (2). Two washing steps were performed at room temperature (2x SSC, 1,5 % SDS) for 10 min followed by a washing step (15 min, 1x SSC, 1 % SDS and 1-2 washing steps (0.1 x SSC, 0.1 % SDS) at 62° C for 10 min each. DNA from some family-members were run on several gels to achieve an overlapping of the different parts of the pedigree. DNAs showing one fragment only were reinvestigated on a gel with 500 bp at the bottom of the gel in order to establish the state of zygosity.

Results

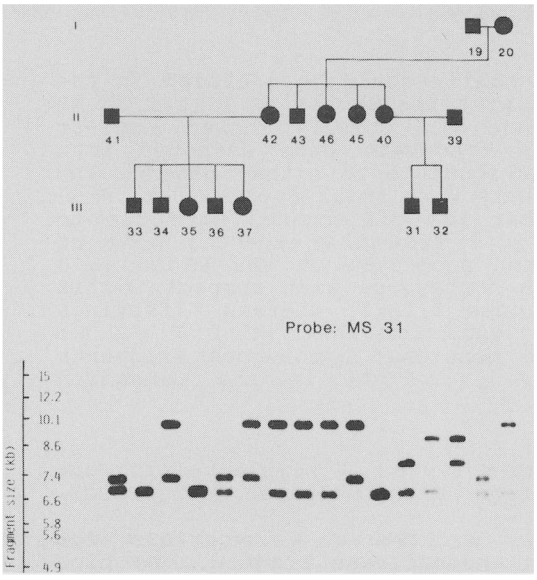


Fig. 1
Excerpt from the pedigree. Homozygosity of members III,33 and III,35 at locus D7S21 is proved

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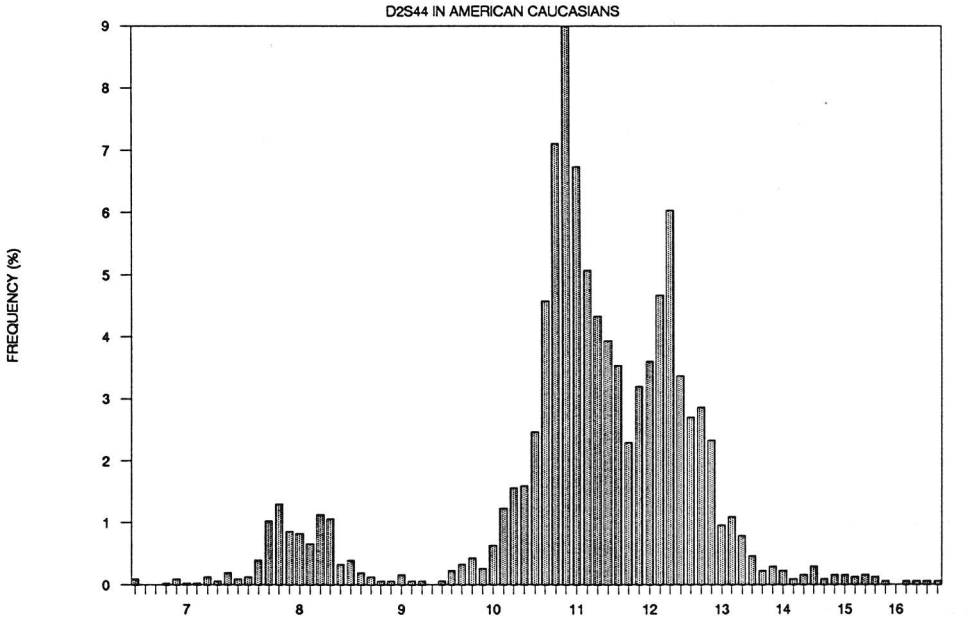
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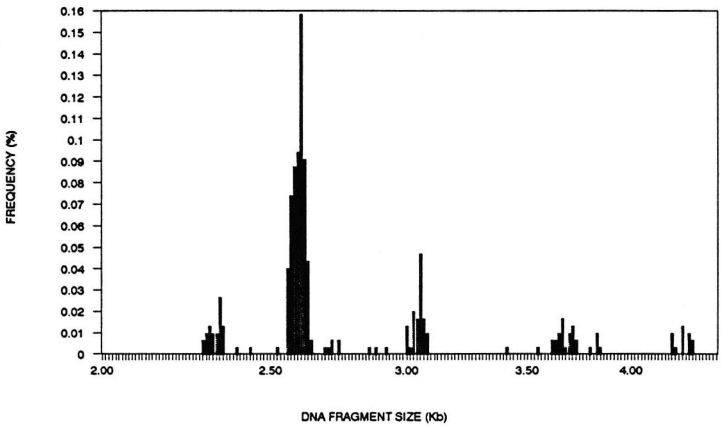
subdivision of the data representing the distribution of DNA fragment sizes, has to be centered around the most frequent DNA fragment sizes. Otherwise, the allele frequency obtained may underestimate of the actual allele frequency and the test for Hardy-Weinberg equilibrium may artificially result in an excess of observed vs expected homozygotes.
When this type of analysis was applied to several VNTR containing loci (e.g. D2S44, D14S13, D17S79), the observed frequency of homozygote and heterozygote genotypes was that predicted from the frequency of the individual alleles. One example of such analysis is illustrated in Fig. 2 for D2S44 using unrelated American caucasian individuals. The accompanying table shows no significant difference between the observed and expected number of homozygotes when the data is divided into bins that encompass the most frequent DNA fragment sizes.



SIZE RANGE(Kb)	7.72 8.00	8.00 8.29	8.29 8.59			10.25 10.62	10.62 11	11 11.4	11.4 11.81	11.81 12.23	12.23 12.67	12.67 13.13	13.13 13.6	
Expected	1.24	1.51	0.12			10.22	75.6	31.37	12.46	30.37	13.94	2.85	0.51	
Observed	1	1	0			13	82	34	13	34	14	5	2	

Fig. 2

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SIZE RANGE(KB) 3.6% increments	>2.27 2.35		>2.54 2.64		>2.96 3.07	(A)
EXPECTED	1.2		53.3		2.2	
OBSERVED	2		54		2	

SIZE RANGE(KB) 3.6% increments	>2.49 2.59	>2.59 2.69	(B)
EXPECTED	13.5	13.8	
OBSERVED	24	30	

SIZE RANGE(KB) 0.6% increments	>2.55 2.56	>2.56 2.58	>2.58 2.59	>2.59 2.61	>2.61 2.62	>2.62 2.64	(C)
EXPECTED	0.2	0.8	5	3.8	2.2	0	
OBSERVED	3	5	16	14	13	2	

Fig. 1

ESTIMATION OF ALLELE FREQUENCY FOR LOCI HAVING A CONTINUOUS DISTRIBUTION OF DNA FRAGMENT SIZES.

Polymorphic DNA fragments can be assigned to discrete alleles as long as the resolution between DNA fragments exceeds the size measurement error. As the number of alleles, within a given distance in a gel increases, the allele frequency distribution becomes a sum of the overlapping normal distribution curves that represent each allele. As boundaries between alleles overlap the distinction between alleles disappears and the result is a continuous distribution of DNA fragments.
When loci exhibit increments in allele size smaller than our ability to measure them, the size measurement error becomes the parameter that best defines the limits of the alleles. In addition, the subdivision of the distribution of DNA fragment sizes should not be arbitrary. The position of the most common alleles has to be taken into account. As shown above for the HRAS locus, the

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Hardy-Weinberg equilibrium analysis of DNA polymorphic loci that exhibit a continuous distribution of fragment sizes

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Hardy-Weinberg equilibrium analysis of alleles at a locus can be readily performed for conventional genetic markers (i.e. where each allele can be assigned a specific property to distinguish it from all others). There are however, many DNA polymorphic loci that demonstrate very large number of allele sizes that reflect variations in the number of tandem repeats (VNTR), of short DNA sequences (Baird 1986; Wong 1986; Dykes 1988; Balazs 1989). Complete separation of these alleles by electrophoresis requires very long gels and fractionation times, which is impractical using current technology. Nevertheless, the approximate size of an allele can be determined by measuring its mobility relative to DNA fragments of known size. The estimation of the size of these alleles allows them to be subjected to the same type of analysis as conventional genetic markers. One such analysis is to determine whether the alleles are in Hardy-Weinberg equilibrium. The following examples illustrate the effect of the allele frequency distribution pattern and the size measurement error in the Hardy-Weinberg equilibrium analysis of DNA polymorphic loci that exhibit a continuous distribution of DNA fragment sizes.

SIZE MEASUREMENT ERROR AND THE DETERMINATION OF ALLELE FREQUENCY.

Repeated measurement of the DNA fragment sizes, after multiple independent fractionations of the same DNA sample, show that the value of the measured sizes follow a normal distribution. Under our conditions of DNA fractionation and size measurement (Balazs 1989) the standard error of measurement is approximately 0.6% of the DNA fragment size, and the individual measurements fall within +/- 1.8% of the average fragment size. Therefore, in order to account for all the observations that represent a particular allele size it is necessary to sum all the events falling within a window or bin size. The boundary of the window is in turn determined by the size measurement error.

When polymorphic DNA fragments that are well resolved from each other are sized (e.g. such as those detected for the HRAS locus), the frequency of each allele can be obtained by summing the events that fall within this 3.6% bin size (+/- 1.8% of the average fragment size). It is important to note that it is not the resolution of the gel that defines the allele size range, but the measurement error. The results presented in Fig. 1 illustrate this type of analysis as applied to alleles of the HRAS locus. Using this criteria, a test for Hardy-Weinberg equilibrium for the HRAS locus does not reveal a significant difference between the expected vs observed number of homozygotes or heterozygotes (see Table A, below Fig. 1). In contrast, an apparent excess of homozygotes is obtained if the measurement error is ignored and the size measurements of a DNA fragment are arbitrarily subdivided into two or more size intervals (see Table B,C below Fig. 1).

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Table 1. Statistical inferences on PstI/YNH24 - YNZ22

	alleles	$\bar{q}$	H	q^2 (2-q)	$2q - q^2$
YNH24	23	0.15	0.85	0.042	0.28
YNZ22	12	0.23	0.77	0.094	0.41
comb.	-	-	-	0.0038	0.11

combined typing with a probe mix in just one hybridization should be easily performed in casework analyses. Due to this fact, and thanks to the yet high discrimination power of the two systems, PSTI/YNH24-YNZ22 is a suitable protocol for paternity investigations.

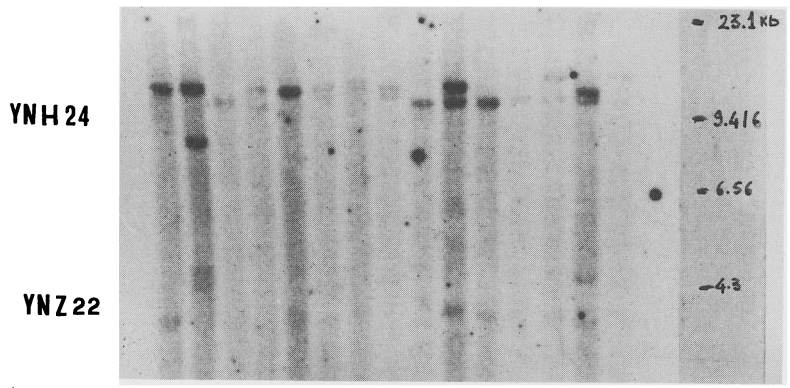


Fig.2. YNH24 and YNZ22 over a PSTI filter blot (two overlapped autoradiographs have been photographed together

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minipreps - were labeled with 32P dCTP by a random exanucleotide priming method. Filters were finally washed at 65 C for 15' with 3xSSC 5%SDS, 15' with 1xSSC 1%SDS, and 15' with 0.1xSSC 0.1%SDS. After rinsing in 2xSSC they were finally exposed for autoradiography (1-6 days).
Mobilities of the restriction fragments was independently measured on the original Xray films by two persons of our staff, using a hand ruler. Allele sizes were then calculated using lambda/HindIII digest bands as reference positioned at the side extremities of each gel. A simple computer program based on algorithms by Elder and Southern (1983) led us convert mobility into fragment lenght values. Gels always contained digests of two selected individuals whose Kb values made us calculate standard deviations and percentages of error in size assignment to alleles of both systems. An average 2% error in alleles size assignment was found for both systems. The maximum resolution in the gels was extrapolated using the nearest bands still discernible in heterozygous samples (1mm corresponding to 250 bp on average for YNH24, and to 50 bp for YNZ22).

RESULTS AND DISCUSSION
Values reported above (% error, maximum resolution in gels) were used to plot the following graphs reproducing the distribution of alleles (Fig.1).

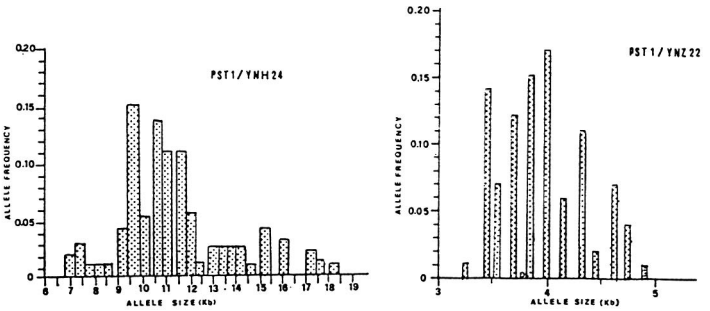


Fig.1. Distribution of YNH24 (left, 73 digests) and YNZ22 (82 digests) alleles

In Table 1, average allele frequencies ($\bar{q}$), heterozygosity (H) and inclusion probability $[(q^2 (2-q)]$ and probability for a given allele to be fortuitously present in an unrelated individual $[(2q - q^2)]$ are reported.
By estimates reported here, YNH24 and YNZ22 are somewhat less polymorphic with PSTI than with other restriction enzymes. However, as shown in Fig.2, band patterns of the two systems do not interfere each other with PSTI digests, so that a

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Allele frequencies distribution of two VNTR markers (YNH24; YNZ22) in PST I digests from random Italian individuals (population of Rome)

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INTRODUCTION

Since the first report of a highly polymorphic locus in Man (Wyman and White, 1980), numerous hypervariable DNA sequences have been described. These markers have been given the name VNTR (Nakamura et al, 1987), and represent nowaday a major tool in forensic biology and paternity testings.

The most systematic investigation on human VNTR has been carried out by Nakamura et al (1987, 1988) who used synthetic oligonucleotides (reproducing core sequences of known hyperpolymorphic loci) for screening a genomic cosmid library. Seventy-two VNTR were this way discovered. Among these, YNH24 (D2S44) and YNZ22 (D17S30) proved to be very polymorphic over several genomic digests (as PSTI, TaqI, HinfI).

Prior to introducing these markers in the routine of the forensic applications, their formal and population genetics need to be studied further.

Here we report the allele frequencies distribution of YNH24 and YNZ22 in about 80 random individuals from Central Italy (Rome). This is our first contribution to a collaborative project for the creation of a repertory of VNTR frequencies in the population of Italy.

MATERIALS AND METHODS

Eighty PSTI genomic digests were prepared according to standard procedures (Maniatis et al., 1982). Seven ug of each digest were run on agarose (0.7% W/V) gels (250 x 150 X 4 mm) using a submarine electrophoretic set. Separations (70 mA /gel) were prolonged as far as the 4 Kb of lambda DNA/Hind III digest marker had migrated beyond the middle of the gels. After capillary Southern transfer on nylon membranes, the resulting filter blots were hybridized overnight under high stringency conditions (Church and Gilbert, 1984). A rotating incubator conditioned to 65 C was used with filters rolled into plastic cylinders. Prehybridization with competitor DNA (250 u/ml for 17 h in the same buffer used for subsequent hybridization) was necessary for YNZ22 only. Cosmid-inserted probes (100 ng) - kindly supplied by Dr. Y. Nakamura, amplified in HB101 host cells and extracted by alcaline lysis

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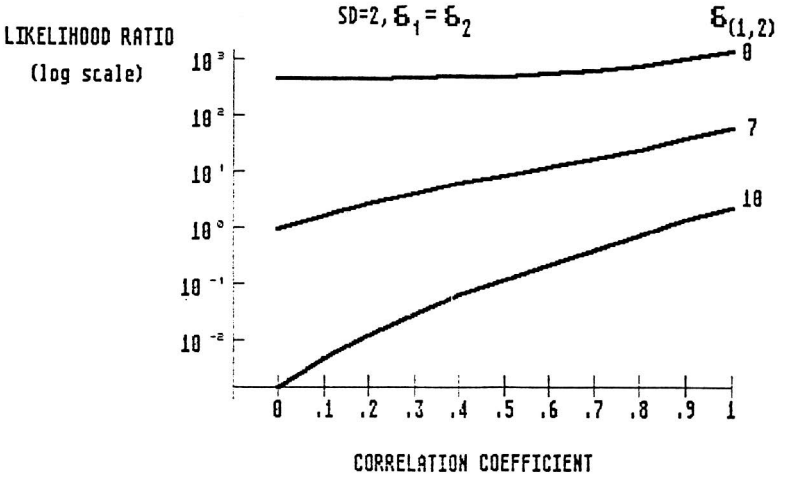


Fig. 5. Likelihood ratio versus correlation coefficient of delta 1 = delta 2 (0, 7, 10 units separation). Band frequencies = 0.005, SD = 2.

two bands match. Using a Bayesian approach the decision of whether 2 profiles could have had the same origin is no longer binary and does not depend upon arbitrary decisions regarding cut-off point. The likelihood ratio is dependent upon band separation between control and suspect profiles. The strength of the analysis relies upon a detailed analysis of characteristics and errors in an electrophoretic system.

