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TECHNICAL NOTE

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STR typing of human telogen hairs – a new approach

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Abstract A new approach to short tandem repeat (STR) typing of DNA extracted from telogen shed hairs is presented. Newly designed primer pairs with annealing positions close to the repeat units of the STR loci HUMFES, HUMTH01 and HUMTPOX were used for amplification. The typing results were compared to those obtained by the commonly used primer pairs by means of success rates. The primer pairs capable of producing very short amplicons (<106 bp with HUMFES, <86 bp with HUMTH01 and <87 bp with HUMTPOX) described in this study significantly increased the success rates when typing telogen hairs.

Keywords Short tandem repeat loci · STR · Degraded DNA · Telogen hairs

Introduction

In recent years, DNA typing of STR loci by PCR has become one of the most convincing instruments in forensic casework. Even the smallest amounts of DNA extracted from saliva cells on cigarette butts [1], as well as degraded DNA obtained from old bloodstains [2] and human skeletal remains [3], have been shown to be suitable for DNA profiling. Thus, forensic scientists have even focused their interests on applying the new technology to the analysis of human hairs.

In the past, single hairs have been used for DNA typing if adhering cells, such as hair root sheath cells were present [4, 5]. However, DNA typing has shown a relatively low success rate [6] or failed if adherent cells are lacking [7]. On the other hand, hairs in the telogen phase represent the majority of trace hairs collected in connection with criminal cases. Until now little was known about the extent to which nuclear DNA persists inside the keratinised cells of the hair material and to what extent degradation of nuclear DNA occurs during keratinisation. Experiments indicated that the nuclear DNA from keratinised cells is highly degraded and in general about 100 bp in size [8].

To evaluate these hairs in routine forensic casework, mitochondrial DNA (mtDNA) has been extracted which is suitable for the analysis of variable regions of mtDNA by sequencing procedures [9, 10, 11]. Unfortunately, the results obtained by mtDNA analysis cannot be compared with DNA profiles obtained in routine casework or those implemented in forensic intelligence databases.

The aim of this study was to improve methods for nuclear DNA profiling adapted for telogen hair material. Because of degradation of the DNA during keratinisation, new primer pairs for FES, TH01 and TPOX producing very small amplicons less than 110 bp in size were used for PCR amplification.

Materials and methods

Samples and DNA extraction

Scalp hairs from 48 individuals were collected with a comb and controlled microscopically (50 × magnification) for adhering root sheath material and from the morphology, only typical telogen hairs lacking adhering tissue as well as the germinal nipple [12] were used for DNA extraction. Basal hair segments of approximately 20 mm in length including the telogen club root were digested in 100 µl of TN_{Ca} buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$, 2% SDS (w/v), 39 mM DTT and 250 µg/ml proteinase K.

The hairs were incubated at 56 °C in a water bath with gentle agitation for a period of 2 h and if undissolved hair fragments were still macroscopically visible after 1 h, further proteinase K was added to a final concentration of 500 µg/ml. Following the digestion procedure, proteins were separated by the addition of equal volumes of phenol/water/chloroform (Applied Biosystems, Weiterstadt, Germany) following standard protocols. The aqueous supernatant was transferred to a Microcon-30 microconcentrator sample reservoir (Millipore, Eschborn, Germany) and the vial centrifuged at 14,000 g for 12 min, 200 µl H_2O was added to the sample reservoir and centrifuged again at 14,000 g for 12 min. The membrane of the microconcentrator was overlaid with 40 µl H_2O, inverted, transferred to a new vial and centrifuged at 1,000 g for

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Table 1 Primer sequences and PCR product size

STR locus	PCR product size range (bp)	Primer sequences	Reference
FES	213–237	[a]5′–GGGATTTCCCTATGGATTGG–3′ 5′–GCGAAAGAATGAGACTACAT–3′	[26]
FESvs	81–105	[a]5′–GTTTAGGAGACAAGGATAGCAGT–3′ 5′–GCGAAAGAATGAGACTACAT–3′	New
TH01	169–193	AmpFlSTR Green I PCR Amplification kit sequence not available	PE Applied Biosystems
TH01vs	61–85	[b]5′–CCTGTTCCTCCCTTATTTCC–3′ 5′–GAACACAGACTCCATGGTG–3′	[14]
TPOX	218–246	AmpFlSTR Green I PCR Amplification kit sequence not available	PE Applied Biosystems
TPOXvs	58–86	[c]5′–GGGAACCCTCACTGAATG–3′ 5′–CAGCGTTTATTTGCCCAA–3′	New

[a] FAM-labelled strand
[b] JOE-labelled strand
[c] HEX-labelled strand

5 min to transfer the extract to the vial. The DNA extract was immediately used for PCR amplification.

