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CASE REPORT

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Identification of the minor component of a mixed stain by using mismatch primer-induced restriction sites in amplified mtDNA

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Abstract We report a case in which STR typing failed to identify the minor component of a mixed saliva stain, but a mitochondrial restriction analysis succeeded in discriminating between the two components. To identify the nt16093 and nt16265 transitions, the template was amplified with the mismatch primers L16092-mm16085 and H16266-mm16269. In the presence of the transitions the mismatch primers created a *Bsa*B I and a *Cac*8 I restriction site, respectively. Subsequently, aliquots were restricted separately using the enzymes *Cac*8 I and *Bsa*B I which clearly identified the minor stain component.

Keywords Mitochondrial DNA · Restriction sites · Mixed stain · Forensic testing · Mismatch primer

Introduction

In most instances STR analyses appear to be superior to mitochondrial DNA (mtDNA) typing as they yield an enormously high level of individualisation and exact computational results even in mixed stain cases [5]. However, there are situations in which an STR analysis of stains fails and usable results can only be obtained by employing mtDNA analysis. We report a case in which STR typing of a mixed stain failed, but a mitochondrial restriction analysis following the mismatch primer technique succeeded in detecting the characteristic features of the minor component of the stain.

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Case history

While breaking and entering, a burglar drank a bottle of cola empty at the scene. This was discovered as the owner could remember that he had already drunk only about three-quarters of it. Hence, it could be assumed that there was a mixed saliva stain on the neck of the bottle caused by two contributors. The police found a suspect and asked us to identify both DNA profiles of the mixed stain but by performing an STR analysis and D-loop sequencing we were only able to establish the profile of the owner. Additional STR peaks did not clearly exceed the background noise level. Therefore, we performed a restriction analysis to identify the assumed mt DNA pattern of the suspect.

Materials and methods

Wipe-off specimens on Q-tips from the cola bottleneck and buccal swabs from the persons involved were used to extract DNA using a phenol-chloroform extraction protocol [9]. First we performed an STR analysis following the established routine methods on samples of the potentially involved contributors and partially also on the stain DNA. Our investigations included singleplex PCR reactions for the TH01, D21S11, FGA, ACTBP2 and D12S391 systems. As we did not succeed in detecting the minor component of the mixed saliva stain with these methods, we stopped the STR analysis and followed the mtDNA analysis strategy.

We started sequencing of the regions HVI and HVII by amplifying the entire D-loop with the primers L15926 and H00580 in compliance with the appropriate cycling protocol as suggested by Orrego and King [10]. DNA isolated from the buccal swab was added in quantities of approximately 10 ng. The saliva stain was amplified by employing different DNA quantities from 10 down to about 0.05 ng in order to find the optimum ratio for the analysis of mixed stains. The DNA quantity was established by means of the QuantiBlot kit (Applied Biosystems, Foster City, CA) as a more specific method of quantifying the mtDNA was not commercially available. In the course of further sequencing we used the RR Dye Terminator Sequencing kit (Applied Biosystems) and followed the protocol of Parson et al. [11] with minor modifications.

Having established that conventional sequencing methods revealed the stain component of the victim only to a certain extent, we checked whether the profile of the suspect could be identified with the help of a restriction analysis. To create restriction sites including the nt16093 and nt16265 transitions, we amplified the samples of both contributors as well as the mixed stain with the mismatch primers L16092- mm16085 (CCATCAACAACCG**A**TATGTAT) and H16266- mm16269 (TTTGTTGGTATCCTAG-

CGGG) resulting in 213 bp fragments. In the presence of the nt16093 transition, the primer L16092- mm16085 created a *Bsa*B I restriction site during amplification and 17 bp could be cut off from the PCR product. In the presence of the nt16225 transition, the primer H16266- mm16269 induced a *Cac* 8 I site which reduced the amplification product by 18 bp, whereas the product amplified from the control DNA remained uncleaved at this site. The PCR set-up (except the primers) was comparable to that used for amplification of the entire D-loop region: 94°C for 180 s (initial soak), 94°C for 40 s, 52 C for 40 s, 72°C for 40 s over 30 cycles and 72°C for 180 s (elongation).

By treating the amplification products with restriction enzymes, we checked whether the stain contained a DNA component with the nt16265 and nt16093 transitions, respectively. To this end 8 µl of the PCR product was mixed with 10 U of each of the restriction enzymes and 1 µl of the appropriate buffer. Digestions with *Cac*8 I and *Bsa*B I (New England BioLabs, Beverly, MA) were performed in separate set-ups.