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band appears at position y1, then the second at y2 must be greater than the resolution interval of the electrophoretic system. In Figure 4 a plot of $\delta_1 = -\delta_2$ is compared with $\delta_1 = \delta_2$. As the

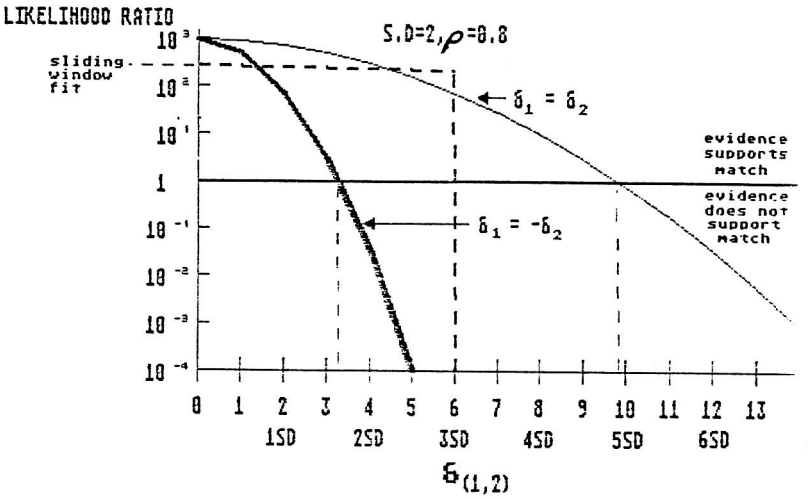


Fig. 4. Plots of likelihood ratio versus delta (1,2) when SD = 2, ρ = 0.8; frequency of 2 bands = 0.005 each. Likelihood ratio calculated using a sliding window Gill et al (1989) is superimposed.

distance between bands in the control and suspect profiles increases, the likelihood ratio falls. The likelihood ratio = 1 when $\delta_1 = \delta_2 = 10$ units (5 SD's separation), contrasting with the alternative plot of $\delta_1 = -\delta_2 = 3$ units (<2 SD's). The equivalent likelihood ratio obtained from a sliding window fit is superimposed, emphasising the problem of using an arbitrary cut-off point where the value drops from 167 to 0 at 3 SD's separation distance. The lower standard deviation, the greater the potential maximum likelihood ratio, demonstrating the need to optimise electrophoretic systems, perhaps by introducing internal markers, for example.

The importance of the correlation coefficient is illustrated in Figure 5. When $\delta_1 = \delta_2$, the likelihood ratio steadily increases with the correlation coefficient for any given value.

CONCLUSION

To conclude, some of the problems of band matching probabilities have been discussed. Lander (1989) has already pointed out the problems of using an arbitrary cut-off point in deciding whether

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The denominator assesses the probability of the evidence if the profile has come drom a randomly chosen individual, and this can be calculated from another probability density function derived from the distribution of bands in a given population. The density function in Figure 3 has been produced using Kernel density

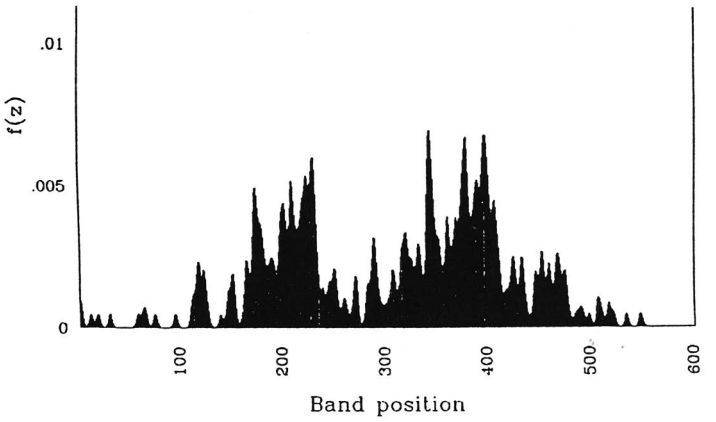


Fig. 3. Frequency histogram of D2S44 produced by Kernel density smoothing.

smoothing. This routine takes raw data and simulates an expected distribution if an infinitely large population had been sampled. The area under the curve is 1. If the recorded band position falls close to a sharp peak then a conservative value could be taken (i.e. the value of the peak itself).

If the profile contains 2 bands at positions y1 and y2 and the probability of obtaining a profile with 2 bands is p(2) then the probability of finding two bands at those positions can be given:-

$$p(2)p(y1,y2 \mid 2)$$

If the profile has only one band at position y1 and the probability of obtaining a profile with 1 band is p(1) then the denominator is:-

$$p(1)p(y1 \mid 1)$$

The advantage of using these expressions rather than the equivalent genetic expressions $2p(y1)p(y2)$ for heterozygotes and $p(y1)^2$ for homozygotes is that the former do not require unambiguous identification of homozygotes and heterozygotes. Theoretically, two bands may appear so close together, that they cannot be distinguished and appear as a homozygote. Alternatively, bands may be scored as homozygotes if a low molecular weight band has run off the end of a gel or it too faint to be visualised. Evett et al (1989) has introduced a correction term to allow for the fact that if a

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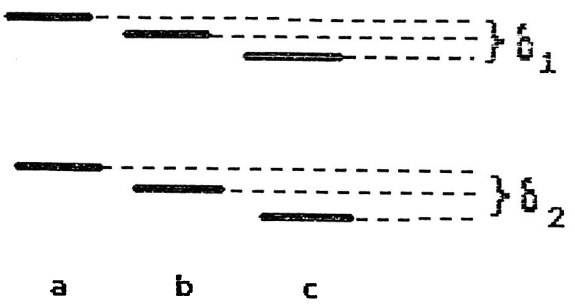


Fig. 1. Unless the correlation coefficient is taken account of, the probability of profiles b and c matching control a are the same.

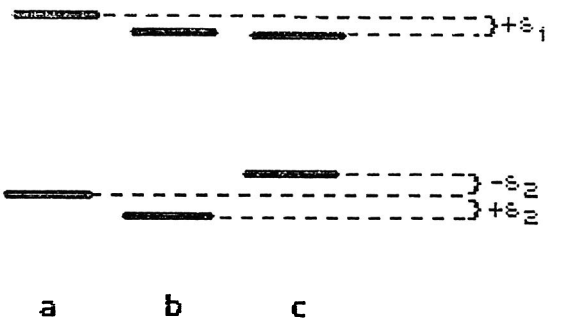


Fig. 2. If profile a is the control, then intuitively, b is a better match than c. This is allowed for in Bayesian statistics but using 'binning' or 'sliding window' routines Gill et al (1989) gives equal probabilities to the results.

the likelihood ratio assesses the relative probabilities that two profiles have originated either from the same or from different individuals:-

$$\frac{\text{The probability of the evidence given that two profiles have the same origin}}{\text{The probability of the evidence given that two profiles have different origins}}$$

The numerator is the probability of obtaining the two profiles given that they have originated from the same source. The demoninator measures the probability that the stain has come from someone else. If the likelihood ratio is greater than 1 it will tend to support the hypothesis that the control and the crime stain have the same origin, whereas a value of less than 1 will support the hypothesis that the stain has come from someone else.

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PROBLEMS ASSOCIATED WITH THE DETERMINATION OF BAND MATCH PROBABILITIES

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INTRODUCTION

Recently Lander (1989), and Evett et al (1989a) have addressed the problem of determining whether two bands match each other in suspect and crime samples. Whereas the statistical treatments discussed by Gill et al (1989) in general provide a conservative answer to the problem of allele frequency estimation, the approach does not give information regarding the probability that 2 bands match, taking into account errors in measuring band position. Gjertson et al (1988) suggested a Bayesian model which addressed the problem of band match determination, although this model did not take account of the correlation of shift in band position.

Gjertson et al (1988) introduced a Bayesian analysis which incorporated measurement error associated with fragment size determination. Evett et al (1989b) introduced a model which took account of the correlation of shift in band position.

A Bayesian Approach

Any attempt to define a clear cut off point in deciding whether two bands match each other is problematical for the following reasons: first, criteria used to decide a cut off point are arbitrary. Second, the probability that the samples are from one individual has occurred must decrease the further away the bands are from each other. Intuitively, the pattern in Figure 1, profile b is a 'better' match than that in Figure 1, profile c yet regardless of this, probabilities are usually assigned to be the same, i.e. 1. If an arbitrary cut off point of 3 SDs is given, this could mean that if bands are greater than 3 SDs apart, the probability that samples have come from one individual suddenly plummets to 0. Clearly the declaration of a match or an exclusion cannot be a binary event. A moderator needs to be introduced into the analysis to take account of bands which are not recorded at precisely the same positions. One approach would be to define a match as occurring when bands are <3 SDs apart. If reported as inconclusive. Alternatively, cut-off points can be avoided by introducing a Bayesian analysis, where

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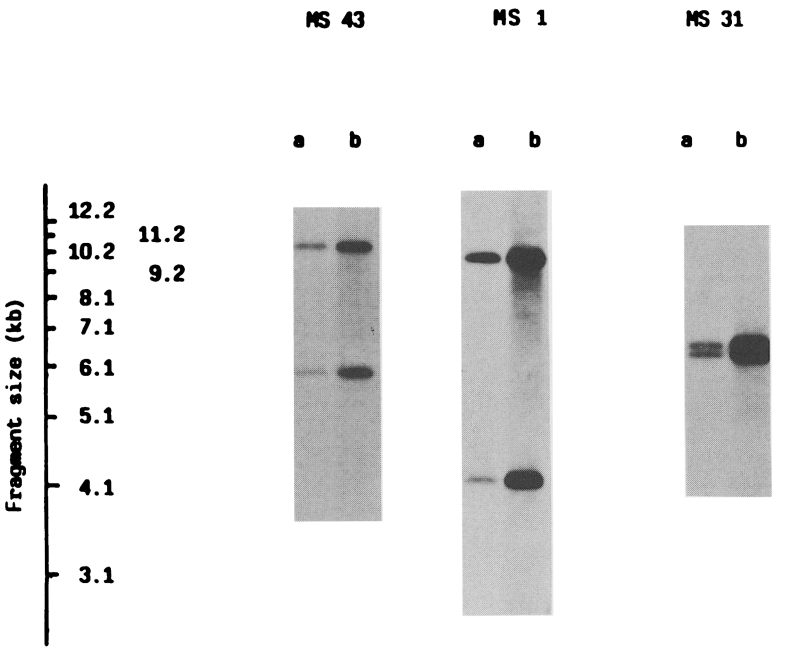


Fig. 1 shows the results of hybridization experiments. A Hinf I digested DNA sample from a fresh blood (a) was electrophoretically seperated side by side along with the DNA from the appropriate aged blood sample (b). After hybridization with one probe and autoradiographic documentation the probe was stripped-off in order to allow hybridization with another probe.

The appearence of artificial extra-bands is not unusual. Smaller and faint extra-bands are obviously due to a certain degree of partial degradation of DNA in aged samples. This means that a normal restriction fragment which still carries the entire minisatellite sequence becomes artificially smaller. Therefore, these extra-bands are both the genuine and unique expression of ageing and decomposition and they are hardly attributable to a contamination of DNA probes.

Last, but not least, it should be mentioned that in 8 = 4,5 % of our 175 cases a genetically conditioned non-identity could be established. All of these non-identities were not due to an erroneous mix-up but to different kinds of criminal manupulations in order to avoid prosecution.

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Investigation of Aged Blood Samples by Means of Single-Locus DNA Probes

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In cases of drunken driving, optimal evidence can be obtained by drawing of blood samples followed by the determination of ethanol. This procedure is superior to any other method.

This is why suspects sometimes challenge the identity of a blood sample and the forensic geneticists is asked to give an expert opinion (3,4).

In the past years, respective hemogenetic analyses have been carried out by means of conventional blood group systems, which means that genetic markers have had to be typed in up to 2 years old blood samples. The results were then compared with those obtained from a fresh drawn blood specimen. The similarity between this task and a stain analysis is obvious. It is not unusual that difficulties arise when aged and more or less decomposed blood samples have to be typed. Bacterial contamination, protein degradation, and hemolysis may have the impact that genetic markers become untypable or worse: genetic markers appear either false positive or false negative (2,6,7,8,10)!

The experienced serologist is acquainted with such problems and extended typings are then required allowing a conclusion whether a non-identity is due to artificial or to genetic reasons. If such a clear-cut conclusion cannot be reached the expert witness should have the integrity and the personal independence to deliver respective testimony.

A powerfull new tool in these investigations are DNA probes (1). We prefer to use the single-locus probes MS1, MS31, MS43, and G3 described by Jeffreys and co-workers (11,12) and also probe YNH24 described by Nakamura and colleagues (5).

In 17 out of 175 expertises on the identity of a blood sample we additionally worked with the probes mentioned. In 12 cases we successfully could extract high molecular weight DNA from the aged samples by means of a phenol-chloroforme extraction procedure (9).The DNAs were then passed on to Hinf I digestion, Southern transfer and hybridization.

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quality and validity of the analytical data is maintained; (5) to maintain adequate documentation and records concerning how the evidence was analyzed, what was done and by whom; (6) to ensure that problems are noted and that corrective action is taken and documented; and (7) to demonstrate to the forensic science community, the judicial system, and the scientific community at large, the laboratory's commitment to maintaining excellence in all areas of its work.

While most forensic laboratories have a tradition of providing reliable and accurate results without the benefit of a formally established QA program, the formal establishment and implementation of a comprehensive QA program would provide essential support to the accuracy and reproducibility of the results presented in court by the DNA laboratory examiner or analyst. The QA program would provide the necessary evidence, through documentation, that the quality control requirements established for the RFLP procedure are being performed adequately and that the DNA analysis process is operating within the established performance criteria; it would also provide a measure of the quality of the results. In addition, the documentation requirements of a QA program would serve as an archive for retrospective scientific inspection, reevaluation of the data, and reconstruction of the DNA procedure.

The QA guidelines ("Guidelines for a Quality Assurance Program for DNA RFLP Analysis", *Crime Laboratory Digest*, April-July 1989, V. 16, No. 2, pp. 40 - 59) were developed by the TWGDAM QA subcommittee using established quality functions (American National Standard ANSI/ASQC C1-1968; ANSI/ASQC Z-1.15-1979; ANSI/ASQC Q90-1987a,b; Juran 1979; Ruzicka 1979) and follow systematically the DNA RFLP typing procedure. These guidelines cover all significant aspects of the laboratory process including: Planning and Organization, Personnel, Documentation, Materials and Equipment, Validation of Analytical Procedures, Evidence Handling Procedures, Internal Controls and Standards, Data Analysis and Reporting, Proficiency Testing, Audits, and Safety. These guidelines provide the necessary documentation to ensure that the DNA analysis process is operating within the established performance criteria and ensure that the quality, integrity, and accuracy of the RFLP data is maintained.

These guidelines form the basis of a quality assurance program for RFLP analysis and are subject to future revisions as the state of the art and experience dictate.

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Elements of a Quality Assurance (QA)/Quality Control (QC) Program for DNA Testing

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With the advent of DNA typing technology in the forensic laboratory, the forensic examiner now has the potential to individualize various body fluids and tissues. Since the results of these tests can have a significant impact on the outcome of a trial, it is important that they be accurate and reproducible. Consequently, the use of appropriate standards and controls, as part of a QA program, is essential in order to ensure scientifically sound and reliable results from the analysis of forensic evidence.

Although often used interchangeably, quality control (QC) and quality assurance (QA) refer to different, specific quality functions (American National Standard ANSI/ASQC A3-1978; Kilshaw 1986; 1987a,b). The QC measures would be employed by a DNA analysis laboratory to ensure that the quality of the product (DNA typing) will meet and satisfy specified criteria. The function of the QA program, on the other hand, is to provide the evidence needed to establish with confidence that the QC function is being performed adequately. This is accomplished in part through the use of proficiency tests and audits.

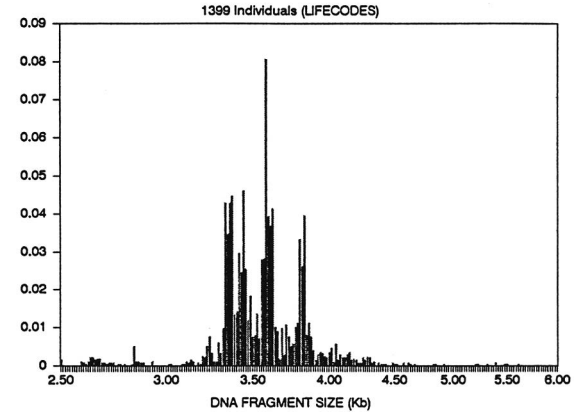
In November, 1988, the first meeting of the Technical Working Group on DNA Analysis Methods (TWGDAM) was hosted by the FBI Laboratory at the FBI Academy. This group consisted of 31 scientists representing 16 forensic laboratories in the United States and Canada and two research institutions. During the first meeting, a subcommittee was established to formulate suggested guidelines for a QA program for crime laboratories conducting restriction fragment length polymorphism (RFLP) analysis of variable number of tandem repeats (VNTR) loci.

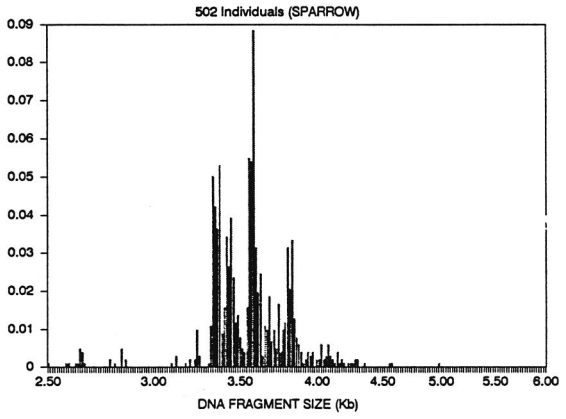
These guidelines represent the minimum QA requirements for DNA RFLP analysis and are intended to serve only as a guide to laboratory managers in establishing their own QA programs for DNA RFLP analysis.