DNA from control saliva samples taken from the donors of the hairs, was isolated with Chelex100 according to standard methods [13].

PCR primers

The primer pairs as standardised for the application in European forensic laboratories are listed in Table 1. In addition three new primer pairs denoted as FESvs, TH01vs and TPOXvs were introduced here. The primer sequences for the FESvs and TPOXvs loci were designed using the PrimerExpress software (PE Applied Biosystems, Weiterstadt, Germany). The primer sequence for the TH01vs locus was described elsewhere [14].

PCR amplification

Singleplex amplification of the STR loci FES, TH01 and TPOX was performed in a total volume of 50 µl consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each deoxyribonucleoside triphosphate (dNTP), 12.5 pmol of each primer, 2 U TaqGold DNA polymerase (PE Applied Biosystems, Weiterstadt, Germany) and 20 µl of hair extract for 34 cycles in a Gene Amp PCR System 2400 thermal cycler (Perkin Elmer). Annealing temperatures were 54 °C for all singleplex PCRs using the newly described primer pairs (FESvs, TH01vs and TPOXvs) and 60 °C for conventional primers (FES). Amplification with the Perkin Elmer AmpF*l*STR Green I PCR Amplification kit was carried out according to the manufacturer's manual for 34 cycles. Reference samples were amplified using 3 µl of saliva extracts in a total volume of 50 µl PCR reaction mix for 30 cycles. Amplicons were separated and detected by 6% polyacrylamide gel electrophoresis in an ABI 377 DNA Sequencer (PE Applied Biosystems, Weiterstadt, Germany). Allelic ladders for the new primer pairs were reamplified from a $1{:}10^8$ dilution of original ladders provided by various manufacturers.

Results and discussion

Hair digestion

Experiments concerning sex determination by staining of the sex chromatin in human hairs have shown that there are reasonable numbers of nuclei detectable in keratinised cortex cells [15, 16]. However, genetic typing from nuclear DNA isolated from hairs mostly failed when using PCR amplification of VNTR loci. Therefore it was suspected that methods of DNA extraction so far described were not capable of isolating sufficient DNA from keratinised hair cells suitable for successful amplification. Thus, a new buffer system containing proteinase K and Ca^{2+} instead of EDTA [17] or Chelex [6, 13, 18, 19] was used leading to total digestion of the hairs examined. The progress of hair disintegration was controlled microscopically in preliminary experiments. In the presence of calcium and proteinase K, the time of digestion could be decreased from overnight to 2 h incubation at 56 °C. In most cases, no hair debris could be detected macroscopically even after 1 h. This efficiency of hair digestion could not be achieved using the standardised methods described above. The activation of proteinase K by calcium was described previously [20]. Experiments relating to DNA degradation during hair digestion using the new buffer system have been performed by adding high molecular weight DNA to the sample. No products of DNA degradation could be observed (data not shown).

DNA isolation and PCR amplification

A method involving phenol-chloroform extraction and microcon 30 purification has been applied to DNA isolation from single telogen hairs. This procedure yielded total volumes of 40 µl of DNA extract, sufficient for two PCR amplifications. Profiling of DNA extracts from 36 hairs in preliminary experiments using the AmpF*l*STR Green and Blue PCR multiplex amplification kits for STR typing under conditions described above, were unsuccessful in most cases. Furthermore, the use of less than 20 µl DNA extract for each 50 µl PCR, or even the reduction of the reaction volumes, decreased the success rates and may be due to insufficient contents of target DNA. Nevertheless, the results gave evidence for increasing typing efficiency with decreasing sizes of the STR amplicons (data not shown). Our findings are supported by observations made

