ADVANTAGES AND DISADVANTAGES OF THE APPLICATION OF NESTED PCR FOR TYPING OF FORENSIC SAMPLES

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INTRODUCTION

Amplifying the VNTR 3′ from APO B using standard PCR procedures with unnested amplification the sensitivity lies between 100-400 pg of genomic DNA. Employing nested PCR after designing a second primer pair that is external to the regular primers but different from the primers described by Vuorio et al (1990) we could increase the sensitivity to single cell level. Using DNA dilutions, DNA mixtures and artificially produced bloodstains we tested the limitations of this method for forensic application. To achieve defined numbers of DNA templates, sperm cells of two heterozygous individuals were purified and combined by micromanipulation.

MATERIALS AND METHODS

Sperm Isolation:
1. Swim-up
-1ml M2-medium or Hepes/Saline to 0,5ml aliquots of semen
-Centrifugation at 600g for 6 min.
-Supernatant discarded, 1ml medium added to the pellet
-Incubation for 60 min. at 37 °C (migration of spermatozoa)
2. Micromanipulation
Isolation of individual sperm or a defined number of sperms from the supernatant of the swim-up under a Leitz microscope (Leitz, Labovert; Germany) equipped with micromanipulators (Leitz, Germany).
3.Microscopic counting and preparation of different ratios of sperm cells
- Counting of the supernatant of the swim-up in a BÜRKER counting chamber.
- Diluting the supernatant to 100 sperms/µl down to 20 sperms/µl
One single sperm was transferred to a PCR tube by micromanipulation. Different dilutions of sperms made by microscopic counting or micromanipulation were added.

Alkaline sperm lysis (Li et al, 1991) and DNA amplification:
-Lysis of the sample (1-2,5µl) with 2,5µl 400mM KOH/ 100mM DTT in 5µl for 10 min. at 65 °C
-Neutralization with 5µl 900mM Tris-HCL pH 8.3/300mM KCL/200mM HCL
First round of amplification:
2U Taq Pol (Promega), 0,5µM each primer (pr.1,pr.2), 150µM dNTPs, 10mM Tris-HCL pH 8,3, 1,5mM MgCl2, 0,1% Triton X-100

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94 °C-1 min., 58 °C-1 min., 72 °C-4 min., 20 cycles
Second round of amplification: (1µl aliquot first round) as above but 0,5µM each primer (pr.3, pr.4) and 50mM KCl
94 °C-1 min., 58 °C-1 min., 72 °C-4 min., 25 cycles

Primer sequences of the outer set:
Pr. 1: 5′ AACCTCTAGAACACATAGTGTGA 3′
Pr. 2: 5′ CCTCAGGATCAAAGTATGTACAA 3′
Primer sequences of the inner set (Boerwinkle et al, 1989):
Pr. 3: 5′ TGGAAACGGAGAAATTATGGAGG 3′
Pr. 4: 5′ CCTTCTCACTTGGCAAATACAATT 3′

Primer 1 and 2 were located 20bp 5′ to primer 3 and 19bp 3′ to primer 4 respectively and designed based on published sequences (EMBL GenBank, Knott et al, 1986). All amplifications were carried out in a Trio-Thermoblock (Biometra).

DNA was extracted and quantified following published protocols (Miller et al (1988), Jung et al (1991), Human DNA Quantitation System, Gibco BRL). DNA amplification of DNA from blood and bloodstains was performed as above except the addition of 8µg of BSA to the reaction mix.

RESULTS

Effect of DNA amount on nested PCR - see figure 1 :

DNA of heterozygous individuals was diluted to achieve varied concentrations, which were verified using the dot blot procedure. As shown in figure 1 on the left side, for a DNA sample with 300 bp length difference between the two APO B alleles, a strong tendency towards preferential amplification of the shorter allelic product is noticeable starting at 50pg. For 200pg the longer fragment produces only a faint band in comparison to the shorter allele. Besides, starting from 100pg an increasing amount of DNA causes additional bands, especially outside of the allelic range. Amplifying 6pg of DNA, which corresponds to the size of a diploid genome of a humane cell, at times only one of the two alleles was detected. Because of the random fluctuation of the alleles in DNA dilutions, this was to be expected.

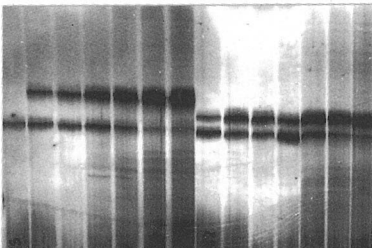

Fig. 1 Effect of DNA amount on nested PCR
lane 1-7, 8 -14:
6, 12.5, 25, 50, 100, 200 and 400pg DNA of different genotype

Ability to type mixed samples

DNA mixtures in varying degrees of magnitude - see figure 2:
DNA of two heterozygous individuals were combined in defined ratios prior to amplification. Genotype 39/51 was always present at higher rate. Genotype 29/49 was added with decreasing amounts down to 6pg.