The objectives of the QA program are: (1) to monitor on a routine basis the analytical testing procedures for DNA typing by means of QC standards, proficiency tests and audits; (2) to ensure that proper equipment, materials and procedures are employed during the analysis of forensic evidence; (3) to verify that the entire DNA typing procedure is operating within the established performance criteria; (4) to ensure that the

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ALLELE FREQUENCY DISTRIBUTION





In order to validate a database of VNTR alleles, it must be shown that the frequency distribution is not biased by sampling error. Allele frequency distributions for PstI derived alleles at the loci D2S44, D17S79, and D14S13 were constructed by independent laboratories (Lifecodes, The Blood Center of Southeastern Wisconsin, Roche Biomedical Laboratories, and Sparrow Hospital). The distributions do not show statistically significant differences. As an example, Fig. 1 shows the data obtained for the D17S79 locus in American Caucasians.

Fig. 1 Allele frequency distributions of D17S79 alleles in PstI-digested DNA from American Caucasians as determined by two independent laboratories.

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Southern transfer and hybridization). The second reason is to provide the means for proper measurement of the allele sizes from the resulting autoradiographs. Accordingly, the sizing of alleles on each membrane includes the measurement of the alleles from the K562 control DNA. Obtaining consistent values in the same laboratory over a period of time for the K562 alleles provides an important quality assurance parameter.

Similarly, consistent sizes of the K562 alleles obtained at different laboratories ameliorates concerns of site-to-site variations in RFLP analysis of DNA for identity and paternity testing. An analysis of the values obtained from a multitude of sizings of K562 alleles in three laboratories is shown in Table 1.

Table 1. Allele Sizes for Three Loci of K562 DNA

Locus	N	Mean (kb)	S.D. (kb)	% Err	Range (kb)
D2S44 (1)	203	11.963	0.058	0.49	0.303
D2S44 (2)	203	10.859	0.068	0.62	0.471
D13S14	112	5.011	0.022	0.45	0.170
D17S79 (1)	230	4.007	0.023	0.57	0.171
D17S79 (2)	203	3.552	0.021	0.60	0.162

The maximum standard deviation corresponds to 0.62% of the DNA fragment size, which is consistent with a previous report which claimed a 0.6% error in the measurement of allele sizes by RFLP analysis (Balazs 1989). The same error is observed over a size range of at least 12 kb to 3.5 kb. Thus, by applying the three standard deviation rule, one is 99% certain that a DNA fragment measured to be 12 kb is between 11.784 kb and 12.216 kb, or a fragment measured to be 3.5 kb is between 3.446 kb and 3.554 kb. Furthermore, the data are highly consistent among the laboratories. The maximum difference between the mean of an allele size determined in one laboratory and the combined mean for all laboratories is 0.4%.

A second approach to determining the level of reproducibility of this technology was to split samples for analysis in two laboratories. Five laboratories participated in the study. Samples submitted for paternity testing to four of the laboratories where split. One aliquot was analyzed in the receiving laboratory and the other was analyzed in our laboratory. Allele sizes for all samples were determined, and the data were subjected to a pairwise analysis. The percent difference in the measured values of each allele was calculated as $[(A_L-A_B)/((A_L+A_B)/2]*100$, where A_L was the size measured in our laboratory and A_B was the size measured at the receiving laboratory. Table 2 compiles the data for the 877 pairs of sizes (two sizings for each allele) that were analyzed, including 59 complete paternity trios. In every case, both laboratories arrived at the same determination of inclusion or exclusion of the alleged father. The average standard deviation of the per cent differences of the data from the four beta sites participating in the study was 0.85.

Table 2. Variations in Two Independent Determinations of 877 Allele Sizes

Beta Site	N	Range of % Difference	Standard Deviation
1	261	(-1.95) - (2.40)	0.86
2	386	(-2.46) - (2.73)	0.74
3	159	(-4.80) - (3.01)	0.95
4	71	(-3.36) - (2.12)	0.85

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ACCURACY, PRECISION, AND SITE-TO-SITE REPRODUCIBILITY IN ANALYSIS OF DNA POLYMORPHISMS FOR IDENTITY TESTING

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INTRODUCTION

Interest in using RFLP analysis of DNA for identity testing is growing rapidly. As an increasing number of diverse laboratories begin practicing the technique, the concerns over the reproducibility of the methodology and over the utility of the data generated will continue to grow. Since the procedure for generating an RFLP analysis is composed of a large number of steps, there are potentially a large number of opportunities to introduce variances from lab to lab. The concerns can be categorized into two broad areas. First, it must be shown that the RFLP result (ie. allele sizes) obtained from any given sample does not vary significantly when determined in various laboratories. Second, we must assure ourselves that data bases used for determining probabilities of identity or paternity are valid. This paper is intended to begin compiling data to address these concerns.

Five different laboratories have used identical protocols and sets of reagents to process blood samples for paternity testing. The data generated have been used to estimate the magnitude of site-to-site variations in RFLP analyses and the degree of coincidence of databases constructed with data from the various laboratories.

ALLELE SIZE

DNA was extracted with a simplified, non-organic method which gives a high yield of intact DNA which is suitable for RFLP analysis (Turck 1989). Samples were restricted with PstI and electrophoresed on a 28 cm 0.6% agarose gel. One lane of each gel contained 500 ng of K562 DNA which had been digested with PstI. DNA was transferred from the gel to a nylon membrane according to a modified Southern procedure (Southern 1975), and the membranes were then hybridized with ^{32}P-labelled DNA probes for the loci D2S44, D14S13, and D17S79. After autoradiographs were prepared, all alleles from the samples and from the K562 control DNA were sized using a digitizing tablet along with sizing software provided by Lifecodes.

The procedures employed included various standards and controls for two reasons. The first is to allow the monitoring of each stage of the analysis (extraction, restriction, electrophoresis,

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three) probes used. Ten forensic specimens failed to detect any readable bands with all probes used.
In two cases only, discordant results were obtained with different probes. In the first case (a blood stain 7 months old absorbed over a cloth) readable pattern resulted from the use of 3'HVR under both low and high stringency; conversely lambda MS1 and YNH24 failed to detect readable patterns. In a further case, two seven months-old blood stains absorbed to a car seat acrylic cover yielded a single-locus pattern with 3'HVR, but not with lambda MS1/31 mix nor with YNH24.
These results are difficult to evaluate. While a marked resistance of 3'HVR/Hinf I polymorphism to degradation processes would be argued, the number of samples examined is too small to allow significant inferences.
If letting unanswered the question as to whether some VNTR systems are more refractory to aging than other ones, results reported here enphasize well that testing forensic DNA digests with several DNA probes is mandatory whenever they derive from very aged specimens.

ACKNOWLEDGEMENTS
We thank Dr. J. Savin, ICI Cellmark (UK) and Dr. Y. Nakamura, Howard Hughes Med. Inst. for providing YNH24 and MS1/31 probes. Pharmacia - LKB (Dr. Dr. Surace and Dr. P. Petri) supported in part this work.

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throughout. Details on forensic specimens worked out have been described elsewhere (Pascali et al. 1989). When beginning this study, most filters had been generally hybridized once by 3'HVR under low/high stringency conditions, and at times reprobed with lambda MS1/31 at high stringency. For reprobing, they were washed in 0.1% SDS at 90°C for 30 min, then rinsed in excess 2 X SSC.
While hybridizing all our filters (referring to as much forensic cases) with 3'HVR, YNH24 and MS1/31 was in our aim, nine out of 15 filters were actually probed for these three systems. The remaining six filters were probed with only two. Labelling was done with a random exanucleotide priming method (Multiprime, Amersham). Hybridization (high stringency buffer, according to Church and Gilbert, 1984) was prolonged overnight (3 x 10^7 cpm/blot) with following high-stringency washes (3xSSC/5% SDS for 15'; 1xSSC/1% SDS for 15'; 0.1 x SSC/0.1% SDS for 15' at 65°C).
Time of exposure to Xray films varied according to the counts/sec over the filters.

RESULTS AND DISCUSSION

The outcome of multiple hybridizations over fifteen filters can be summarized as follows. Ten out of 23 forensic samples analyzed revealed coherent bands with all (either two or

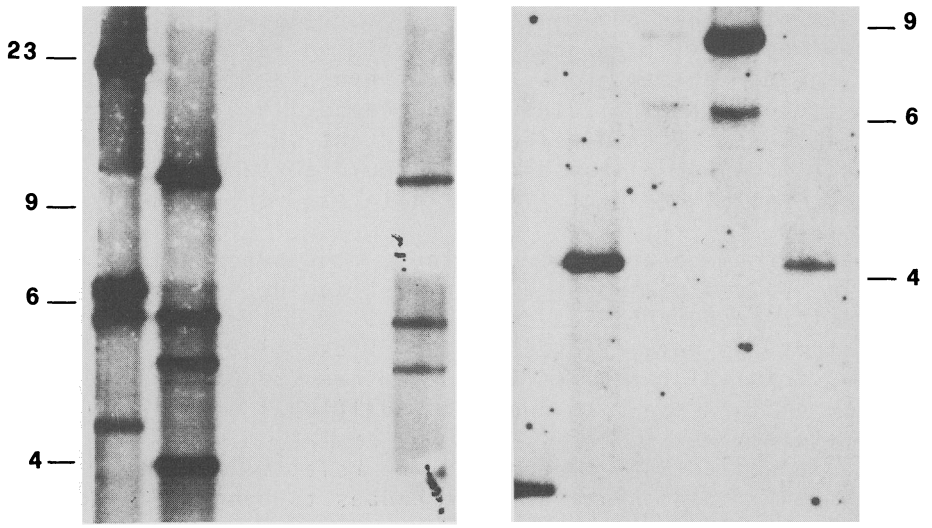


Fig. 1. Discrepant hybridization experiments with MS1/31 (left) and 3'HVR over the same filter. From left: a, reference; b, suspect; c,d, car seat bloodstains; e, shirt bloodstain.

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On the effectiveness of three DNA probes in hybridization experiments over HINF I blots containing aged/degraded samples

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INTRODUCTION

Several DNA probes recognizing variable number of tandem repeats (VNTR) polymorphisms have been recently introduced in the analysis of forensic specimens. They include lambda MS1/31/43/G3 (Wong et al, 1987), alpha globin 3'HVR (Fowler et al, 1988) and YNH24 (Nakamura et al, 1987).
These probes belong to different though related families of tandem repeats with different core sequences, and recognize clusters of alleles with distinctive molecular sizes.
Little is presently known on the liability of these VNTR polymorphisms to aging/degradation damages.
A general notion is that on aging restriction fragments of a given digest undergo fragmentation. However, reduction in size of DNA is known not to be merely correlated to aging. Endogenous hydrolytic processes underlying size reduction largely depend on factors as sample hydratation, and the relative content in pirimidine bases (Paabo, 1989).
If this is true, then a different content in C and T bases within distinctive classes of VNTR could perhaps affect a selective resistance of VNTR alleles to hydrolytic degradation.
Even apart from general speculations, the hypothesis that some VNTR polymorpshisms could resist aging better than others merits to be verified as such.
In this study, we have reinvestigated filter blots pertaining to several criminal cases recently examined for DNA profile, by rehybridizing them to one/two additional single-locus probe systems.
Our purpose was to know further on the relative ability of three commonly used hypervariable probes to hybridize aged samples.

MATERIALS AND METHODS

Southern blot analysis of various forensic specimens (fifteen criminal cases) was performed according to standard procedures (Maniatis et al, 1982). HINF I digests were used

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three hours. During this "ramp-up" period an increasing amount of light is emitted. By the third hour a constant amount of light is emitted which persists for several days. A one hour exposure of a fully ramped blot clearly reveals the DNA type of a microgram or less of human gemomic DNA. 5) the sensitivity of the radioactive procedure is such that YNH24 alleles could be read from one microgram of human genomic DNA after overnight autoradiography. The nonradioisotopic method which used BCIP/NBT was found to be at least five-fold less sensitive (24 hour detection). The chemiluminescent method yielded an image in one hour that was comparable to that obtained from overnight autoradiography. 6) the chemiluminescent method described here could not be used to distinguish alleles in samples that contained much less than one microgram of genomic DNA. For although light is emitted for several days, the background light precludes any greater sensitivity with the system that was studied. Nevertheless, the steady state emission of light afor a long time allows the investigator to take multiple exposures as with autoradiography in order to secure the highest quality image.

SUMMARY

1) radiolabeled YNH24 probe can detect VNTR alleles in as little as 40 nanograms of genomic DNA following a Southern transfer to nylon, probing and prolonged (weeks) autoradiography.
2) the chemiluminescent procedure described here routinely detects YNH24-specific VNTR alleles in one microgram of genomic DNA after a single hour of imaging. The result is comparable in quality and sensitivity to that obtained from the analysis of the same amount of genomic DNA by overnight autoradiography.
3)compared to the radioactive and chemiluminescent methods, colorimetric detection with BCIP/NBT is less reproducible, less sensitive, and slower since it requires a 24 hour enzymatic reaction.
4) the chemiluminescent detection of alleles is quickly and easily done. The method is currently suitable for applications where as little as one microgram of genomic DNA is available to be run in a single lane of the analytical gel. Most significantly, the hazards of working with radioactivity and of its disposal are avoided.

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Nonradioisotopic Detection

Probe sequences labeled with digoxigenin-dUTP (Boehringer Mannheim) were hybridized to blots of genomic DNA. Detection was effected by the subsequent binding of anti-digoxigenin polyclonal antibody conjugated with alkaline phosphatase (Boehringer Mannheim), and the action of this enzyme on the substrate 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Promega Corporation) in the presence of the enhancer nitro blue tetrazolium (NBT, Promega Corporation). The sensitivity of the method is such that it is necessary to have five micrograms of genomic DNA per lane to obtain a satisfactory result. Alkaline transfer methods were curiously incompatible with the nonradioisotopic procedure and a six hour neutral transfer method was utilized instead (Maniatis et al, 1982).

Chemiluminescent Detection

The use of a chemiluminescent alternative to the BCIP/NBT substrate/enhancer combination was recently described (Beck et al, 1989). The new substrate is a phosphorylated dioxetane which upon dephosphorylation by alkaline phosphatase forms an unstable intermediate. The intermediate decays emitting light over time in a pH and temperature dependent manner. It was unnecessary to alter the nonradioisotopic procedure until the last step where the phosphorylated dioxetane chemiluminescent substrate (PDCS) was used instead of the BCIP/NBT combination. Thus blots which had been previously probed and treated with the immunoglobulin-alkaline phosphatase conjugate were then immersed in a solution of PDCS for five minutes, sandwiched between sheets of thin, transparent plastic and placed in direct contact with X-ray film. The chemiluminescent method is faster and more sensitive than the colorimetric procedure.

DISCUSSION

The DNA typing procedure is evolving rapidly becoming faster, easier, cheaper, and more sensitive. Technological improvements will radically push the procedure toward these objectives.

Some general conclusions from this work are: 1) it was necessary to use a neutral transfer method in conjunction with the nonradioactive procedure. However, the overnight transfer was shortened to six hours with no apparent loss of signal. 2) depurination is unnecessary for the quantitative transfer of DNA with the size range of alleles detected by the probes used in this study. 3) the greatest source of variation is the membranes themselves. Membranes from the same lot, and more rarely different parts of the same membrane, showed a five-fold range of binding efficiency. 4) in the chemiluminescent method, steady state emission of light is not achieved for

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Comparison of Chemiluminescent and Radioactive Methods of DNA Typing

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INTRODUCTION

DNA typing has the potential to completely replace the immunological and biochemical genetic tests that have been used over the past decade in parentage testing and forensic analysis. The method is easily performed, but there are several incubation periods which result in the procedure taking a number of days to complete. Nevertheless, DNA typing is attractive because: 1) it is a single technique, 2) with the exception that identical twins cannot be distinguished, conclusive results are always obtainable since the same blot of genomic DNA can be sequentially stripped and tested with different probes, 3) once a blot has been made, the reprobings are extremely quick and easy to perform, 4) it is cost-competitive with the other group of tests, 5) it is more sensitive than the other methods in that far less sample is required for the analysis.

RESULTS

The genomic DNAs used in this study were isolated via an organic extraction method. HaeIII restriction endonuclease (Promega Corporation) was used to at five units per microgram to completely digest human genomic DNA in five hour reactions.

Radioisotopic Detection

Following the electrophoresis of the digested genomic DNA in a 1X TEA, 1% agarose gel, a Southern blot was made. The transfer of the DNA profile resulting from electrophoresis was accomplished by a six hour alkaline transfer (GenePrint™ Technical Manual). It was found that overnight hybridization with ^{32}P-labeled YNH24 probe (D2S44) invariably led to an enhanced signal from the VNTR loci when compared to the results from a three hour hybridization. Nevertheless, it is important to note that the three hour hybridization always yielded a satisfactory result when five micrograms of genomic DNA per lane were being probed.

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TABLE 5. PI VALUES FOR NON-EXCLUDED CASES

	2 PROBES 2 MEMBRANES		2 PROBES SAME MEMBRANE		3 PROBES	
PI	FREQ	AVERAGE	FREQ	AVERAGE	FREQ	AVERAGE
<100	0.38	49	0.42	28	0.18	76
100-1000	0.50	415	0.48	278	0.44	342
>1000	0.12	1955	0.10	2432	0.38	10,372

* pL336 on one membrane and two probes on second membrane.

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probing in conjunction with single probing demonstrated a five fold increase in PI values.

Considering the cost of isotopic techniques and the time required to rehybridize membranes (days), the use of simultaneous probing will maximize results at a considerable savings. Careful selection of non-overlapping loci will enable the testing laboratory to provide clients the best results at a reasonable price.

TABLE 4. EXCLUSION RESULTS FOR PROBE COMBINATIONS *

PROBE	NO. EXCLUSIONS			
COMBINATIONS	0	1	2	3
336 + 194	1/18	5/18	12/18	
336 + 427-4		6/13	7/13	
336 + 144-D6		4/12	8/12	
336 + 159-1		1/1	0/1	
194 + 427-4		0/3	3/3	
194 + 144-D6		1/2	1/2	
194 + 365-1		0/1	1/1	
427-4 + 144-D6	4/7		3/7	
THREE PROBES		5/15	5/15	5/15

* Results taken from Table 3 using both two and three probe combinations. 336 + 194 and 427-4 + 144-D6 include "0" exclusions with these combinations but alleged father was excluded by a third probe.