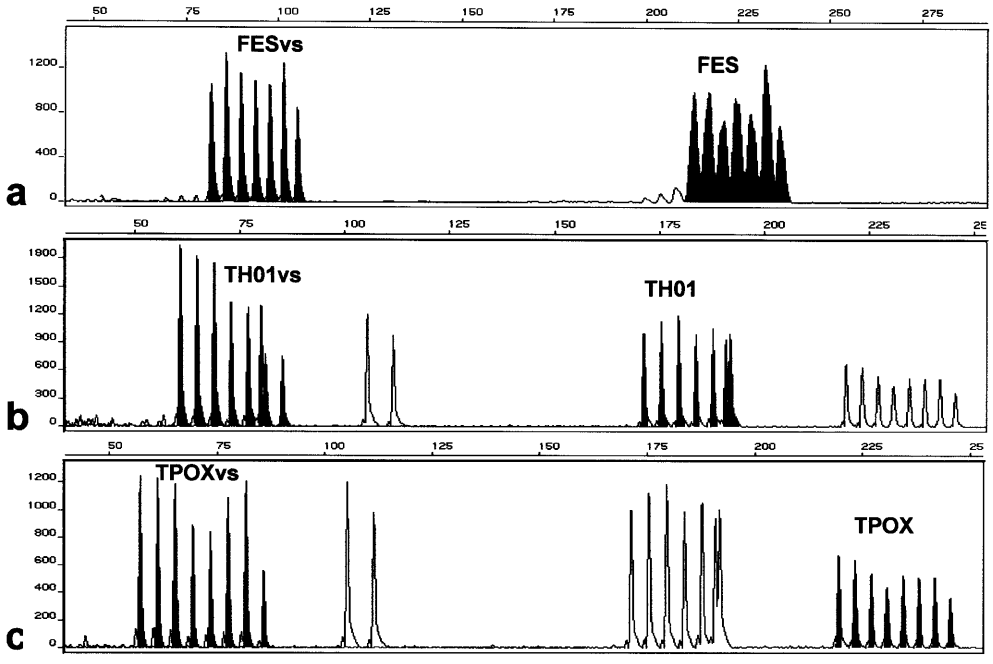

Fig. 1 a–c Electropherogram of allelic ladders. Profiles shown were obtained by separation of **a** sample containing reamplified (*FESvs, alleles 8–14*) and conventional ladders (*FES, alleles 8–14*) for HUMFES, **b** sample containing reamplified (*TH01vs, alleles 5–11*) and conventional ladders supplied by the AmpF*l*STR Green I PCR Amplification kit (*TH01, alleles 5–10*) for HUMTH01 and **c** sample containing reamplified (*TPOXvs, alleles 6–13*) and conventional ladders supplied by the AmpF*l*STR Green I PCR Amplification kit (*TPOX, alleles 6–13*) for HUMTPOX

by various authors. TH01, representing one of the small STR loci yielding amplicons ranging from 150 bp to 200 bp depending on the primer pairs used, has been described for DNA typing of single hairs [21]. Typing of VNTR loci with large fragments such as D1S80 mostly failed or gave only poor results [6]. These observations indicate that not only the low contents but mainly the quality of the DNA extractable from hairs are the reasons for the low success rates. Another important hint was given by Matsuda et al. [8], who described a degradation of nuclear DNA to small fragments with a size mainly around 100 bp in the hair shaft.

The size of an amplified STR fragment is characterised by the number of the tandemly repeated motif plus the flanking regions and the lengths of the primers themselves, whereas in general the length variations of the alleles only are determined by the repeat numbers. A suitable strategy to decrease the sizes of amplicons is to reduce the length of the flanking regions by looking for primer pairs binding adjacent to the repeat units. Thus, it is principally possible to amplify the same alleles of a given locus independently of the positions of the primers. However, it has to be considered that allelic drop-out can occur due to sequence mutations at the primer binding sites as described for some STR loci [22].

For DNA typing presented in this study, PCR was carried out with DNA extracts from single shed hairs of 48 individuals. One extract was used for two amplifications yielding small and large fragments for the same STR locus, respectively. Data obtained from preliminary experiments concerning the typing efficiency of the AmpF*l*STR Green I PCR Amplification kit were included in the presentation. The purpose of the study was to compare all results in DNA typing with respect to the size of the amplified DNA fragment. The newly described primer pairs bind directly adjacent to the tandem repeat sequence thus eliminating any flanking regions. In consequence, all amplicons produced by PCR are characterised by a size of less than 110 bp, leading to the suggestion that these primers be named vs (very small) primers as introduced here (e.g. TH01vs). The reamplification of the allelic ladders produced DNA fragments in the range 81–105 bp for FESvs, 61–85 bp for TH01vs and 58–86 bp for TPOXvs, whereas the conventional fragments range was 213–237 bp for FES, 169–193 bp for TH01 and 218–246 bp for TPOX, separated by the method described above (Fig. 1).

The utilisation of these new vs-primer pairs for DNA typing of single telogen hairs increased the amount of correctly typed hairs from 21.7% to 65.3% whereas the amount of hair extracts producing no detectable PCR products decreased from 71.1% to 8.2% for TH01 (Fig. 2).

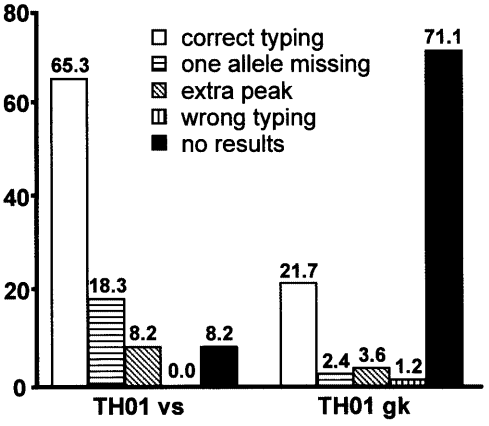

Fig. 2 Typing efficiency (%) of single telogen hairs using vs-primer pairs (*TH01vs, n = 49*) in comparison to primer pairs supplied by the AmpF*l*STR Green I PCR Amplification kit (*TH01gk, n = 48 + 36 from preliminary experiments*) for the HUMTH01 locus