To examine amplification products and restriction results, 6 µl crude PCR product and 6 µl enzyme-treated PCR product were separated by horizontal electrophoresis and silver stained as described previously [13].

Quality assurance

At our institute all stains and samples from suspects and victims are analysed by different members of staff in different laboratory areas. The laboratories are located in separate buildings and never exchange any reagents or equipment. Data are exchanged by electronic media without any paper transfer therefore cross-contamination is excluded. Hence, it was not critical in this case to deviate from the recommended sequence of operations in preparing the samples [14].

Results

The STR technique failed to identify components of the mixed saliva stain from the neck of the bottle. As shown in Table 1 for this particular case of a mixed stain analysis, the systems TH01, D21S11 and FGA were informative only to a limited extent or were not informative at all. An STR analysis of the stain in the systems ACTBP2 and D12S391 only revealed the alleles of the victim. The intensity of additional peaks under consideration did not significantly rise above the level of background noise. Table 2 depicts the results obtained by mtDNA sequencing of the same samples. Deviations from the Cambridge Reference Sequence (CRS) in the sequences of the victim and the suspect were concordant in positions nt00073, nt00263, nt00309.1, and nt00315.1 and differences between both persons were established in 5 positions. The sequence of the suspect was characterised by the transitions nt16093 and nt16265, but these features could not be detected when sequencing the saliva stain from the bottleneck. This sequence however, was directly indicative of a mixed stain at position nt16224. The other sites revealing a difference between the victim and the suspect did not yield conclusive proof of the existence of a minor component. Figure 1 depicts three sections of the mtDNA sequences in the stain, including the positions nt16093, nt16224, and nt16265.

To identify the minor component in question, the stain was checked for the presence of templates containing the nt16093 and nt16265 transitions by using the mismatch primer technique. As a result the saliva stain PCR product yielded 196 bp and 159 bp bands after *Bsa*B I and *Cac*8 I digestion, respectively. (Figs. 2 and 3).These patterns are compatible with the assumption of a mixed stain comprising a major component from the victim and a minor component corresponding with the pattern of the suspect.

As could be established by using the database http://www.d-loop-base.de [15], the pattern of the nt16093 and nt16265 transitions is unique among 1,182 data sets from Germany (frequency 0.0008). A pairwise comparison offered only one sequence with only one additional different base pair. In comparison with other databases on an international scale [7], which offered at least three or more differences, this showed the closest similarity and led to the assumption that the German database was the relevant population sample as proposed by Forster et al. [3].

A pairwise analysis of complete profiles was carried out in the database and resulted in a mean sequence divergence (suspect versus database) of 4.37. The overall haplotype diversity of this dataset was 0.975 with a variance of 0.003. We calculated the match probability according to Balding and Nichols [1, 14] as 0.0017.

Table 1 STR typing results of the stain and two alleged stain contributors

STR	Victim	Suspect	Saliva stain
TH01	7/9.3	9.3/9.3	Ø
D21S11	29/30	28/29	Ø
FGA	21/24	20/24	Ø
ACTBP2	16/23.2	26.2/27.2	16/23.2/(26.2?)/(–)
D12S391	17/20	18/22	17/20/(–)/(22?)
VWA	14/18	14/20	14/18/(–)

(Ø) Stain STRs were not investigated when the alleles of the suspect were expected to superimpose the alleles and/or the stutter peak areas of the victim's pattern.

Table 2 Mitochondrial DNA profiles analysed in suspects and victims oral swabs and the saliva stain

HV2	Victim	Suspect	Stain
L00073	G	G	G
L00152	T	c	T
L00263	G	G	G
L309.1	C	C	C
L315.1	C	C	C
L16093	t	C	t
L16224	C	t	C(t)
L16265	a	G	a
L16311	C	t	C (t)

Nucleotides in capital letters indicate deviations from the CRS, nucleotides in parentheses indicate low intensity signals.