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TABLE 3. OBSERVED EXCLUSIONS USING CRI AND EPITOPE PROBES

336	194	427-4	144-D6	159-1	365
yes	yes				
yes			yes	no	
yes	no	yes			
yes	yes				
yes			yes		
yes	yes				
yes		no	no		
yes		no	no		
no		yes	yes		
yes	yes				
yes		yes			
yes	no	yes			
no	no		yes		
yes	yes				
no	yes				
yes		no	no		
yes		yes	yes		
yes		yes	no		
yes	yes				
yes	yes				
yes			yes		
					yes
yes	yes		yes		
yes					
yes	yes				
yes	yes				
no		yes	yes		
yes	yes	yes			
yes	yes	yes			
yes	yes				
	yes		yes		yes
(a) 25/29	14/18	9/13	8/12	0/1	2/2
(b) 86.2%	77.8%	69.2%	66.7%		100%

(a) = exclusion count, (b) = % total exclusion

single and double exclusions. Simultaneous probing of a single membrane also proved effective, for example pS194 and pL427-4. Using three probes clearly demonstrated the best combination due to multiple exclusions.

Table 5 lists the observed PI values in the non-excluded cases. Using simultaneous probing on the same gel was as effective as a single probe on two separate membranes. Using a simultaneous

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man was based upon a summation of all band frequencies +/- 1% from obligatory band shared by the child and alleged father.

RESULTS

Table 1 shows the probes used to detect the various polymorphic loci detected in this study. Although pL336 was always run alone on a single gel because of it's size range, various combinations of multiple hybridizations were used for the second gel, for example pS194 and pL427-4 or pL355-8, pL159-1 and pL427-4 (data not included). 85 referral cases and 29 private cases were reviewed which demonstrated an exclusion rate of 27-28%, Table 2.

In Table 3 are the 31 observed exclusions seen in the 114 cases tested. In all but one case pL336 was probed on one gel whereas the second gel contained either a second probe or a combination of two non-overlapping loci which could be detected with simultaneous probing. The heterozygosity of pL427-4 was expected to demonstrate more exclusions than observed but we suspect that sample size affected the lower exclusion rate. Table 4 breaks down the results of the probe used in table 3 into two and three probe combinations. pL336 probed on one gel and either pS194, pL427-4 or p144-D6 on the other gel all showed similiar rates of

TABLE 1. DNA LOCI, PROBES AND SIZE RANGES

LOCUS	PROBE	SIZE RANGE	SOURCE
D1S47	pL336	2-21	Collaborative Res.
D7S107	pS194	5.2-12.4	" "
D18S17	pL159-1	5-7	" "
D21S112	pL427-4	1.4-3.4	" "
D11S129	pR365-1	1.5-3	" "
D20S15	pL355-8	6-8	" "
D17S34	p144-D6	3.4-7.9	Epitope

TABLE 2. TABULATED RESULTS FROM PRIVATE AND REFERRAL CASES

	REFERRALS	PRIVATES
EXCLUDED	23	8
NON-EXCLUDED	62	21
TOTAL	85	29
% EXCLUDED	27	28

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Simultaneous DNA Probing of Paternity Cases Using Non-isotopic Methods

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INTRODUCTION

The use of DNA restriction fragment length polymorphisms (RFLP's) in the field of parentage testing has recently become a reality. Commercial availability of probes and the introduction of non-isotopic methods of detection (Dykes et al. 1986, 1988) has gradually made phenotyping of RFLP's more practical for the normal laboratory setting. The common complaint is that non-isotopic methods do not permit probe stripping and rehybridization. Historically the real problem was due to the fact that most polymorphic loci had broad overlapping ranges of band sizes. However, with careful selection of DNA probes it has become possible to identify highly polymorphic loci with non-overlapping band sizes which permit simultaneous probing.

This study was performed to determine the number of exclusions and the level of inclusion estimates when testing single versus multiple hybridizations. Multiple hybridizations were considered to be two to three probes applied to the same membrane. The results confirm the expectation that multiple probing is an advantage over rehybridization.

METHODS

Blood samples were supplied to Analytical Genetic Testing Center for complete parentage testing using our routine testing protocol of 2-4 DNA probes, serum protein and red cell enzyme polymorphisms, exclusion probability >99%, or as referrals from other laboratories. Referral cases involved non-excluded fathers with PI<100 which required further testing. DNA was extacted using the non-toxic extraction method of (Miller et al. 1988). Probes were selected from Collaborative Research and Epitope and labeled with biotin using the ONCOR labeling kit. DNA was restricted with Pst I and electrophoresed 36 hours at 1.2 V/cm in 0.8 gm% agarose. Each sample was run on two separate gels to permit hybridization with different probes. DNA was blotted to Sureblot and detected using the non-isotopic alkaline phosphatase-streptavidin detection system of ONCOR. All paternity cases were run using the mother, child, alleged father and a child/alleged father mix.

Size markers were a mixture of lambda restricted with Hind III, Bst EII and Sph I. Population data provided by Collaborative Research, Epitope and our laboratory and generally ranged from 500-1200 individuals. PI values were calculated using a binning system where the frequency of the obligatory gene of the random

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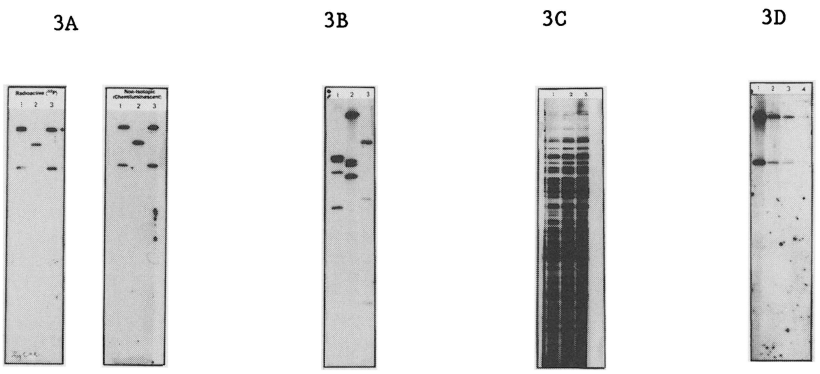


Fig. 3A. Comparison of ^{32}P with non-isotopic system. The non-isotopic system used the protocol described in Fig. 2. The same filter was subsequently probed with ^{32}P using the random hexamer method to label a purified 6.3 kb insert followed by overnight hybridisation and 6h film exposure. Probe MS43 was used.

Fig. 3B. Use of a cocktail of two single locus probes. The protocol was as described in Fig. 2 except that two probes were used simultaneously, each at 0.25 nM. Probes MS1 and MS31 were used. Tracks 1-3 represent different individuals.

Fig. 3C. A multilocus fingerprint using chemiluminescence. The protocol was as described in Fig. 2. Probe 33.6 was used. Tracks 1, 2 and 3 represent genomic DNA loadings of 2, 4 and 6 μg respectively.

Fig. 3D. Sensitivity with a single locus probe. The protocol was as described in Fig. 2 except the exposure time for the film was increased to 24h. Tracks 1-4 represent genomic DNA loadings of 500, 100, 50 and 10 ng respectively.

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We are grateful to the following scientists at ICI Diagnostics for their contributions to this project : J. Carlton, C. Arnold, D. Taylor, K. Bowness, N. Robertson and S. Little.

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CONCLUSION

We conclude that the system described exceeds the criteria listed above with superior sensitivity, simpler protocols, sharp bands, easy reprobing and faster processing time. We believe this technology will be the basis of the next generation of DNA fingerprinting tests.

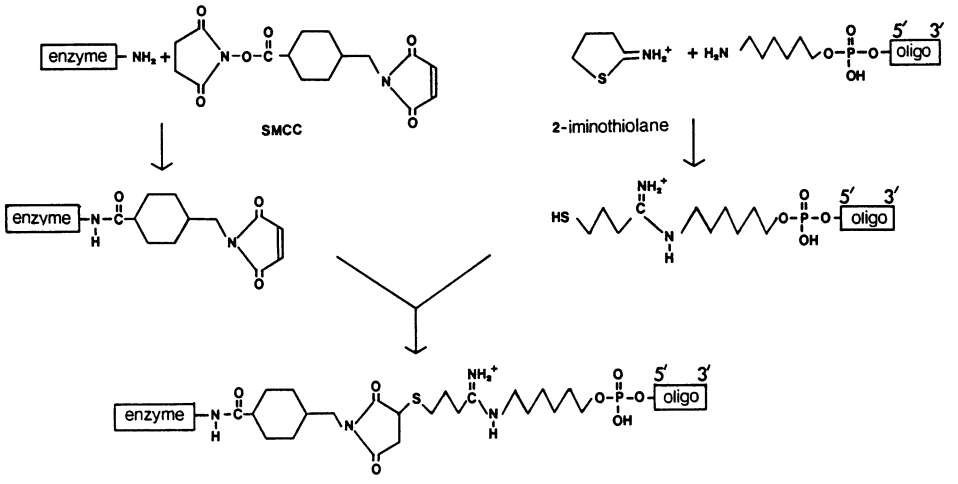


Fig. 1. Synthesis of alkaline phosphatase oligonucleotide conjugates. Aminoalkyl oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser using the reagent "Aminolink 2" (Applied Biosystems). After reaction with iminothiolane, the product was reacted with the maleimido-derivatised enzyme and the resulting conjugate purified by gel filtration on Biogel P-100F (Bio-Rad).

(1) Prepare Southern blot as standard with 2-5 μg Hinf1 digested genomic DNA per track
(2) Prehybridise for 1h at 50°C in 5 x Denhardt's, 5 x SSC, 1% SDS
(3) Hybridise in prehybridisation buffer containing 0.25 nM probe (@ 3 ng/ml) for 20 min at 50°C
(4) Wash 4 x 5 mins at 50°C in 1 x SSC, 1% SDS (multilocus probes) (for single locus probes the last 2 washes contained 0.25 x SSC)
(5) Wash 2 x 5 mins at room temperature in 1 x SSC
(6) Apply substrate, place filter in cassette with film and leave for 2 h at 37°C
(7) Develop film

Fig. 2. Hybridisation and signal development protocol.

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Rapid, Simple, Non-Isotopic Probing of Southern Blots for DNA Fingerprinting

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INTRODUCTION

DNA fingerprinting, as pioneered by ICI Cellmark Diagnostics using the Jeffreys' probes (Jeffreys et al. 1985, Wong et al. 1987) is a powerful technique for identification in paternity and forensic cases. The technology has previously been based upon ^{32}P. We have developed a non-isotopic alternative using a chemiluminescent enzyme substrate from Lumigen Inc (Schaap et al. 1989). The technology was designed to satisfy the following criteria:- (1) sensitivity - at least equivalent to ^{32}P, (2) simplicity - no additional procedural steps, ideally fewer, (3) sharp bands -to aid interpretation of profiles, (4) sequential probing - at least as convenient as ^{32}P, (5) speed - faster processing time desirable.

METHODS

The probes consisted of the enzyme alkaline phosphatase (as a label) coupled to the 5' end of synthetic oligonucleotides derived from the Jeffreys' minisatellite sequences. Probe synthesis is described in Figure 1. Stability for several months at 4°C has been achieved.

The hybridisation and signal development protocol is described in Figure 2. The rapid hybridisation was facilitated by a high concentration of single stranded probe. The temperature could not be raised significantly above 50°C without inactivating the enzyme label. The room temperature washes removed SDS which interfered with signal generation. Light emission continues for several days so the user is not committed to exposing the film immediately.

RESULTS

Identical results were obtained when conventional ^{32}P probes were compared with the system described above (Fig 3A). Probes could be combined as cocktails without backgrounds increasing (Fig 3B) and high quality multilocus profiles were obtained (Fig. 3C). Filters were able to be re-probed at least 7 times using hot SDS as the stripping agent. Preliminary results on casework filters were presented. By extending the exposure time to 24h with the single locus probe MS1, high molecular weight alleles were still detected with only 10 ng of genomic DNA (Fig 3D). For a 10 kb band in the 50 ng track this represents only 170 fg of target or 10 attomoles of enzyme (assuming 100% hybridisation). Note that in practice much less enzyme remained bound after washing. This was more sensitive than a conventional overnight hybridisation with a purified 4.6 kb insert labelled with ^{32}P by the random hexamer method followed by film exposure for 6 days.

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Fig. 3. The assembled components of a chemiluminescent identity test. Lane 1 contains the Lifecodes sizing standard. Lane 2 contains 1 μg of Pst I digested human genomic DNA. The blot was hybridized with alkaline phosphatase labeled oligonucleotides complementary to part of the sizing standard and to the D2S44 and D17S79 loci.

The Lifecodes ^{32}P identity test consists of two labeled probes and a set of molecular weight markers on each blot. Figure 3 shows preliminary results of an equivalent chemiluminescent test thus confirming that all the components of a ^{32}P identity test can be assembled into a chemiluminescent test. The extra bands in the middle of lane 2 have been shown to result from non-optimal stringency conditions for the membrane wash. This locus presented similar challenges while optimizing stringency conditions for the ^{32}P based procedure.

Three important advantages of the chemiluminescent detection system are that it requires few changes in the existing identity test format, the probes have been shown to be stable for >1 year, and it is extremely sensitive. VNTR signal can be detected from <40 ng of human genomic DNA which is the level required for forensic samples. Other non-isotopic systems work well for paternity testing (Dykes 1988) but do not have the required sensitivity for forensic casework.

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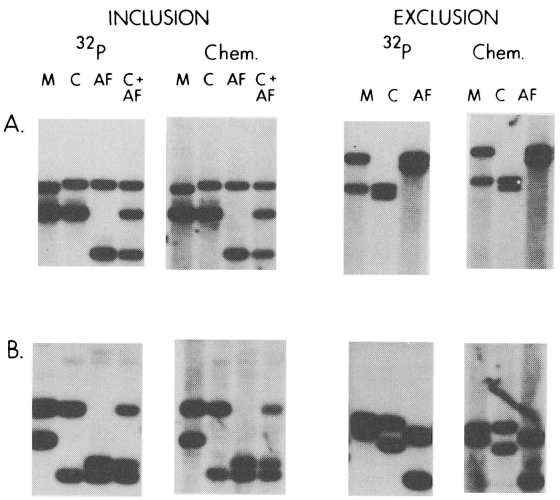


Fig. 1. A comparison of the ^{32}P and chemiluminescent detection systems for paternity identifications. One μg of DNA from each individual was digested with Pst I, separated on a 1% agarose gel, and transferred to an MSI Magna Nylon membrane. The DNA was from the mother (M), child (C), alleged father (AF), or a mixture of child plus alleged father (C+AF). The blots were hybridized with oligonucleotides to the D2S44 (A) and D17S79 (B) loci. Each of the cases was done on duplicate blots using either ^{32}P labeled probes or probes directly coupled to alkaline phosphatase (Chem.) as indicated. The alkaline phosphatase labeled probes were detected using a phosphorylated chemiluminescent substrate (Bronstein and Voyta 1989; Schaap et al. 1989). The ^{32}P detection system was exposed for 16 hours to x-ray film and the chemiluminescent system was exposed for 2 hours.

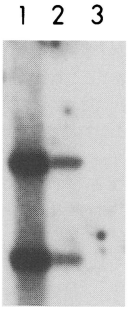


Fig. 2. A genomic DNA titration showing the sensitivity of the chemiluminescent detection system. 400 ng, 40 ng, and 4 ng of Hae III digested human genomic DNA (lanes 1-3) was separated on a 1% agarose gel and hybridized with alkaline phosphatase labeled probe to the D2S44 locus. The blot was exposed to x-ray film for 16 hours.

detected the VNTR sequences from 40 ng of DNA (Fig. 2) which is equivalent to the amount of DNA found in 1-2 μl of blood. On the original autoradiograph, the probe could weakly detect the VNTR pattern from 4 ng of DNA which is similar to the results obtained with ^{32}P labeled probes. This high sensitivity is required for forensic samples because they are often degraded.

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A Non-Isotopic DNA Detection System With the Sensitivity of ^{32}P: Applications For Paternity and Forensic Identifications

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INTRODUCTION

DNA blot hybridization is used for identity testing because the DNA of every individual (except for identical twins) is unique. In forensic casework where the amount of material is limited, a sensitive detection system such as ^{32}P must be employed to discern variable number of tandem repeat (VNTR) polymorphisms in the sample. However, ^{32}P labeled probes have numerous shortcomings including: a short biochemical half life, and the requirements for safety shielding, constant monitoring and a radioactive license. The short biochemical half life effectively means that fresh probe must be labeled once a week, thereby creating a possible quality control problem of lot-to-lot variation. To avoid these problems, we have developed a non-isotopic system for paternity and forensic identity testing.

EXPERIMENTAL AND DISCUSSION

The non-isotopic system utilizes an alkaline phosphatase conjugated DNA probe to hybridize specifically to VNTR's in the sample. The probe is detected using a phosphorylated 1,2-dioxetane chemiluminescent substrate (Bronstein and Voyta 1989; Schaap et al. 1989). When the substrate is dephosphorylated by the alkaline phosphatase labeled probe, light is emitted at 470 nm. The light is localized to the site of the reaction and can be detected on x-ray film. Light is steadily emitted by the enzyme/substrate reaction for at least 6 days (data not shown).

Alkaline phosphatase labeled probes in a chemiluminescent system detect the same VNTR sequences as equivalent probes labeled with ^{32}P (Fig. 1). An example of a paternity inclusion and an exclusion using two different probes is shown. In these cases, the chemiluminescent signal can be detected in 2 hours from 1 μg of genomic DNA with total hybridization and detection times of 3 to 5 hours. Similar results with a ^{32}P detection system require longer exposure times (Fig. 1).