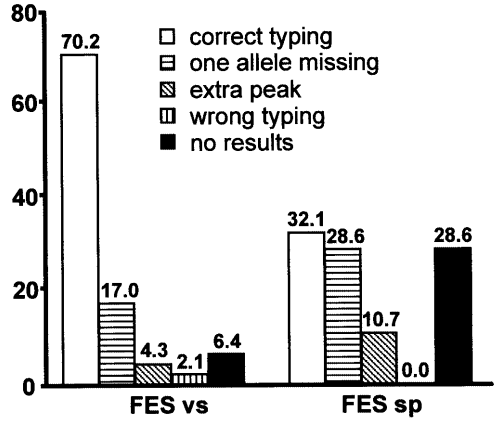

Fig. 3 Typing efficiency (%) of single telogen hairs using vs-primer pairs (*FESvs, n = 47*) in comparison to conventional singleplex primer pairs (*FESsp, n = 48*) for the HUMFES locus

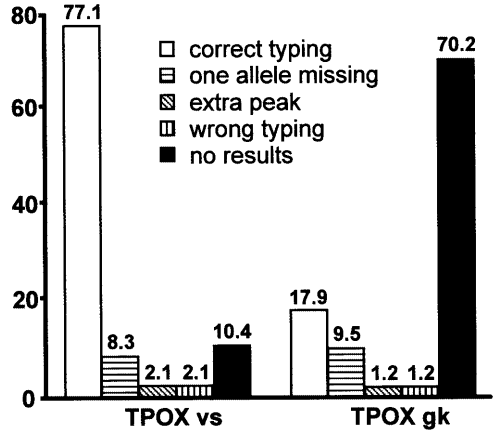

Fig. 4 Typing efficiency (%) of single telogen hairs using vs-primer pairs (*TPOXvs, n = 48*) in comparison to primer pairs supplied by the AmpF*l*STR Green I PCR Amplification kit (*TPOXgk, n = 48 + 36 from preliminary experiments*) for the HUMTPOX locus

The success rates of STR typing increased from 32% to 70% and from 18% to 77% if the FESvs and TPOXvs primer pairs were used, respectively (Figs. 3 and 4). In general, the typing efficiency could be at least doubled by the use of the new primer pairs when compared to conventional primers, regardless of whether singleplex or multiplex PCR was performed. Nevertheless, a low percentage of preferential amplification of only one allele of a heterozygous individual (one allele missing), extra peaks and wrong typing results could be observed for all STR loci. Similar results have been described for STR typing of undegraded DNA with very low content in the picogram range [23, 24]. The extra peaks probably resulted from slippage processes during the first amplification cycles. In most cases they revealed a relatively low peak height of less than 15% of the highest peak corresponding to the correct alleles known from typing the saliva reference sample. Some extra peaks as well as wrong typing results may be caused by contamination of the hair material. Taking into account the very low DNA content of telogen hair roots in the picogram range, even contamination by single cells on the hair surface not detected microscopically prior to DNA extraction could cause wrong typing results. This explanation is supported by the observation that incorrect STR typing in relation to the rates of correct typing occurred to a lesser extent when STR loci were amplified using vs-primers. Taking into account the degradation of nuclear DNA one can expect a higher proportion of target DNA extracted from the hair for the amplification of short fragments.

These findings coincide with the assumption of a dramatic degradation of nuclear DNA in keratinised cells, not only in the hair shaft material as described [8] but also in the telogen hair root. Thus DNA typing will lead to a low success rate or even fail if conventional STR primer pairs producing large amplicons are used for PCR, as experienced in forensic case work. The new primer pairs presented here gave excellent results due to the need for only short strands of target DNA of less than 110 bp.

Nevertheless, false positive results must be avoided or at least be recognised, especially the preferential amplification of only one allele, before application of the method described in routine casework. Special prior washing steps should be taken into consideration for hair decontamination as suggested for mtDNA sequencing [25].

The results presented in this study again emphasise the difference between trace material consisting of saliva, semen or blood containing undegraded DNA and the problematic trace material of telogen hairs containing highly degraded DNA.

The newly described vs-primers in combination with effective extraction procedures, can be used as a powerful tool in DNA typing of single shed hairs. In addition it can be expected that vs-primer systems can be applied to any highly degraded stain materials of human origin.

Further studies have to be carried out, firstly to develop additional primer systems in order to increase the numbers of loci suitable for hair analysis and secondly to develop methods to enable analysis of more than two loci from one single hair extract.

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