Discussion

In some cases it is not possible to differentiate between the minor component alleles and the major component stut-

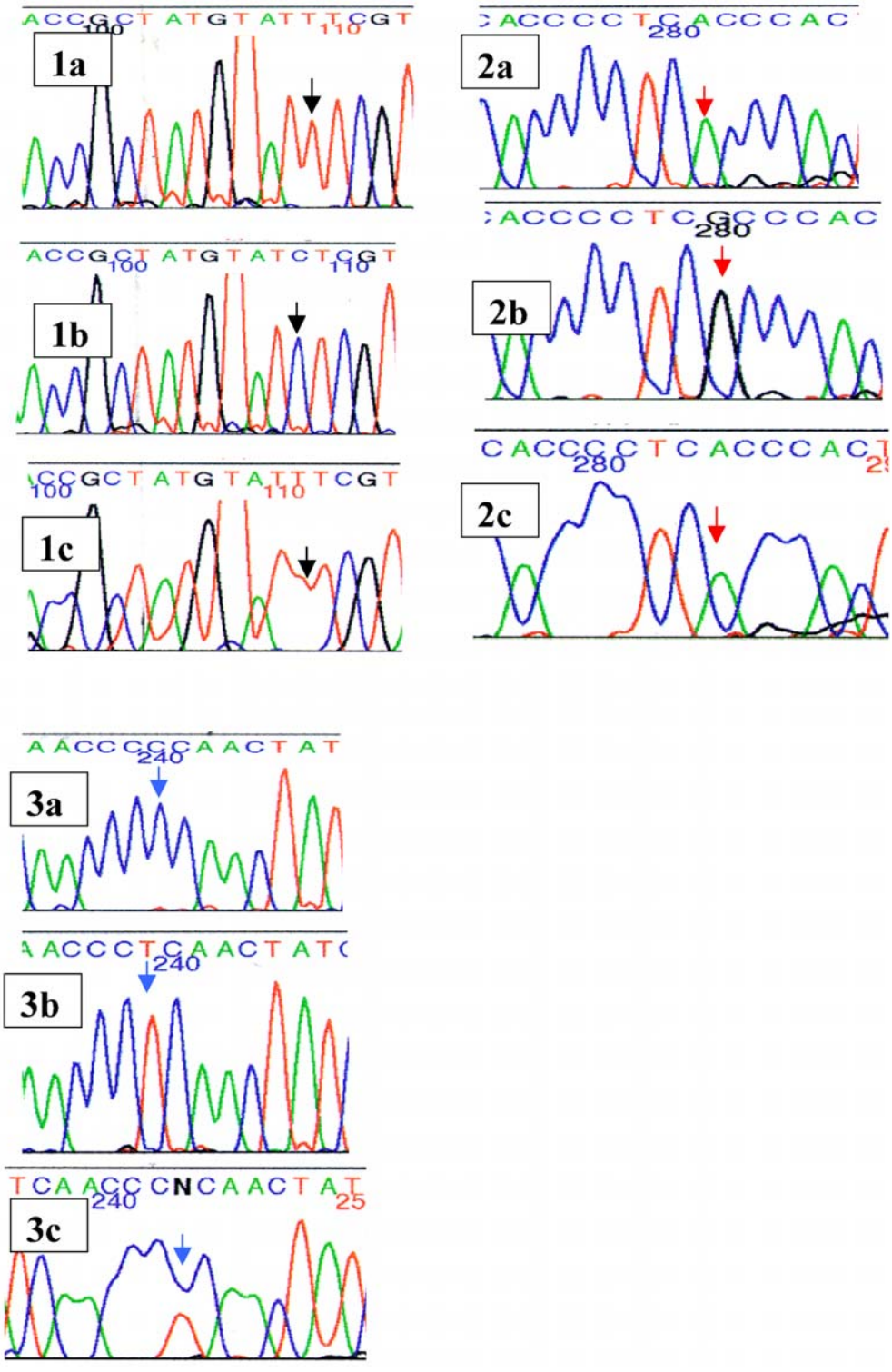

Fig. 1 Sequence sections of interest in the case under investigation. Victim: 1a, 2a and 3a; suspect: 1b, 2b and 3b; stain: 1c, 2c and 3c. The arrows indicate the positions L16093 (1a–c), L16265 (2a–c) and L16224 (3a–c)

ter peaks using the STR typing method [2, 12]. The literature on experimental studies in quantified mixed stains suggests that the detection limit of the minor component may vary to a major extent in STR analysis, depending on the locus under investigation, the mixing ratio and the absolute DNA quantities of the stain components [4, 12]. Possibly, minor components cannot be detected due to a competition between DNA components during PCR. Such competition does not occur when mixed mtDNA stains are amplified as there is only a minimum difference between the lengths and sequences of major and minor components. However, the detectibility in sequencing is limited because minor component signals could possibly be masked by the background of the major component, although this