The sensitivity of the chemiluminescent system was examined by hybridizing an alkaline phosphatase labeled probe to varying amounts of genomic DNA. The probe to the D2S44 locus easily

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DISCUSSION

The aim of this study was to see if VNTR polymorphisms from different electrophoresis can be compared. For this purpose the size of the fragments defined by pYNH24 were calculated by image processing using size markers. The results from 186 blood donors out of the 461 tested could be compared. The parallel analysis of the other 275 samples were not comparable because of a weak hybridization signal with the smallest fragment in the size marker. This problem could be solved with size markers where the number of copies for each fragment increases with decreasing size. However, the linear regression analysis of the 186 duplicates has a correlation good enough ($r = 0.999$) to make conclusions about samples tested on different electrophoresis.

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RESULTS

The genomic DNA reference was analyzed 41 times and the hybridization with pYNH24 gave two bands, one within the range of 7 211 - 7 638 bp and the other within 2 823 - 2 927 bp with means and standard deviations of 7 352 ± 96 and 2 864 ± 27 respectively. Thus the standard deviations were approximately 1 % of the size of the fragments.

In 186 DNA duplicates the fragments were compared by linear regression analysis and the correlation coefficient was r = 0.999. The scattergram of these duplicates is shown in Fig. 1.

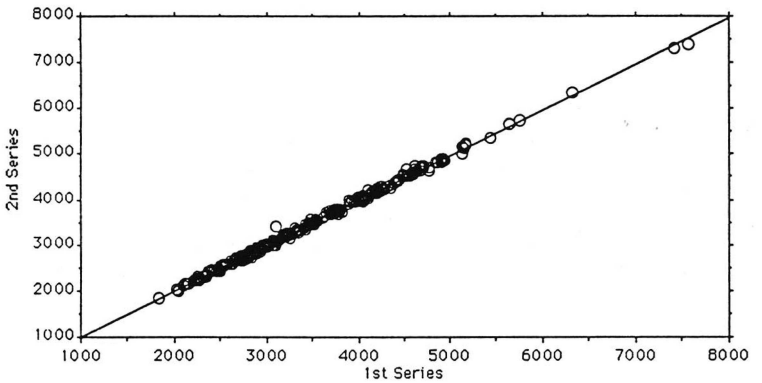


Fig. 1. Linear regression analysis of fragment sizes (bp) defined by pYNH24 from 186 DNA saples tested in parallel.

The allelic distribution was analyzed in 326 single tested samples. The bimodal distribution is shown in Fig. 2. 294 (90 %) of the tested individuals were heterozygous with two clearly separated bands. The most frequent "allele" had the size of 4 000 - 4 100 bp and a gene frequency of 0.077.

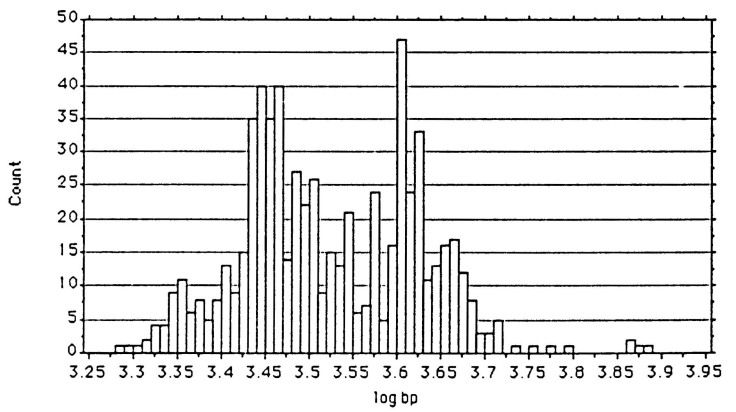


Fig. 2. Distribution of fragments (log bp) defined by pYNH24 in 326 blood donors.

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VNTR Polymorphism: Reproducibility in techniques and interpretation

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Variable number of tandem repeat (VNTR) markers, first described by Wyman and White (1980), are highly polymorphic DNA regions of great value for paternity and forensic investigations. The polymorphism is displayed by the relative migration of individual bands, alleles, according to the number of repeats. Some highly polymorphic VNTR systems include alleles so close to each other in size that they are difficult to distinguish in RFLP. In order to determine the technical reproducibility and the reliability of the interpretation of such systems duplicate DNA samples on parallel electrophoresis were analyzed with the probe pYNH24. The size of the fragments was calculated and the results compared.

MATERIALS AND METHODS

DNA was prepared from 461 blood donors according to the SDS-urea method described by Lindblom and Holmlund (1988). Two series of 3 ug DNA from each blood sample were cleaved with 6-9 units Hinf I (Boehringer Mannheim, West Germany) and the fragments separated in parallel electrophoresis. Four lanes with fragment size markers (10 ng of Hind III and 15 ng of Eco RV cleaved Lambda DNA) and one lane with a genomic DNA reference were included on each gel. The DNA was transferred to Zeta-Probe membranes (Bio-Rad Laboratories, USA) by vacuum blotting (VacuGene, Pharmacia-LKB, Sweden). The fragment size markers were visualized by adding 20 ng Lambda DNA to the probe pYNH24 (Nakamura 1987) before radioactive labelling. Hybridization and washes were performed under normal stringencies. The autoradiograms were analyzed by image processing using a computer system (LabEye DNAE, Innovative Vision AB, Linköping, Sweden), by which the size of the individual fragments was calculated according to the fragment size markers included. For these calculations four bands (9 416, 5 376, 2 884 and 1 921) in each size marker lane had to be clearly visible for the results to be included in the study. The distribution of "alleles" was analyzed using the logarithm of the number of base pairs (bp) calculated for each fragment.

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Extraction time being the same, the LGT agarose method has the advantage of involving less work-consuming steps, so that it is possible to handle many samples simultaneously. Since the band shifting is not that severe, the accuracy should be sufficient for certain clinical applications of DNA typing, although for forensic use even the smallest degree of band shifting has to be avoided or at least controlled. Till now this problem has not been satisfactorily solved. There are several improvements to be considered. A binning marker can be added to the samples as a control. To improve the mobility of DNA embedded in agarose variations of different electrophoretical parameters have to be tested. Using thinner slots already had a positive effect, and other possibilities are e.g. the gel concentration and the applied voltage. Another approach is the embedding of the material using insert moulds, and thereby avoiding the addition of enzymes and reagents, which may have an adverse effect on the mobility.

To summarize the results achieved so far, the LGT agarose method still has deficiencies in the quality of the resulting RFLP typing, but the quantity of recovered DNA is higher than that of conventional phenol/chloroform extraction.

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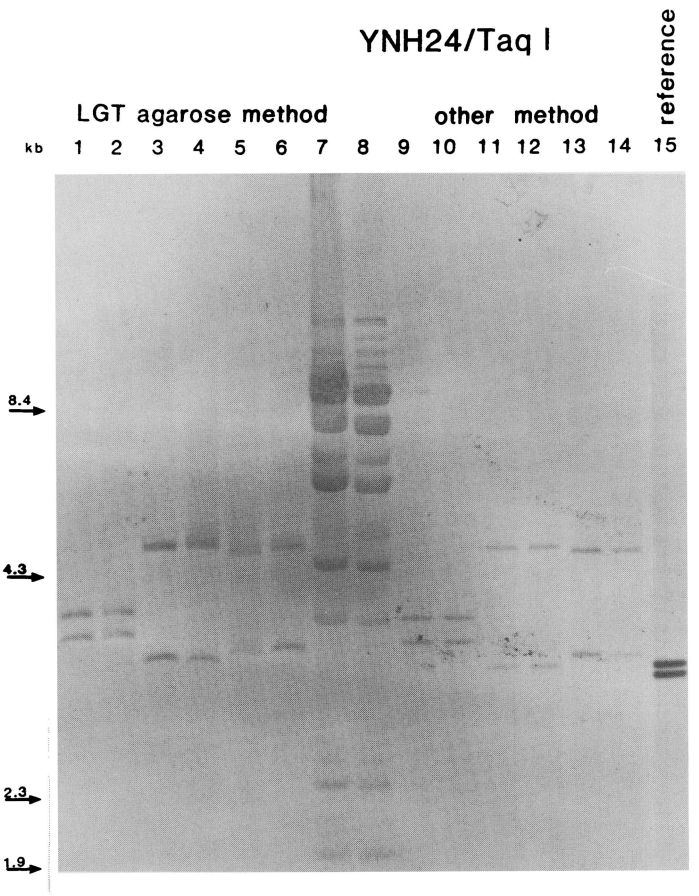
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Figure 2. Hybridization result of DNA extracted from 20 µl bloodstain on glass (lane 7,8 lambda DNA)



DISCUSSION

The comparison of two methods presented here confirmed the theoretical expectations concerning the quantity of DNA. The DNA extraction in LGT agarose showed higher yields. The rate of severe loss of DNA using the conventional method however, turned out to be lower than had been expected after the initial experiences with phenol/chloroform extraction of minute samples. This can be attributed to improved handling through practical experience and due to the introduction of modifications to increase the efficiency of DNA recovery, as adding glycogen as inert carrier to the ethanol precipitation and prolonged centrifugation times (Zeugin and Hartley 1985).

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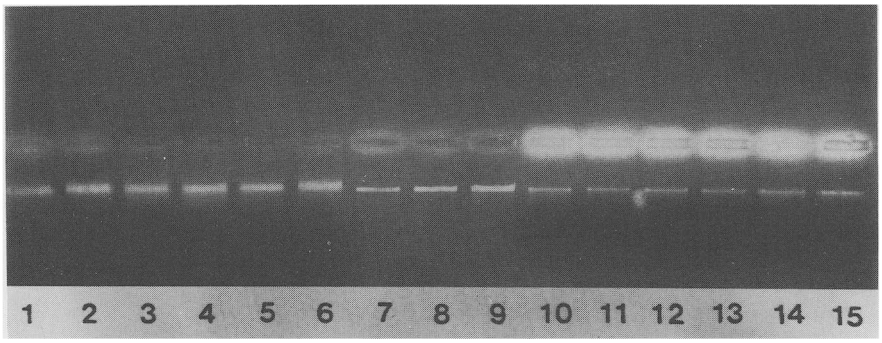


Figure 1. Concentration test gel for DNA extraction from 10 µl blood on cotton fabric, lane 1-6: LGT agarose samples, lane 7-9: 50, 100, 300 ng lambda DNA, lane 10-15: phenol extraction samples

The LGT samples were suitable for restriction enzyme digestion and RFLP typing. Different restriction enzymes show different degrees of sensitivity to the presence of LGT agarose in the reaction. While TaqI and HaeIII were unproblematical, the samples could not be completely digested with PstI, despite controlled dialysis (compare Feddersen and Van Ness 1989).

Compared to the conventionally extracted DNA samples on the same blot, the intensity of hybridization signals was increased for the LGT agarose samples (fig 2). For DNA concentrations close to the detection limit, which for the digoxigenin system is stated to be 0.1pg target sequence the rate of successful RFLP typing was increased in the LGT agarose samples. For 3 kb long DNA fragments, 0.1pg target sequence correspond to 200 ng of genomic DNA. Approximately this amount could be extracted from 1 hair root, 5 µl semen stains, and 10 µl blood stains. For 10 µl blood stains on cotton fabric it was for example possible to type all of 8 samples using the LGT agarose method and only 6 of 8 samples using phenol/chloroform extraction. Testing 4 samples of 5 µl semen stains on cloth and glass, hybridization signals were visible for all the LGT agarose samples and 3 of the conventional method.

A problem connected with DNA extraction in agarose is the occurrence of band shifting as on the membrane shown in fig. 2. The positions of the DNA fragments differ not only compared to the conventionally extracted samples (lane 1 and 2 to lane 9 and 10), but also between different extractions in LGT agarose (lane 5 to 6). To achieve more homogenous electrophoretic qualities two modifications have been tested. Firstly, the reaction volume for the restriction enzyme digestion was increased to lower the final agarose concentration of the sample. Secondly, a different slot-forming comb was used to get thinner slots. The first modification did not influenc the band shifting, while using the different comb had a positive effect.

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DNA extraction using phenol/chloroform: DNA was extracted according to Gill et al. (1985). The stain carrier was removed as above. DNA was precipitated by the addition of 0.1 volume of 4M lithiumchloride, 10 µg of glycogen (Boehringer Mannheim) and 2.5 volumes of ice cold ethanol (-20°C, overnight, 25 min,14000 xg). The samples were dialysed as described by Gill (1987).

The amounts of recovered DNA were estimated by comparison with ethidiumbromide stained lambda DNA markers of 20, 50, 100, 300, and 600 ng DNA after minigel electrophoresis.

Samples prepared by phenol/chloroform extraction were digested with 20 U of restriction enzyme (Hae III, Taq I and Pst I, Boehringer Mannheim) according to the instructions. The LGT agarose samples were melted at 65 °C after the dialysis buffer had been carefully removed. After adding reaction buffer and 40 U of restriction enzyme the samples were vortexed, briefly centrifuged, and incubated with the other samples. Before electrophoresis the LGT agarose samples were melted at 65 °C, 4 µl of gel loading buffer were added, and each sample was briefly vortexed, centrifuged and loaded onto the gel using a micro- pipette. Each slot was filled to the top with 0.6% melted LGT agarose. The samples were allowed to solidify for 5 min before the gel was placed in the electrophoresis chamber, and covered with TBE buffer. Electrophoresis was carried out for 20-28 h at 40 V. The DNA was blotted on nylonmembranes (Hybond N, Amersham) following the original method by Southern (1975).

DNA probes YNH24 (Nakamura et al,. 1987) and ps194 (Barker et al. 1987) were labelled with digoxigenin using the Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim) and hybridization was carried out following the Boehringer manual. Reaction was scaled up so that the final concentration of labelled probe in the hybridization mix was 200 ng/ml.

RESULTS

For comparison of the DNA yields the results from 45 sets (2 per set) of performed DNA extractions from bloodstains of various sizes and/or donors were evaluated. The amount of DNA that could be recovered using the LGT agarose method was in 24 cases higher, in 19 cases equal and in 2 cases lower than that of the conventional method. For both types of stain carrier the percental difference in yields was higher for the 10 and 20 µl stains. An example for a concentration test gel is shown in fig 1. The samples in lane 1-6 have been extracted using the LGT agarose method and show a distinctly higher intensity of fluorescence.

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DNA Extraction from small samples using a microextraction method with low gelling temperature (LGT) agarose

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The method of embedding biological material in low gelling temperature agarose, as is employed for pulsed field gel electrophoresis, does not require further transfer steps of the DNA, so that the unavoidable loss of DNA with the conventional methods is reduced to a great degree. Consequently, different authors have employed the LGT agarose method for working with minimal numbers of cells (Williams 1987; Mage et al. 1988; Feddersen and Van Ness 1989). After having presented technical modifications for the application on dried bloodstains (Prinz et al. 1989), our goal in this work was a quantitative and qualitative comparison of the LGT agarose method to the frequently used phenol extraction.

MATERIALS AND METHODS

1,5,10, and 20 µl amounts of blood, as well as 1,5, and 10 µl of semen were spotted on glass and cotton fabric, and after two days storage used for DNA extraction. Anagen hair roots were cut off freshly plucked hairs and DNA was isolated immediately.

DNA extraction using LGT agarose: Stains were shredded or scraped off and presoaked in 10-25 µl of 0.001M EDTA at 4°C, for 2h in 0.7 ml eppendorf tubes. A 2% low gelling temperature agarose (type VII, Sigma) solution was prepared in DNA-lysis buffer (0.05M TrisHCl pH 8.0, 0.01M EDTA, 0.5% sodium lauroyl sarcosinate) and after the solution cooled down to 50°C, 500 µg/ml proteinase K was added. 20-50 µl of this solution and 10-25 µl of double concentrated lysis buffer were given to the samples and thoroughly mixed in. DTT in a final concentration of 0.039M was added only to the semen samples. Incubation took place at 50°C for 2h, for hair root samples the incubation time was increased to 4h. For stains on cotton the stain carrier had to be removed prior to dialysis. This was achieved by melting the samples at 65°C, piercing the bottom of the tube and centrifuging the extraction liquid down to a second tube (5 min, 3000xg). The agarose was then allowed to solidify. Samples without stain carrier were briefly centrifuged and allowed to solidify on ice for 10 min. Dialysis was carried out by covering the agarose with 500 µl of cold TE buffer and incubation at 4°C for two days (6 buffer changes).

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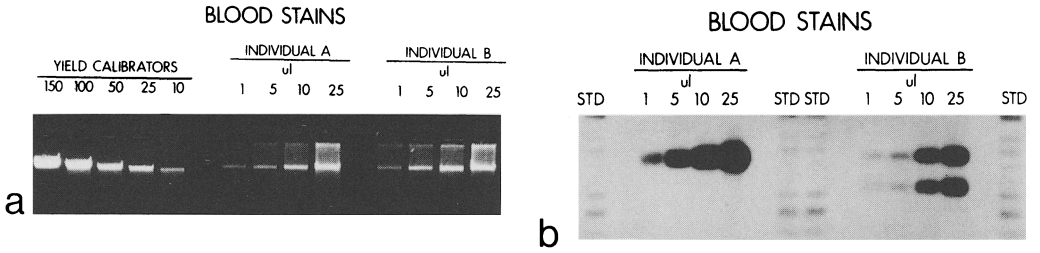


Fig. 1. (A) Isolation of high molecular weight DNA from bloodstains of two individuals. The DNA was fractionated in a 0.8% agarose gel containing ethidium bromide and photographed under UV light. The amount of sample loaded onto the gel ranged from 50% of the 1ul stain to 20% of the 5, 10 and 25 ul stains. (B) Southern blot analysis of DNA from bloodstains. The total amount of DNA obtained from each stain was digested with HaeIII and fractionated on a 1% agarose gel. Following Southern transfer, the blot was hybridized with an oligonucleotide probe localized to the human locus D2S44.

