MIXING AND THERMIC TREATMENT OF MITOCHONDRIAL PCR FRAGMENTS REVEAL SEQUENCE DIFFERENCES BY HETERODUPLEX FORMATION –A RAPID METHOD FOR FORENSIC IDENTITY TESTING

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INTRODUCTION

For extracting the full information about stains or an identification situation, it is advisable to investigate karyotic STR systems as well as mitochondria (mt) DNA. Each of the above strategies is associated with special advantages and disadvantages:
1. Due to the high number of mt copies per cell, it is a much higher sensitivity what distinguishes mt testing from other methods.
2. Since mt concerns maternally inherited markers, it avoids wrong results due to illegitamate paternities.
3. mt testing can be superior if mixed stains have to be investigated. Stain sorting by cloning is possible.
Within the human mt D-Loop, a considerable potential for information concerning individualization is condensed on an assessable DNA region of about 800 bp.There are two hypervariable regions designated HV1 and HV2 usable for individualization by the sequencing technique. Within a group of 100 unrelated white Caucasians, 91 different sequences were seen (Piercy 1993).
We suggest that the application of mt testing needs a simple screening method for detecting identities and nonidities if a mass of stains has to be investigated or if stain sorting has to be carried out.
In the genomic diagnostic of human diseases, sequence heterogenity between alleles can be recognized by using techniques such as heteroduplex (HD) analysis (HDA), single strand confirmation polymorhism (SSCP) investigation and chemical cleavage.
The PCR product of a karyotic gene fragment produces a HD if the genotyp is heterozygous with regard to the sequences and / or to the repeat numbers in STRs. Leaving aside the fact that there are some infrequent mt heteroplasmatic disturbances, e.g. deletions, mt population of a human induviual is usually uniform. Therefore, D-loop amplification of polymorphic regions of a certain proband or a pure stain normally cannot produce any HDs. Likewise, if D-loop products stemming from the same source are submitted to mixing and thermic treatment, they should provide only homoduplexes. Thus, if D-Loop products from a stain and the stain causer or from a proband and his maternal relatives are treated in the mentioned manner, it is expected that no HD formation takes place. On the other hand, if nonidentical sequences were mixed and thermically treated, reciprocal rearrangements - beside homological reassociation - take place resulting in HD formation. HDs migrate markedly slower than homoduplexes if native PAGE is used for separation.Therefore, HDA can reveal sequence differences in D-Loop fragments.

MATERIAL and METHODS:

For amplifying the fragments of interest we use the following primers:
HV 1* L 15996 / H 16498 (544 bp) HV 2* L 29 / H 408 (431 bp)
Whole D-loop* :L 15926 / H 580 (1280 bp)[*PCR was carried out according to Sullivan (1991)

mt-HDA PROTOCOL:

1. Amplify the D-loop fragments from the different sources and check the PCR success by PAGE.
2. Mix the samples of question (e. g. stain vs. possible causer or corpse vs. maternal related relatives).
3. Carry out 5 PCR cycles omitting the enzyme and chill immediately on ice.
4. Separate the mixtures as well as the pure mixture components using vative PAGE and silver staining.

For introducing mt HDA, mixing experiments were performed using amplified HV1 as well as HV2 fragments. Mother/child and sibling/sibling combinations were regarded as mixtures of identical sequences. Father/child and wife/housband pairs were seen as mixtures of non identical fragments.

Sequences were readed using the A373 sequencer and the Taq dye terminator cycle sequencing kit (ABI).

RESULTS and DISCUSSION

If HV1 fragments stemming from 50 mother/child pairs were mixed and thermically treated, HDs could never be seen. In opposite, if the procedure was carried out in 50 father / child pairs, HDs were seen in 48 cases. As it is demonstratad by mixing HV1 fragments of siblings with those from their father, each mixture provides the same HD shape. (Fig.1)
In parallel, when we mixed HV2 products within 100 mother/child pairs we did not see HDs, but we did in 85% of father/child pairs. HDA in families using HV2 is depicted in Fig. 2.
If the HV2 fragments of any proband are mixed with those of unrelated persons, about 85 % of mixtures produce HDs again (Fig. 3). Furthermore, there is a high variability in the HD patterns.
The following conclusions can be drawn from Fig. 4 and 5:
1. HV fragments of different source but with equal sequences (e.g. siblings) produce identical HD patterns if they are mixed with their counterpart fragments stemming from the same probands.
2. A prominent HD is caused by a high number of missmatches, no or a discrete HD indicates that there are no or only sporadic missmatches. Additionally, we suppose that the missmatch positions may play a significant role, too (This assumption has not been systematically investigated until now).
3. HDA in the HV 1 and the HV2 region have an independent chance to indicate nonidentities of mt of persons or stains.
In addition to HDA, for the same purpose, we tried the SSCP technique. Here is again a good chance that heterogenity in mixed fragments becomes visibel by doubleband formation. However, in our hands, the SSCP technique seems to be much less effective than HDA.

Ending Statement: HDA is a suitable technique to detect the majority of the nonidentities of sequences if mtDNA stemming from different sources is mixed. Thus, if a mass of stains has to be investigated or if stain sorting has to be done by HV1 and HV2 cloning, this method may help to focus the more expensive sequencing techniques onto highly interesting samples or stains.