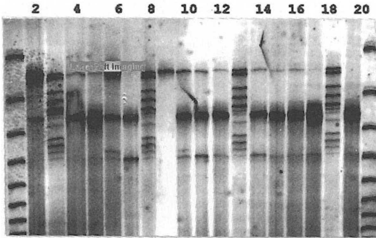

Fig.2 Different mixtures of DNA from two heterozygous individuals
lane 2,4,5:
1ng+1ng;0,1ng;0,01ng
lane 6,7:
0,1ng+0,1ng;0,01ng
lane 9: 6pg;
lane 10-12,14-17,19:
6pg+120;180;240;300;420;600;1200;
1800pg

The DNA typing results for the mixed samples are also influenced by preferential amplification. With a total amount of 2ng of DNA, even with a DNA ratio of 1:1 only the two shorter alleles of each genotype could be detected (lane 2). Using 0,2ng of mixed DNA with a ratio of 1:1, all four alleles were amplified (lane 6). The ability to detect one PCR template with different backgrounds of human DNA also depends on the total amount of DNA in the reaction and on the length of the allele. With a DNA input of 6pg Figure 2 shows how the shorter allele gets fainter with increasing amount of added DNA but still can be detected up to a ratio of 1:200 (lane 9-20). The longer fragment can only be seen up to a ratio of 1:30.

Defined number of sperm cells - see figure 3:

Different numbers of sperm cells (20, 30, 40, 60, 100) from a heterozygous male (Genotype 29/35) were added to single sperm cells that stemmed from a second heterozygous male with a different APO B genotype (37/47). Up to a ratio of 1+40 the allele of the single sperm could be detected. The signal intensity of the PCR products of equal sperm cell numbers showed a high degree of variation, which leads to the conclusion that the true amount of sperm cells in the reaction shows a high fluctuation. Although another cause could be unequal distribution of the two alleles.

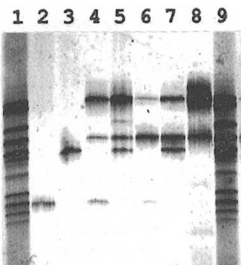

Fig.3 Defined numbers of sperm cells
Mixture of a single sperm and sperms of another genotype
lane 2,3: single sperm
lane 4: 1+20 sperm cells
lane 5: 1+30 sperm cells
lane 6,7: 1+40 sperm cells
lane 8: 1+60 sperm cells
lane 1,9: allelic ladder

Results of nested PCR for contaminated stain material

Bloodstains were prepared on one piece of cotton cloth by applying 0,1µl of blood (genotype 39/51) as discrete stains or faint smears and dried. To simulate possible contaminations the cotton cloth was touched (genotype 37/49), coughed on (genotype 31/32) or spitted on (genotype 37/49). For extraction each bloodstain was cut out. The quantitation of the total yields of some extractions gave DNA amounts from 300pg to 20ng/bloodstain. Ten of fourteen bloodstains showed contamination. Sometimes the contamination was only detected by different dilutions of one extraction. It was nescessary to reduce or increase the DNA input to identify the DNA mixtures. After strong contamination with saliva only the contamination could be found and e.g. three different dilutions of one extraction showed three different results: either one or both alleles of the contaminant were detected. Typing the moderately and lightly contaminated material, different genotypes also could be obtained. One extraction showed only the genotype of the contaminant (total DNA amount: 20ng). In four other extractions only the original genotype was found otherwise a combination with one or both alleles of the contaminant was detected.

DISCUSSION

Our results show that, as has been described for other loci (Li et al, 1988), the approach using nested primers for the APO B VNTR increases the sensitivity of the PCR to the single cell level. Working with small forensic samples the higher sensitivity is a big advantage, e.g. we suceeded in typing very degraded DNA from bones that didn't yield signals otherwise. A main problem of nested PCR is the occurence of preferential amplification if overly high total amounts of DNA are used. Probably the second round enhances the amplification of unequal amounts of short and long PCR products that are produced in the first round (Ruano et al, 1991). As a consequence one may get allelic drop out and/or wrong typing results. Exact quantitation of DNA is therefore absolutely necessary to achieve correct typing results.

The correct typing of mixtures is also influenced by preferential amplification, dependent on the respective allele length. In the presence of overly high amounts of DNA even a proportion of 1:1 could not be correctly typed for the longer alleles. The same effect could be demonstrated using 6pg amounts (equivalent to one diploid genome) with the genotype 29/49. Similar results were obtained from the defined numbers of sperm cells.

The high sensitivity causes an increased susceptibility to contamination. Even only superficial contact with bare hands left amplifiable alleles. It was possible to obtain three different results from one piece of evidence: Only the genotype of the blood donor, the genotype of the contaminant, or both genotypes could be detected in separate extractions. The typing of minute quantities of stain material or DNA, as it is possible with nested PCR, is prone to sampling error if working with DNA dilutions from one sample (Navidi et al, 1992). Therefore, DNA-ty-

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ping results from such small amounts of DNA are not necessarily reliable. Dealing with mixed samples in casework, the amplification of different dilutions increases the chances of detection of minor components. Otherwise false results may be caused by excessive DNA (allelic drop out) or too little DNA (unequal distribution of templates). The increased danger of contamination, preferential amplification, allelic drop out and sampling error also concerns other sensitive PCR approaches such as amplifying STR's.

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