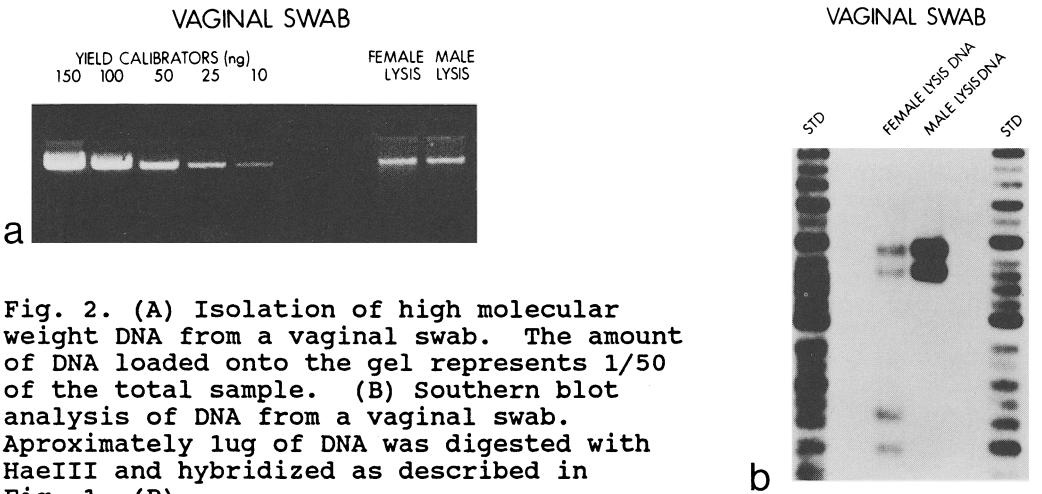


Fig. 2. (A) Isolation of high molecular weight DNA from a vaginal swab. The amount of DNA loaded onto the gel represents 1/50 of the total sample. (B) Southern blot analysis of DNA from a vaginal swab. Aproximately 1ug of DNA was digested with HaeIII and hybridized as described in Fig. 1. (B).

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The lysate was incubated on ice for 5 minutes (occasional vortex) and centrifuged 13,000 x g for 30 seconds. This process was repeated once more with CLB and then once with Protein Lysis Buffer (PLB, 10mM Tris-HCl pH8.0, 10mM Nacl, 10mM EDTA). Following the final centrifugation, residual PLB was removed from the tube by micropipetting. The fabric was then submerged in 40ul to 300ul (depending on size of fabric) of PLB containing 1mg/ml proteinase K. The tube was incubated at 65°C for 2 hours in a heatblock. Upon completion of the 2 hour incubation, the DNA lysate was removed from the fabric with a micropipet and transferred to a new tube.

Vaginal Swabs: The possibility of female cell contamination in samples obtained postcoitus necessitates a 2 step differential lysis to obtain DNA derived solely from sperm. The first lysis involves the isolation of female DNA obtained by incubating the fabric in a PLB/Proteinase K solution at 65°C for 2 hours as described above for bloodstains. Following the removal of the female DNA lysate, the fabric was washed 3 times with 1.5ml of PLB prewarmed to 65°C to remove trace amounts of female DNA from the fabric. At this point the sperm were still intact and could be lysed in a PLB/DTT/Proteinase K solution (PLB, 5mM DTT, 1mg/ml Proteinase K) at 65°C for 2 hours. Upon completion of the 2 hour incubation, the male DNA lysate was removed from the fabric.

RESULTS AND DISCUSSION

Several parameters are examined when DNA is recovered from evidentiary samples: 1) the quantity of DNA (yield), 2) the molecular weight of the DNA (integrity) and 3) the ability to digest the DNA with restriction enzymes. In order to obtain a reliable RFLP pattern from evidentiary samples an isolation procedure must be optimized for these three parameters.

Agarose gel electrophoresis indicates high molecular weight DNA was isolated from bloodstains 1-25ul in volume with total yields of approximately 25ng-1ug of DNA (Fig. 1A). Southern blot hybridization of this DNA indicates that the yield from all stains was sufficient for detection of RFLP patterns (Fig. 1B).

High molecular weight DNA was also obtained from the vaginal swab in both the female and male differential lysis steps (Fig. 2A). Southern blot hybridization demonstrates that the male lysate was free of contaminating female DNA. In contrast, the female lysate contained male DNA (Fig. 2B). This contamination was probably due to immature sperm which are less resistant to the female lysis step.

The recovery of DNA from biological samples requires that the DNA be of sufficient quantity and quality to perform RFLP analysis. Compared to existing methods, the DNA isolation technique described in this paper should significantly improve the recovery of DNA from evidentiary samples.

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A SIMPLE AND EFFICIENT NON-ORGANIC PROCEDURE FOR THE EXTRACTION OF DNA FROM EVIDENTIARY SAMPLES

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INTRODUCTION

The isolation of human genomic DNA from evidentiary samples is often time consuming and cumbersome. Existing methods typically involve the use of one or more of the following extraction techniques: digestion with a combination of Proteinase K and SDS or deproteination with organic reagents such as phenol and chloroform (Kanter 1986). Additional purification steps such as dialysis, precipitation with a saturated solution of NaCl and/or absolute ethanol are then required for enzymatic analysis (Miller 1988). The DNA isolation procedure described here is simple and efficient, eliminating the need for hazardous organic reagents or additional purification techniques.

This non-organic method for DNA isolation from evidentiary stains involves the incubation of the stained fabric with a high concentration of proteinase K at 65°C for 2 hours. It has previously been shown that proteinase K is at least seven fold more active on denatured protein and that prolonged incubation at high temperatures results in autoinactivation (Jeanpierre 1987). Following the 2 hour incubation the DNA extract can be used directly for restriction digestion.

This article describes the recovery of high molecular weight genomic DNA from dried blood stains and vaginal swabs and its subsequent use in determining RFLP patterns from stains as small as 1ul.

PREPARATION OF BLOOD STAINS AND VAGINAL SWABS

Blood stains were prepared from freshly drawn blood which was micropipetted onto white cotton cloth. Stain sizes of 1ul, 5ul, 10ul and 25ul were prepared, dried and stored at room temperature. Vaginal swabs were obtained from volunteers by swabbing the vagina approximately 10 minutes postcoitus. The cotton swabs were stored at -20°C until processing.

ISOLATION OF DNA

Blood Stains: Blood stained fabric was cut into small pieces (3x3mm) and placed into a 1.7ml microcentrifuge tube. Cells were lysed by the addition of 1ml ice cold cell lysis buffer (CLB, 0.32M sucrose, 10 mM Tris-HCL pH7.6, 5mM $MgCl_2$, 1% Triton X-100).

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The electrophoresis of the uncleaved DNA (not shown) as well as the distinct bands in the autoradiogram with a high signal to noise ratio in each lane show that this rapid method yields genomic DNA pure enough for RFLP analysis and fingerprinting.

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RESULTS

The results from 101 DNA preparations are given in Table 1.

Table 1. A 260 / A 280 ratios and the DNA yields in 101 samples prepared on nine different occasions.

Series	n	A 260 / A 280	ug DNA / ml blood
1	12	1.7 ± 0.03	9.5 ± 2.1
2	12	1.7 ± 0.11	8.4 ± 4.2
3	16	1.8 ± 0.06	10.7 ± 3.6
4	16	1.5 ± 0.05	11.4 ± 3.0
5	14	1.5 ± 0.19	8.2 ± 4.2
6	5	1.6 ± 0.08	7.7 ± 3.3
7	10	1.8 ± 0.07	12.2 ± 3.7
8	7	1.6 ± 0.05	10.5 ± 2.8
9	9	1.8 ± 0.06	7.4 ± 2.4
Total	101	1.7 ± 0.15	9.7 ± 3.7

The uncleaved DNA in each preparation has also been tested by electrophoresis and ethidium bromide staining. For RFLP analysis 3 ug DNA were cleaved with Hinf I (Boehringer Mannheim, West Germany) and the fragments separated by electrophoresis on a 20 x 20 cm 0.7 % agaros gel. After transfer to Zeta-Probe blotting membranes with a VacuGene Vacuum Blotting System (Pharmacia-LKB, Sweden) the membranes were hybridized with the VNTR probe pYNH24 (Nakamura 1987) kindly provided by Dr Y. Nakamura (Howard Hughes Medical Institute, Salt Lake City, USA). An autoradiogram of such a membrane with DNA from 16 blood donors and with four size marker lanes is shown in Fig. 1.

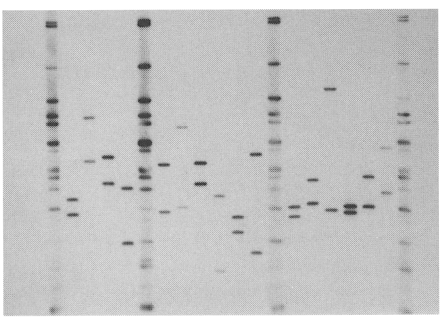


Fig. 1. RFLP with size markers of DNA from 16 blood donors cleaved with Hinf I and hybridized with pYNH24

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Rapid Preparation of DNA for RFLP Analysis and DNA Fingerprinting

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The standard procedure for preparation of eucaryotic DNA includes several time consuming steps such as digestion of proteins with proteinase K and subsequent extractions of aminoacids and protein fragments with phenol (Maniatis 1982). The method described here is based on protein disintegration with SDS-urea (Meinke 1974) instead of proteinase K.

The procedure is fully described by Lindblom and Holmlund (1988) and is shortly as follows: Five milliliters of blood samples collected in vacutainer tubes containing EDTA are lysed with a sucrose Triton X-100 buffer, pH 7.5 (1 % Triton X-100 in 320 mM sucrose, 1 mM Tris-HCl and 5 mM $MgCl_2$). The cell nuclei are pelleted by centrifugation for 15 min at 2 000 g at 4°C and washed once with the same buffer. The proteins are disintegrated with SDS-urea, pH 6.8 (2 % SDS in 8 M urea, 240 mM Na_2HPO_4 and 1 mM EDTA) by incubation at room temperature for 15 min. Most of the proteins are extracted by the addition of 700 ul phenol: chloroform:isoamylalcohol, in the volume ratio 25:24:1, and a separation of phases by centrifugation (10 000 g, 5 min, 1°C). 500 ul of the DNA phase is further purified from remaining proteins and SDS-urea by gel chromatography on Sephadex G25 (Nap-5 or Nap-10 columns, Pharmacia, Sweden). To avoid any contamination the DNA is eluted from the Nap-5 columns with 700 ul 1 x TE buffer, pH 7.5 (10 mM Tris-HCl and 1 mM EDTA) and from the Nap-10 columns with 1.10 ml. The elution buffer is easily adjusted to the salt concentrations recommended for any restriction enzyme and the DNA is ready to be cleaved within a few hours from start.

For RFLP analysis and DNA fingerprinting the DNA is cleaved over night with 2-3 units enzyme per ug in a total volume of 200 ul enzyme buffer. The fragments are precipitated in ethanol (20 ul 3 M NaAc and 400 ul 99.6 % ethanol) by centrifugation for 15 minutes at 10 000 g at 1°C. The ethanol is carefully decanted and the DNA dried by vacuum desiccation for a few minutes. A few ul of electrophoresis buffer 1 x TBE, pH 8.3 (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA) is added to each sample and the DNA is dissolved by agitation at room temperature for one hour.

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The blood-group and DNA findings produced the following W values:

	real father is legal father	real father is uncle by marriage	real father is unknown man
blood group opinion	15%	3%	82%
DNA analysis: 2 single-locus probes	16,7%	16,7%	66,6%
DNA analysis: multilocus probe 33.15	0,2%	1,2%	98,6%
combined:	0,008%	0,01%	99,98%

The combined value for the findings of the blood-group opinion and those of the 2 single-locus probes is W = 90%, that is, it is 'probable' that an unknown man is the child's real father. The multilocus probe 33.15 brought the real breakthrough. With the combined W value of 99,98% it is 'practically proven' that an unknown man is the child's real father, and 'practically excluded' that his legal father or his uncle by marriage is.

I should like to very briefly explain the mathematics of the biostatistical evaluation. The blood-group findings as well as the radiograms with the two single-locus probes were evaluated with an extended version of the kinship algorithm Ihm and I developed in 1975 (that is, 14 years ago). This gives the frequencies for the 3 pedigrees. The ratios between them give the probability values for the three hypotheses; the sum of these values is 1 (= 100%).

In principle, the fingerprints obtained with the multilocus probe 33.15 are treated in the same way. However, there is one pecularity: the pherograms gave 12 different band constellations for the five people in the case, with frequencies of occurence between 1 and 8. A 13th constellation was calculated theoretically with n = 33. Each of the 13 band constellations gives a probability value for each of the 3 hypotheses.If a constellation occurs more than once (in the present case: up to 33 times), this affects the probabilities of the hypotheses accordingly. Combining the probabilities obtained from all 13 fingerprints, we get W = 0,2% for the hypothesis 'the legal father is the real father of the child', W = 1,2% for the hypothesis 'the uncle by marriage is the real father of the child', and W = 98,6% for the hypothesis 'an unknown man is the real father of the child'.

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using a mean band frequency, one calculates the frequency of the pedigrees. In the 2-hypotheses case these are f(X) and f(Y). They are inserted in the Essen-Möller formula:

$$W = \frac{1}{1+[f(Y)/f(X)]}$$

which gives the appropriate probability of paternity, maternity, grandmaternity, grandpaternity or sibship. In a case of disputed grandmaternity (= mother of the deceased putative father), to which the multilocus probe 33.15 was applied, the biostatistical result was a plausibility of W = 99,2%. The value speaks very much in favour of real grandmaternity (predicate: 'highly probable'). Moreover, this value is extremely typical of real grandmothers for the given child-mother constellation: their mean value is W = 77%, that is, well below 99,2% (false grandmothers have a mean of W = 29%).

The scatter of the W values for real grandmothers in the present case lies between W = 77% and at least W = 99,2%; in the other direction values of less than W = 50% may be expected. Hence, only high W values clearly indicate kinship; low values speak neither for nor against.

The correctness of this evaluation of W values in deficiency cases was borne out of 190 cases of disputed paternity without findings for the mother. In 95 cases the real father was involved, and in 95 cases a non-father. In 125 analogous cases blood-group and HLA-A,B findings were also available. Practically all non-fathers were excluded from paternity in the blood-group or - more often - the HLA opinion. However, there was not a single exclusion with the multilocus probe, only probability values. Most of these were below 50%; those in the positive range were as high as W = 90% and above. The distribution of the W values for real fathers from blood-group and HLA findings is similar to those for the DNA analysis with the probe 33.15. However, closer examination reveals that the serological expertise provides values in the highest W-values range, and hardly any W values below 50%. For the multilocus probe 33.15, on the other hand, W values for real fathers of below 10% cannot be excluded, and there are fewer results in highest W value classes than for the blood-group and HLA opinions.

The following is an example of a relatively complicated deficiency case - a 2-man case.

A child - now adult - doubts whether his deceased legal father is his real father. Two men are eligible for paternity: his uncle by marriage, previously married to his mother's deceased sister, and an unknown man. Blood-group findings and DNA pherograms are available for 5 persons:

- for the child;
- for his brother (whose parentage is not in dispute);
- for their mother;
- for the daughter of the mother's deceased sister and the uncle by marriage; and
- for the daughter of the mother's deceased sister and another man.

There are three alternatives for the child's paternity (= 3 hypotheses):

1. the child's father is the legal father;
2. the child's father is his uncle by marriage;
3. the child's father is an unknown man.

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Kinship plausibilities from DNA fingerprints

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In our previous paper on the biostatistical evaluation of multilocus pherograms for normal mother-child-?father cases we dealt with two points we should like to apply to a practical question in this paper: firstly, how to determine both the total number of bands, visible and invisible, recorded by the probe and their mean frequency with the aid of the maximum likelihood technique, and, secondly, how to calculate a probability of paternity using the Essen-Möller formula, assuming that every band belongs to a corresponding di-allele system with a silent antithetical property and further that each is genetically independent. With these two steps we can biometrically evaluate pherograms of multilocus probes in deficiency cases, that is, in cases without findings either for the mother or for the putative father (though findings for close kin may compensate). For an exclusion expectation in deficiency cases one must have findings for both parents of either the child's mother or the putative father. Only in this case can a non-father be excluded without any reservations, in other words, will the W_A value (= probability of paternity for non-excludable men for a given child-mother constellation) be fully valid. In practically all other cases proof of non-kinship through "exclusion" (= genetic incompatibility) is either rare or, as the following cases, impossible:

- questionable paternity without findings for the mother;
- questionable maternity without findings for the father;
- questionable paternity without findings for the putative father, but with findings for his siblings and/or real children;
- questionable grandmaternity without findings for the putative father and the putative grandfather;
- questionable grandpaternity without findings for the putative father and the putative grandmother; and
- disputed full or half-sibship without findings for one or both of the parents.

In all these cases only biostatistics can provide positive or negative proof. To establish parentage a second or third probe may be necessary in some cases. The biostatistical results from the probes are combined with one another and with the results of the blood-group and HLA opinions. This gives an overall W value for the case in question.

As a rule, deficiency cases are 2-hypotheses cases: the allegation is true or not. However, there are also cases with more than two hypotheses, e.g. when neither the one nor the other hypothesis, but a third possibility is correct (= 3-hypotheses case). For every hypothesis there is a pedigree. With a special kinship algorithm, and

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$m_0 = 57$
$m_1 = 23$
$m_2 = 11$
$m_3 = 3$
$m = 94$

Using the maximum-likelihood method, the total number of visible and invisible bands recorded by the probe 33.15 is $N = 71$

with a mean band frequency of $a = 0.11$.

One could similarly run the other 5 unequivocal M-C-PF constellations. Of these, the only really significant one is the constellation + - + because in it, as in constellation + + +, one band occurs in two non-related individuals.

Once the maximum number of bands recorded by the probe is known by 71 it is not difficult to calculate the frequency of the constellation - - - for the individual case: $n_7 = N-n$.

Using a = 0.11, the formula for W_{EM} will give Essen-Möller's probability of paternity for a M-C-PF constellation. W_{EM}% tells one how accurate one could be if in 100 serologically analogous cases (= cases with the same fingerprints) one would categorically decide that in every case the putative father was the real F of the C. 100-W% would give the expectation of error for such a categorical decision.