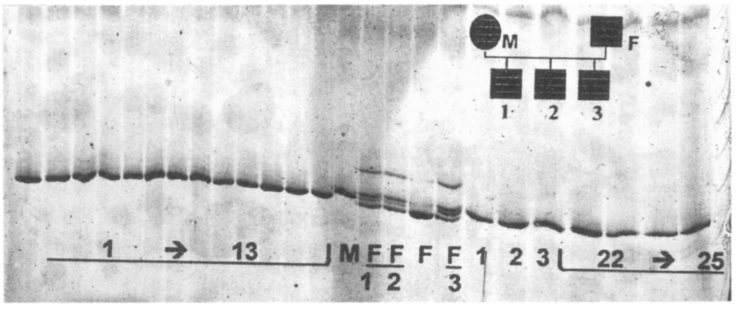

Fig 1: HDA experiment using VH1 fragments of mother/child pairs and of a famliy depicted in the pedigree. lanes 1–13 and 22–25 contain mixed HV 1 fragments of 17 mother/child pairs. In the middle of the gel, there are pure and mixed HV1 fragments stemming from the pedigree. Clear HDs were formed in father/child pairs. Due to the identity of the father/child combinations, all HDs show the same shape.

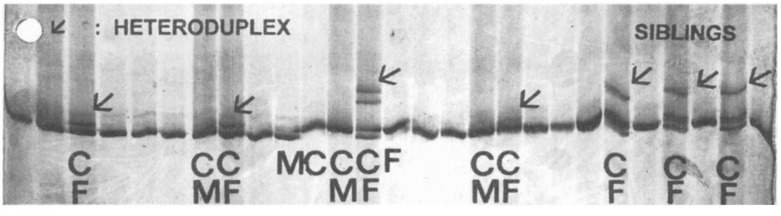

Fig 2: HDA of the D-loop HV2 region in five families. Full lettering is given only for the family in the middle of the gel: M (unmixed fragment of the mother), F (unmixed fragment of the father), C (unmixed fragment of the child), letter combination (mixed fragments).

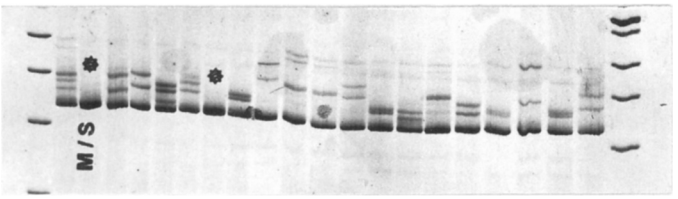

Fig 3: HDA in HV2: All lanes contain the same HV2 product of a woman mixed with the counterparts stemming from her son (M/S) and 19 unrelated donors. *= none HD formation.

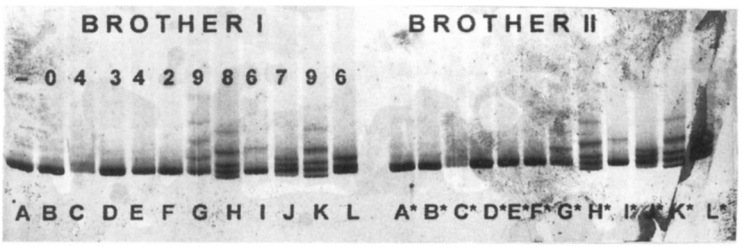

Fig 4: HDA in HV2: PCR fragments stemming from two brothers were mixed with those from 100 unrelated probands giving two identical series of HV2 mixtures. Out of both series four mixtures were chosen producing no or weak HDs (C–F and C*–F*) and six pairs creating powerful HDs (L–G and L*–G*). Sequencing revealed the number of missmatches between the mixture components (figures). Lanes A and B contain the pure and mixed HV2 fragments of the brothers, respectively.

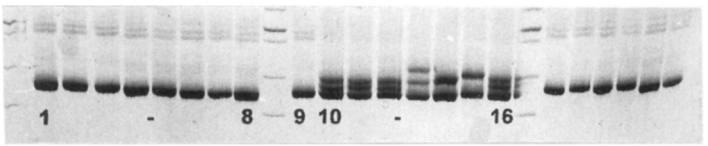

Fig 5: HDA in HV1: Fragments were mixed using products from probands wich were chosen bacause of lacking HDs in mixtures of HV2. (Compare with Fig 4 !) Lanes 1 to 8 contain pure HV1 products, lanes 10 to 16 show pairs which were tested with regard to HD production in HV2 (equal tolanes C–I in fig. 4).

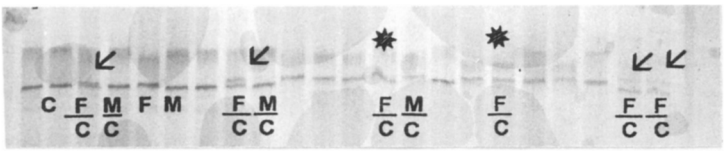

Fig 6: SSCP study in five families using HV2: . Heterogenity in mixed fragments becomes visibel by doubleband formation in three of five nonidentical mixtures.
↙= sequence heterogenity detected, *= sequence heterogenity not detected.

References :

Piercy R, Sullivan KM, Benson N, Gill P (1993) The application of mitochondrial DNA typing to the study of white Caucasian geneticc identification. Int J Med 106: 85-90

Sullivan KM, Hopgood R, Gill P (1991) Automated amplification and sequencing of human mitochondrial DNA. Electrophoresis 12: 17-21