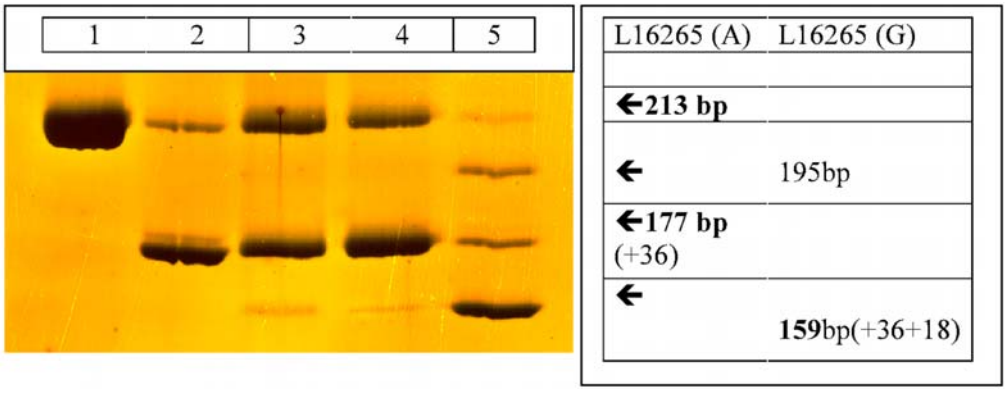

Fig. 2 PCR with the primers L16092-mm16085 and H16266-mm16269 yields fragments of 213 bp in length. First in lanes 2–5 the amplification product was cleaved by the enzyme *Cac*8 I, yielding 177 bp and 36 bp fragments as a result of a constant site. The 36 bp fragments do not appear in the electrophoretic picture. In the presence of a L16265 transition, the PCR with the primer H16266-mm16269 creates a *Cac*8 I site cutting the 177 bp fragment into 159 bp and 18 bp. Due to partial digestion, residual 213 bp bands (*lanes* 2–5) and a 195 bp fragment (*lane* 5) survive. *Lane 1* Untreated PCR product, *lane* 2 *Cac*8 I restricted PCR product of template with allele L16265 A, *lane* 5 *Cac*8 I restricted PCR product of template with allele L16265 G, *lanes* 3 and 4 *Cac*8 I restricted PCR product of template from the mixed stain. In addition to the main bands of the major component, the 159 bp band of the minor component can be identified

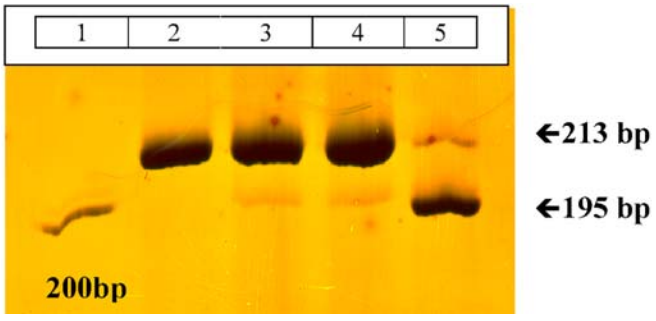

Fig. 3 PCR with the primers L16092-mm16085 and H16266-mm16269 creates a polymorphic *Bsa*B I site through mismatch with the primer L16092-mm16085. *Bsa*B I cleaves 17 bp from the PCR product in the presence of the L16093 transition (*Lanes 1* 100 bp ladder, *2 Bsa*B I-treated PCR product with allele L16093T not restrictable by the enzyme, *3 Bsa*B I-treated PCR product with allele L16093C restrictable by the enzyme, *3* and *4 Bsa*B I-treated PCR product from the mixed stain with L16093 transition in the minor component showing the 195 bp band of the minor component

phenomenon is also dependent on the sequence location. Morley et al. [8] reported a systematic study on mixed stains using the minisequencing technique and observed a similar phenomenon when DNA/DNA mixtures were prepared in a ratio of 1:10. Unfortunately, dot-blot DNA quantification kits that are commercially available at present are not appropriate for mtDNA quantification. Hence, the sensitivity of methods for detection of a minor component in a mixed stain cannot be estimated exactly but new quantification methods using real-time amplification [16] may help to overcome this dilemma in mtDNA typing in the future. Nevertheless, for investigating mixed stains with unknown component ratios, several typing techniques can be considered. When SNPs form polymorphic restriction sites, they can be easily identified using restriction enzymes. This approach allows us to recognise the minor component on a gel without any background. It is known that natural and mismatch primer-induced restriction sites can be used for examining heteroplasmic mtDNA populations [6, 13]. The present case report demonstrates that the application of this method under suitable circumstances may contribute to solving a case of mixed stains.

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