Obviously, the calculation of W_{EM} presupposes formal genetics of band heredity. Regardless of features, some experts regard all formal genetics for multilocus band patterns as arbitrary. This explains why they hesitate to calculate Essen-Möller probabilities from multilocus probes.

Instead of the "general" solution (using W_{EM}), one can choose a "limited" solution. This is based on the M-C-PF constellation - + + alone. The likelihood ratio Y/X of a single - + + constellations has the value a (= mean band frequency). If n such constellations appear in the pherograms, then $Y/X(n) = a^n$.

Substituting in a formula based on the Essen-Möller principle gives a probability of paternity

$$W = \frac{1}{1+Y/X} = \frac{1}{1+a^n}.$$

The subscript A (in W_A) signifies that W_A is not based on all band constellations (like W_{EM}) but only on those which could produce an exclusion in the event of non-paternity (that is, on the M-C constellation - +). W_A(%) tells one how often one would be accurate if in 100 "analogous" cases - that is, cases with the same number of - + M-C constellations - one would regard all non-excludable men as real fathers; 100-W_A(%) would be the quota of error for such a categorical decision.

In most cases W_A is less than W_{EM} because W_A omits the M-C-PF constellation - - - that also strengthens the positive indication. The "advantage" of W_A is that it is based only on the principle that the child must have inherited from the PF a certain number of bands. There are no other, least of all formal genetic, prerequisites for W_A. The disadvantage of W_A is that its application is restricted to trios (normal as well as incest trios) and grandparental cases. If the exclusion expectation for non-kinship is very low or even zero - which is almost always the case in deficiency cases - the value for W_A will not be very helpful. In such cases only the probability value W_{EM} can provide useful information. - Our computer print-outs for trios and grandparental cases include both W_{EM} and W_A.

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Essen-Möller's probability of paternity is then calculated as follows:

$$W_{EM} = 1/(1+(f_1^{n_1} \cdot f_2^{n_2} \cdot f_3^{n_3} \cdot f_4^{n_4} \cdot f_5^{n_5} \cdot f_6^{n_6} \cdot f_7^{n_7}))$$

where $n_1 \ldots n_7$ denote the respective "frequencies" of the different band constellations found.

To obtain a W_{EM} value in the individual case with the formula given above one must know

1. the typical mean band frequency for the probe, a; and
2. the total number N of visible and invisible bands recorded by the probe in question.

The values a and N characterize a multilocus probe sufficiently for the biostatistical evaluation of band patterns. These values are determined from the band constellations of, say, 100 M-C-PF cases ("families"). The method is as follows:

We proceed from the M-C-PF constellation + + +. The phenotype is based on genotype M-C-PF constellations with the overall frequency

$$p = x^2(3-2x),$$

where x = mean frequency of the "alleles" coding the bands.

We regard the appearance of the band constellation + + + for a M-C-PF trio as a "success" in a Bernoulli Test. The probability of this success is p. The number of times + + + constellation appears for a M-C-PF trio - null, one, two, three and more times - is given by the probabilities P(0), P(1), P(2), P(≧3).

Then, for P(i):

$$P(0) = (1-p)^N$$
$$P(1) = N \cdot p(1-p)^{N-1}$$
$$P(2) = 1/2\,(N(N-1)) \cdot p^2(1-p)^{N-2}$$
$$P(\geq 3) = 1-(P(0)+P(1)+P(2)).$$

Among m real M-C-PF trios the + + + constellation may not appear, m_0, appear once, m_1, twice, m_2, or three or more times, m_3;

$$m = m_0+m_1+m_2+m_3.$$

The probability of this series is proportional to

$$L = P(0)^{m_0} \cdot P(1)^{m_1} \cdot P(2)^{m_2} \cdot P(\geq 3)^{m_3}.$$

If M denotes the mean number of visible bands per person in the pherograms of n M-C-PF trios then

$$M = a \cdot N.$$

M can be determined from readings of the pherograms without difficulty; if p is known, the equation $p = x^2(3-2x)$ can be solved for a:

$$a = x(2-x).$$

One uses the maximum-likelihood method to obtain parameter p, solving for that value that will maximize the likelihood function:

$$\ln L = m_0 \cdot \ln P(0)+m_1 \cdot \ln P(1)+m_2 \cdot \ln P(2)+m_3 \cdot \ln P(\geq 3).$$

Using probe 33.15 on 94 M-C-PF trios gave the following results:

The mean number of the visible bands per person was 7.78 (M = 7.78). The frequencies of the + + + constellation were

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Biostatistical approaches using minisatellite DNA patterns in paternity cases (mother-child-putative-father trios)

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The calculation of probabilities of paternity from fingerprints of a multilocus probe is relatively simple in two groups of cases: normal mother-child-putative-father (M-C-PF) trios, and cases where findings for the mother or for the PF are lacking but findings for their respective parents are available. Cases where there is no exclusion also lend themselves to biostatistical evaluation. A M-C-PF trio has 6 genetically compatible fingerprint patterns; one determines their frequencies from the pherograms by counting. If each visible band is regarded as a detectable property of a di-allele system (heterozygous or homozygous), than an invisible band represents the homozygous phenotype of the system's silent property.

In Essen-Möller's formula for the probability of paternity, $W = \frac{1}{1+Y/X}$,

X is the frequency of the trios with the real father,
Y with the false father.

Taking $\underline{a}$ is the mean band frequency, the following formulas are to use to calculate the likelihood ratios for the 6 unequivocal M-C-PF band constellations:

running no.	M C PF	Y/X	
1	- + +	a	$= f_1$
2	+ + +	$a(a+\sqrt{1-a})/(2a+\sqrt{1-a}\ -1)$	$= f_2$
3	+ + -	$a+\sqrt{1-a}$	$= f_3$
4	+ - -	$\sqrt{1-a}$	$= f_4$
5	- - +	$1+\sqrt{1-a}$	$= f_5$
6	+ - +	$1+\sqrt{1-a}$	$= f_6$

Furthermore it is possible that all three persons are homozygous in respect of the silent antithetic "allele" which means there is a 7th constellation which cannot be quantitatively determined from the pherogram:

7	- - -	$\sqrt{1-a}$	$= f_7$

To obtain it, one subtracts the number of obvious constellations ($n = n_1+n_2+n_3+n_4+n_5+n_6$) from the total of all the visible and invisible bands recorded by the probe (= N).

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For the real data the discrimination power of the likelihood ratio approach was not as good as that of the bandsharing method. The smallest ratio calculated under paternity was 9.55, but 3 (3.4%) of the likelihood ratios with non-fathers were higher. The highest ratio under non-paternity was 97.1 and 9 (8.4%) of the ratios under paternity were smaler.

Table 3. Distribution of likelihood ratios (real data)

paternity	n	mean	minimum	maximum	> 1	< 1
yes	107	1.1×10^{4}	9.6×10^{0}	4.2×10^{8}	107	0
no	87	4.2×10^{-2}	1.1×10^{-5}	9.7×10^{1}	19	68

For the simulated data our modified likelihood approach seems to be better than the bandsharing. The smallest value under paternity was 6.31×10^{-3} and 46 (0.9%) likelihood ratios for non-fathers were higher. The maximum ratio for non-father trios was 2.9×10^{2} and 1272 (25.4%) of the ratios under paternity were smaller.

Table 4. Distribution of likelihood ratios (simulated data)

paternity	n	mean	minimum	maximum	> 1	< 1
yes	5000	5.0×10^{3}	6.3×10^{-3}	6.4×10^{10}	4919	81
no	5000	6.9×10^{-8}	6.8×10^{-16}	2.9×10^{2}	2	4998

DISCUSSION

The discrimination power of both methods seems to be very good. The better results for the real data in comparison to the simulated data may be due to the construction of trios out of the same families, which means dependence of bandsharing rates and likelihood ratios. The results for the two methods are similar. The cutpoint of likelihood ratio 1 is not an optimal cutpoint as chosen for the bandsharing. For both methods one should use a region of uncertainty, where no decision is made. These results do not confirm the calculated likelihood ratios. They only show, that the qualitative decision is correct in most cases.

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product of all possible band positions. It is based on the level of band frequencies and the probability to inherit a specific band. This requires several assumption for the genetic model. Independent treatment of the different band positions requires linkage equilibrium, the absence of linkage and no allelism. To derive the transmission probabilities for the bands from the bandfrequencies, the assumption of Hardy-Weinberg equilibrium is necessary. Additionally we assumed, that the band frequencies are equal for all band positions. Eight different combinations of having a band or not are possible for mother, child and alleged father. In their paper Evett et al. did not consider the combination: child has a band, which mother and father do not have. If the alleged father is the true father, this has to be interpreted as a mutation. Because in our real data this kind of "mutation" occurred quite often (6% of the children), we modified Evett's procedure allowing for this band combination and taking it into account with an estimated probability for mutation. Another critical point of the method as proposed by Evett is the use of the band combinations with no band either for mother, alleged father or child, as an suggestion for paternity. This means, by increasing the number of "possible" band positions the likelihood ratio changes towards paternity. Because the number of possible band positions usually is unknown we decided not to use the information from such band positions. This results probably in an underestimation of the "true" likelihoodratio. To get a reliable estimate of the band frequencies we subdivided the range of occurring kb-value in 100 equal sized intervals for the real data as well as for the simulated data.

RESULTS

The distributions of bandsharing rates for the real data (paternity, non-paternity) were only scarcely overlapping. Only two bandsharing values under non-paternity (44.5% and 45.7%) were larger than the minimal bandsharing under paternity (44.2%). An optimal cutpoint for this data may be found 45%.

Table 1. Distribution of bandsharing (real data)

paternity	n	mean	minimum	maximum	> 45%	< 45%
yes	107	65.4%	44.2%	80.0%	106	1
no	87	18.6%	0.0%	45.7%	1	86

For the simulated dataset the result is different. The smallest bandsharing value for true fathers was 25.1%. 1330 trios (26.6%) with non-fathers were highger than this value. The highest value under non paternity was 45.3%. 262 (5.2%) of the bandsharing values under paternity were smaller than this value. An optimal cutpoint may be found with 39%.

Table 2. Distribution of bandsharing (simulated data)

paternity	n	mean	minimum	maximum	> 39%	< 39%
yes	5000	58.0%	25.1%	81.6%	4944	56
no	5000	20.9%	0.0%	45.3%	11	4989

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Likelihood Calculations in Paternity Testing on the Basis of DNA-Fingerprints

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INTRODUCTION

DNA-fingerprints seem to be a powerful tool in paternity testing. But the quantification of the results in terms of likelihood and likelihood ratios is a problem, because in most cases the correct genetic model and its parameters are not known. Two approaches have been suggested to circumvent these problems. The use of band sharing rates to distinguish between pairs of relatives and pairs of unrelated individuals, and the calculation of likelihood ratios on the basis of simplifying assumptions. The first approach reduces the available genetic evidence to "phenotypic" similarities. The second one makes unjustified simplifying assumptions. These two decision strategies have to be examined with respect to their correctness. We applied both strategies to a set of real data and to a set of simulated data. The bandsharing rates and likelihood ratios for paternity were calculated for mother/child/father and for mother/child/non-father combinations, and the distributions were compared.

DATASETS

The real dataset consists of 27 families which were typed for a multilocus probe (Zischler, 1989). The probe was $(CAC)_5$ / $(GTG)_5$, the restriction enzyme was Hinf I. From these 27 families with 107 children we obtained 107 mother/child/father trios. By substituting one of the parents by an unrelated individual typed on the same blot, we got 87 mother/child/non-father trios out of 25 families.

Additionally we simulated two (father / non-father) sets of 5000 trios each. We allowed 200 band positions for 40 independent loci, which produced visible bands (bands within these 200 positions) with a probability of about 50%. The allele frequencies were chosen to be unequal.

METHODS

As stated above, we tested two different strategies. The first one was based on bandsharing. The bandsharing for a pair of individuals (here alleged father and child) was calculated as the average of the relative number of mutually shared bands for both individuals. The bandsharing was calculated for all possible combinations father/child and non-father/child in the four datasets.

The likelihood calculation was done according to a modified version of the method of Evett (Evett et al., 1989). This method calculates a likelihood ratio (paternity / non-paternity) independently for each possible band position. The total likelihood is the

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modification with the Quattro spreadsheet program. A template such as this could also be used in conjunction with templates designed to perform statistical analysis.

While dedicated software could probably perform analysis of multi-allelic systems more expediently than this template, these experiments demonstrate that analysis of small amounts of data can be accomplished using a well designed spreadsheet template and "state of the art" microcomputer system.

Table 1. Time studies of Macro vs Data Table function for determining phenotypes.[a]

		Lotus 123™				Quattro™	
		-Speed-up		+Speed-up			
Computer Model	Table[b] Size	Macro	Data Table	Macro	Data Table	Macro	Data Table
80286,10	4x4	:04	:47	:04	:08	:10	:07
	7x7	:10	2:17	:10	:19	:28	:15
	26x26	2:22	30:39	2:22	5:12	5:53	4:21
	66x66	14:58	114:05	14:59	51:21	52:20	39:31
80386,20	4x4	:01	:10	:01	:02	:04	:04
	7x7	:03	:29	:04	:06	:10	:07
	26x26	:49	6:47	:49	1:35	2:20	2:02
	66x66	5:13	46:46	5:12	21:25	14:14	8:26

[a] Times are shown in Min:Sec.
[b] Table sizes represent a 4, 7, 26 and 66 allele system respectively.

Table 2. Time for calculating frequencies of 500 and 1000 records using 3 computers.[a]

	Computer Models					
	8088-4.77MHz		80286-10MHz		80386-20MHz	
	500	1000	500	1000	500	1000
Gene frequencies[b]	:17	:47	:10	:12	:04	:06
Phenotypes:						
4 alleles	:13	:23	:04	:06	:01	:02
7 alleles	:37	1:01	:10	:16	:04	:08
26 alleles	4:40	16:00	2:22	3:30	:49	1:22
66 alleles	26:57	95:31	14:59	20:35	5:12	7:16

[a] Times are shown in Min:Sec.
[b] Time given is the total time to calculate 4 systems with 4, 7, 26 and 66 alleles.

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Use of a Spreadsheet Template and Microcomputer to Calculate Gene Frequencies of Multi-allele Systems

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INTRODUCTION

Using Lotus 123™, we have developed a macro driven template with simplified data entry and menu driven functions to analyze population data for multi-allelic systems. Four systems, with 4, 7, 26 and 66 alleles respectively were evaluated. In designing the template, various approaches of analyzing data were evaluated to determine the best method for giving expedient results and using the least amount of memory.

METHODS

Timed studies were run on sample data using two versions of Lotus - with and without speed-up, and the Quattro™ program. The built-in Data Distribution function was used to count allele numbers and gene frequency was calculated using cell formulas. Two methods of determining the frequency of phenotypes for the 4 DNA systems were evaluated. Each system was set-up in a two-way table format, then calculated, using the program's built-in Data Table function and with a programmed macro using the spreadsheet's command language. The same formula, @DCOUNT(DATABASE,OFFSET,CRITERIA), was used for both methods. Both spreadsheet programs and methods were initially tested on two microcomputers, each having a different central processing unit (CPU) and speed.

RESULTS

Results of the timed studies on the above variables are shown in Table 1. When using Lotus 123™ the programmed macro was considerably faster than the program's internal Data Table function on both models of computers tested. Quattro™ produced results more rapidly with the internal Data Table function versus the programmed macro, but was still slower than the faster method using Lotus.

Table 2 shows a comparison of time tests using the final template design and Lotus 123™ with speed-up, to process a 500 and 1000 record database on three computer models. As would be expected, the more powerful computer is able to complete the task in a more expedient manner.

SUMMARY

The program chosen to process the final template was Lotus 123™ with speed-up. The Data Distribution function and cell formulas were used to determine gene frequencies. Macro driven commands were used to determine phenotype frequencies using a two-way table format. The template incorporates custom menus enabling persons with minimal computer experience to enter data, perform calculations and generate printed reports. The template will accommodate up to 2000 records using conventional memory (640K). Additional records can be added by using expanded memory. Although the template can be used on a PC (8088,4.77MHz) computer, a more powerful model processes records in a much faster time. The template will also run with-out

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estimated by σ^2_ϵ, and $\hat{\sigma}^2_\epsilon$ = .00041 (again, ≈2% of fragment size) with a 95% CI of (.00037,.00045).

CONCLUSIONS

Using D14S1 replicates, we have illustrated a method of determining the size of random measurement error for an RFLP system. Our rather crude (ruling) measuring technique for DNA bands produced an error rate of approximately 2% of band size. Further refinements in measuring will reduce these errors, but determining their size is necessary for assessing probabilities of identification and parentage in criminal and civil disputes.

Systematical variability between gel lanes was controlled by translating all measurements to the left-most standard lane via 2-dimensional linear regression with the right-most standard lane. Based on our same-subject replicate data, a linear function appears appropriate when both standard lanes are proximal to unknowns. Recently, for reasons of possible degradation of heterozygotes and observed isolated lane irregularities, Landers (1989) and Morris (1989) have suggested that positive controls be placed in each lane bracketing the region of mobility to improve quality control. With such controls in place, translating measurements has even simpler form (set x=1 in the formula [2] for y^*).

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Table 2. Estimates of variance components for D14S1 fragments (log scale), N = 99

$\hat{\sigma}^2_{subj}$	= .00625	
$\hat{\sigma}^2_{tech}$	= .00000	
$\hat{\sigma}^2_{aliq}$	= .00004	
$\hat{\sigma}^2_{reps}$	= .00031	s.e. ≈ .02
$\hat{\sigma}^2_{\epsilon}$	= .00009	

Actual true D14S1 allele size plus error constituted the underlying model for an observed fragment size y (log scale). Further, the model was decomposed into overall (μ), subject (S), aliquot (A), replicate (R), technician (T) and error (ϵ) effects,

$$y_{ijkl} = \mu + S_i + A_{j(i)} + R_{k(ij)} + T_l + \epsilon_{ijkl}, \qquad [3]$$

where i=1,...,99, and j,k,l=1,2. The assumptions of the model included (1) Y_{ijkl} are observable random variables; (2) μ is an unobservable constant; and (3) $S_1,...,S_I$ (subjects), $A_{1(1)},...,A_{J(I)}$ (aliquots within subjects), $R_{1(11)},...,R_{K(IJ)}$ (replicates within aliquots), $T_1,...,T_L$ (technician), and $\epsilon_{1111},...,\epsilon_{IJKL}$ (error) are unobservable random variables that are pairwise uncorrelated, have zero means, and var[S]=σ_S^2, var[A]=σ_A^2, var[R]=σ_R^2, var[T]=σ_T^2 and var[ϵ]=σ_ϵ^2. Also, the unobservable random variables are distributed jointly normally on the log scale. The results of the analysis of variance components performed by BMDP8V (Dixon, 1983) are summarized in Table 2. As expected, the polymorphic nature of D14S1 was demonstrated by the relatively large subject variance estimate ($\hat{\sigma}^2_S$=0.00625). Also, variability due to technicians ($\hat{\sigma}^2_T$=.000002), and interactions (e.g., between subjects and technicians, $\hat{\sigma}^2_{ST}$=.00003, and between technicians and aliquots, $\hat{\sigma}^2_{TA(S)}$=.00001) were quite small. Thus, an estimate of total fragment size measurement error ($\hat{\sigma}^2$) was obtained by summing the remaining components:

$$\hat{\sigma}^2 = \hat{\sigma}^2_{A(S)} + \hat{\sigma}^2_{R(SA)} + \hat{\sigma}^2_{TR(SA)},$$

$$= .00004 + .00031 + .00009 = .00044.$$

Therefore, $\hat{\sigma} \approx 0.02$ or D14S1 gel measurement error is approximately 2% of fragment size with the aforementioned methodology. Noticing that replication was the largest factor contributing to σ^2, the analysis was rerun using a more parsimonious model, $y_{ij} = \mu + S_i + \epsilon_{j(i)}$, where i=1,...,99 and j=1,...,8. Under this simple repeated measures model, σ^2 is

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65 individuals, all replicates measured consistently with 34 (52.3%) "heterozygotes" (i.e., two resolvable bands) and 31 (47.7%) "homozygotes" (i.e., only one distinguishable band). The 396 exposed bands represented 99 independent observations, replicated 4 times each.

The absolute difference (D) between the two technicians' converted log fragment size determinations of the 396 recorded bands was used as the experimental unit in the analysis of gel and lane effects. Before testing for these effects, diagnostic plots (results not shown) from BMDP2V (Dixon, 1983) suggested that observations be transformed since cell means and cell standard deviations appeared related (R^2 = .6290). The square root transformation [i.e., $D^* = D^{\frac{1}{2}} + (D+1)^{\frac{1}{2}}$] alleviated ($R^2$ = .0229) the observed linear relationship. The analysis of variance for gel and lane effects following the square root transformation performed by BMDP2V (Dixon, 1983) is summarized in Table 1. Table 1 shows cell counts and transformed cell means of the absolute difference between the technicians' fragment size determinations for the 12 different combinations of gel and lane effects. Included at the bottom of Table 1 are ANOVA tail probabilities for gel (p=.34), lane (p=.12), and replicate (p=.89) effects. No strong evidence exists to refute claims that gels were manufactured uniformly (i.e., possible gel impurities affecting migration are spread throughout and across gels).

Table 1. Results of analysis of gel and lane effects: mean and number per cell of square root transformed absolute differences in log fragment size measurements

	reps adjacent		reps apart	
	edge	middle	edge	middle
same gel	1.112 (6)	1.083 (7)	1.072 (9)	1.072 (7)
aliquots different gels	1.109 (10)	1.103 (9)	1.088 (11)	1.095 (4)
replicates different gels	1.101 (10)	1.079 (11)	1.086 (8)	1.071 (7)

p-value for gel=0.34, lane=0.12, and reps=0.89.

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$$y^* = \frac{x(y_{21}y_{10}-y_{11}y_{20}) + [(1-x)(y_{21}-y_{11})+(x)(y_{20}-y_{10})]y}{(y_{21}-y_{11})} \quad [2]$$

Analyzing the mobilities of four standard fragments showed that, in the size region 14 kb to 24 kb where D14S1 markers are generally found, log of fragment size and mobility are nearly linear. (The average R^2 of 24 sets of measurements equaled .995 with range .986 to .999.) Thus, following translation, unknown band mobilities were converted to fragment size from the functional log relationship of standards on the same gel.

DNA extracted from the white blood cells of 72 unrelated putative fathers and mothers was split into containers and enzyme-digested with EcoRI via the methods listed above. Each enzyme-digested aliquot was replicated once (i.e., a total of four samples on each subject was generated). The 288 samples were systematically allocated into 12 gels (24 lanes each). The allocation scheme regarded the 12 gels as 6 replicates of 2 gels (A and B), 12 subjects in each. Four of the 12 subjects had all replicates placed within the same gel (e.g., 2 subjects in gel A and two in gel B). Four subjects had their replicates placed across gels with one aliquot-replicate pair in gel A and the other in gel B. The remaining four subjects had their replicates placed across gels with each aliquot-replicate pair also split across gels. In addition, the four subjects at each level of gel assignment were assigned to lanes such that one of the following conditions held: (1) replicates were adjacent near edge of gel; (2) replicates were adjacent in middle of gel; (3) replicates were apart near edge; or (4) replicates were apart dispersed in middle. In total, the allocation scheme placed 6 subjects in each of the 12 cells formed by the 3 levels of gel and the 4 levels of lane position effects.

Next, gels were electrophoresed, DNA-probed with pAW101, and developed onto film according to the techniques mentioned above. Each exposed band on the finished film was read by two independent readers who were blinded to the allocation scheme. Band migration was assigned by measuring the distance (mm) from baseline to the center of the band. Finally, migration distance was converted to fragment size.

RESULTS

A total of 396 exposed bands were translated. Seven (10%) subjects exhibited no bands in any of their replicates (i.e., the measured mobility was 0.0, 0.0). Three of these subjects were retested under more stringent conditions whereby double enzyme-digested samples exhibited bands. The most probable explanation for the initial missing bands is incomplete digestion with enzyme (Rittner et al, 1989). The seven individuals were dropped from the analysis. In the remaining

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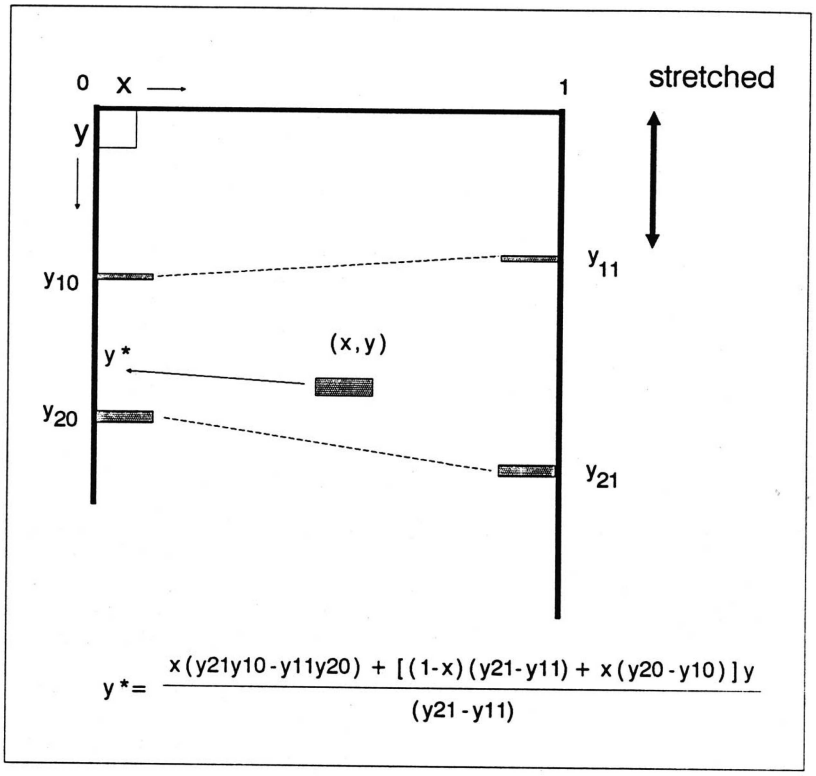


Fig. 3. Translation of RFLP gel bands. X,Y are the coordinates of an untranslated band bracketed by standards y_{10},y_{20} at x=0 and y_{11},y_{21} at x=1. Y^* is the value of y translated to x=0.

Before converting mobility to fragment size, all measurements were translated to a standard lane (usually the left-most lane), accounting for gel irregularities such as membrane stretching during Southern (1975) transfer. As diagrammed in Fig. 3, let (x,y) be the coordinates of an untranslated band bracketed by standards y_{10},y_{20} at x=0 and y_{11},y_{21} at x=1. Here x represents distance (normalized to one) across lanes of the gel and y represents band migration distances. Y^* is the value of y at x=0. If x=0, then, obviously, y^*=y. Next, assume gel irregularities cause linear disturbances across gels (i.e., gels are stretched from one edge in a technique analogous to pulling on a sheet of rubber). Thus, when x=1, y^*=c+dy. So, with c and d increasing linearly in x on [0,1],

$$y^* = xc + [1 + x(d-1)]y. \qquad [1]$$

Solving for c and d in terms of y_{10}, y_{20}, y_{11} and y_{21} gives the desired result for translating mobilities,

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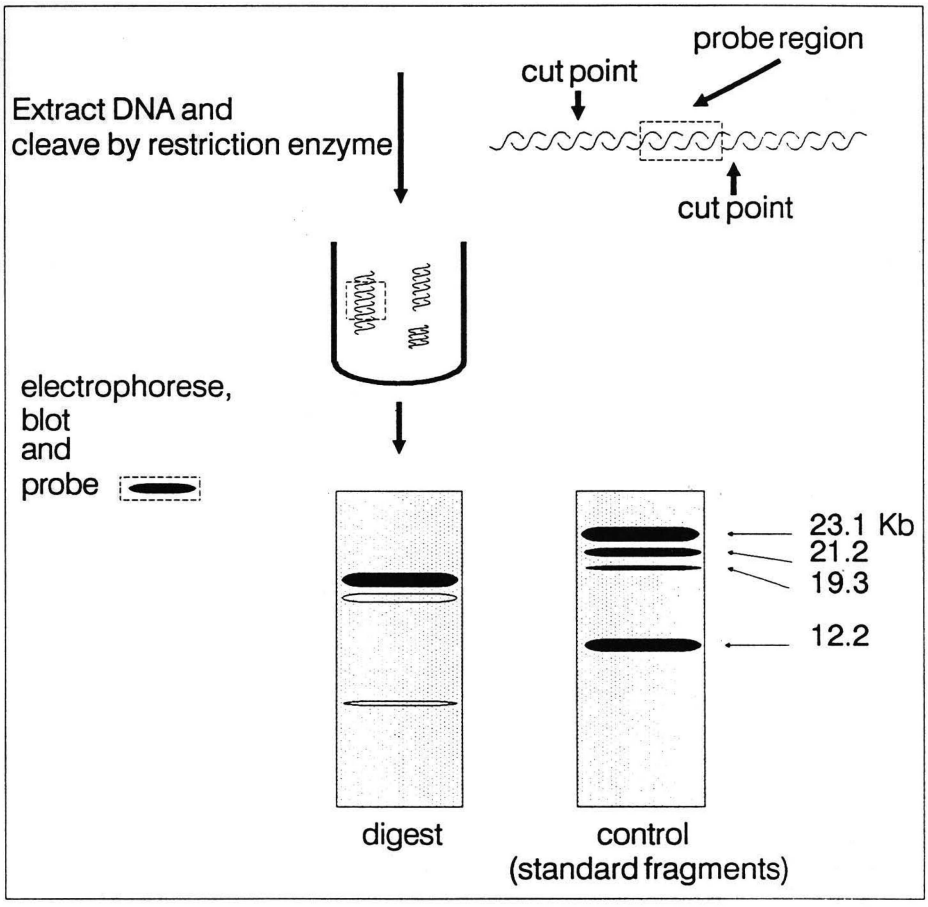


Fig. 2. D14S1 RFLP production. Extracted DNA is cleaved with restriction enzyme EcoRI, electrophoresed on agarose gel, hybridized with ^{32}P-labeled D14S1 probe, pAW101, and visualized by autoradiography. D14S1 fragment sizes are determined by calibrating the gel using known standards.

labeled D14S1 probe, pAW101 (kindly provided by Dr. Ray White, University of Utah). Dried membranes were exposed overnight on X-Omat-AR film (Kodak) between two intensifying screens (Li+) at -80C.

The mobility of D14S1 fragments and standard bands was measured from a common baseline to the nearest mm by two technicians.

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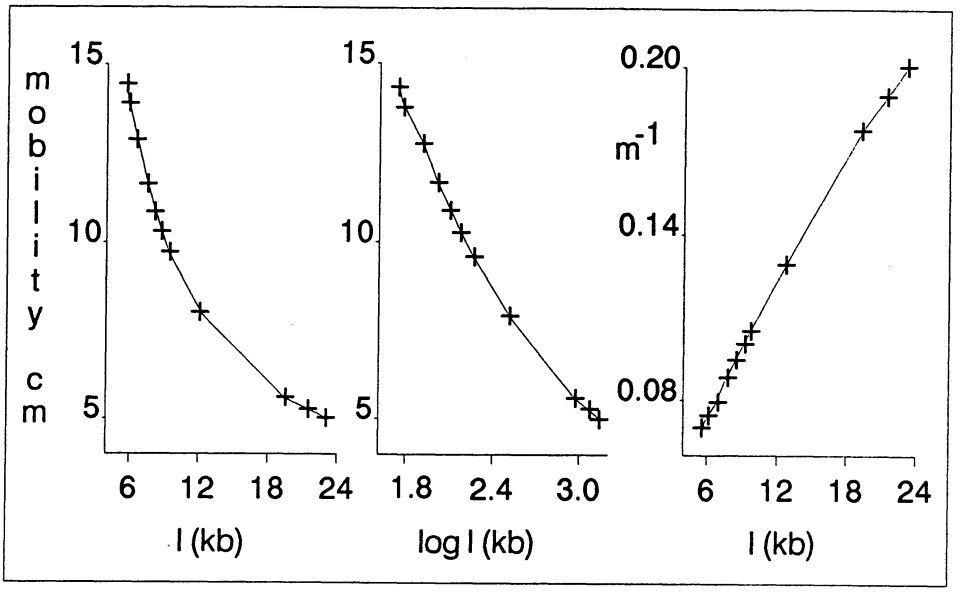


Fig. 1. Three plots of gel band mobility versus length of 11 standard DNA fragments. Ruled mobilities (cm) are 5.0, 5.3, 5.6, 8.0, 9.7, 10.4, 10.8, 11.7, 12.8, 13.9 and 14.4. The respective DNA fragment sizes (kb) are 23.130, 21.226, 19.397, 12.220, 9.416, 8.614, 8.271, 7.421, 6.682, 5.804 and 5.643.

high voltage gradients. He proposed a correcting factor (m_o) to give the best fit to a line of the form: $L = k_1/(m-m_o) + k_2$. Schaffer and Sederoff (1981) justified the correction as a means to account for factors which may affect the apparent location of the origin and further derived a "mechanism of migration", based on kinetic principles, which is consistent with the inverse model. Elder and Southern (1983) compared several methods (all variations of the log or inverse models) for relating mobility to fragment length. They concluded that the reciprocal relationship was the most accurate (maximum error < 0.1% of fragment size). The accuracy in mobility measurement, under any of the above methods, can be further enhanced by densitometers and computer processing techniques (Agard et al, 1981 and Elder et al, 1983).

MATERIAL AND METHODS

Figure 2 illustrates the general procedure for producing D14S1 RFLPs used in the subsequent empirical study. The extraction of high-molecular-weight genomic DNA and digestion with restriction enzyme EcoRI were performed by the Maniatis et al method (1982) with slight modification (Gjertson et al, 1988). Hybridization followed for 36 hours in the presence of ^{32}P

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1.1 Biostatistics

Measurement Error in Determination of Band Size for Highly Polymorphic Single-Locus DNA Markers

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INTRODUCTION

When assessing single-locus DNA information in reference to forensic and parentage problems, most proposed statistical methods (Baird et al, 1986; Gjertson et al, 1988; Morris et al, to be published; and Berry, submitted for publication) incorporate continuous measurement errors (ϵ) into their calculations. Errors arise while distinguishing length of enzyme-cleaved fragments from mobility in gel electrophoresis. Statistically, actual true gene size plus measurement error constitutes the underlying model for an observed allele (sometimes on the log scale) where errors are usually assumed to be distributed normally with mean zero and variance σ^2.

The following is a report of an experiment conducted to determine the standard error in measuring RFLP alleles (log scale). The DNA sequences of the D14S1 region (Wyman and White, 1980) provided the practical example.

Background

Differences among individuals in the lengths of their DNA fragments (or, equivalently, their size measured in number of base pairs) are inherited characteristics that can be recognized by altered mobility of bands in agarose gel electrophoresis (Botstein et al, 1980). To estimate the length of unknown DNA fragments (L) from mobility of bands (m) in gel electrophoresis, a relationship is established between the mobilities and lengths of standard fragments whose lengths are known. Figure 1 depicts three plots of band mobility versus length for 11 standard fragments. The log model (Fisher and Dingman, 1971) and inverse model (Southern, 1979) are the two basic relationships proposed to estimate L from m. Even in their initial studies, Fisher and Dingman (1971) recognized the inadequacy of the log model over a wide range of fragments where plots of log L versus m showed marked departures from linearity for fragments with high molecular weights. Likewise, Southern (1979) observed curvature in plots of the inverse model (i.e., L = k/m) when electrophoresis was carried out at

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