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CASE REPORT

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Forensic mass screening using mtDNA

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Abstract At the forensic autopsy of a sexual murder victim, some trace hairs, possibly belonging to the perpetrator, were saved. Initially, the analysis of a pubic hair shaft only revealed the presence of the mitochondrial (mt) DNA haplotype profile consisting of the $(CA)_6$ allele and the complete hypervariable region 1 (HV1) and 2(HV2) sequence. Later, typing of some further telogene trace hairs, which had been stored for several years, yielded a nuclear short tandem repeat (STR) profile. We used both the mtDNA haplotype and the STR profile to start a DNA mass screening project involving 2,335 male citizens of the relevant communities. MtDNA screening was carried out by using the CA repeat amplification in combination with an SNP typing procedure based on the restriction site analysis of amplified d-loop sequences. The aim of our paper is to put mass screening with mtDNA up for discussion.

Keywords MtDNA · Mass screening · Dinucleotide repeat · Sexual murder · Restriction sites

Introduction

All over the world, the most highly developed countries now have government-operated databases containing nuclear DNA profiles of known offenders. Many crimes have been solved where a database search revealed a match between the DNA profile of a blood, hair, saliva or semen sample left by the perpetrator at a crime scene and the profile of a known individual in the database. Otherwise, where the DNA profile of a perpetrator is known to the police but identity cannot be established, a DNA mass screening or "dragnet" involving the voluntary submission of samples by large numbers of individuals in the relevant community for DNA testing, has been helpful method in several cases. In Germany to date, participation in such forensic mass screening, performed independently from any initial suspicion, has always been voluntary. Nevertheless, data protection activists are trying to restrict such procedures. To our knowledge, dragnet strategies in the past were always based on nuclear DNA profiles and there are no reports in the literature on mass screening using mitochondrial (mt) DNA profiles. From a statistical point of view, a full nuclear DNA profile including eight (or more) short tandem repeats (STRs) is unique in several millions or even billions of people; therefore, accidental matches can be excluded in all probability. In contrast, mtDNA is not individual. Due to its transmission, free of recombination through female lines, a certain mtDNA haplotype will regularly occur several times in a population. Therefore, the evidential power of mtDNA profiles, compared to nuclear DNA profiles, is much lower. Nevertheless, it is well established that mtDNA typing can be a very useful tool in forensic casework [13, 16, 34, 35]. In particular, in cases where only hair traces are available, mtDNA analysis is a promising method [28]. Recently, we have successfully performed an mtDNA screening in a sample of 2,335 men and solved a case of sexual murder. In our case report we refer to this particular case and put the mtDNA dragnet procedure up for discussion.

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Case history

In 1995 a 7-year-old girl fell victim to a sexual murder. Her body was dumped in a pond and found 13 days later. At the forensic autopsy, some hairs, possibly belonging to the perpetrator, were recovered and stored. As a result of

several singleplex amplifications in analysing pubic hair DNA, we were able to generate an STR profile; however, this profile was not sufficient to identify the perpetrator. The analysis only yielded a highly vague pattern. Only some TH01, SE33 and CD4 signals were found, which could be also interpreted as background noise. Moreover, the victim's alleles in the mixed stain also contributed to the confusing results. Finally, an mtDNA analysis was successful in that it provided a haplotype profile consisting of the $(CA)_6$ allele and the complete hypervariable region 1 (HV1) and 2(HV2) sequence. Hence, the police asked 211 men with close links to the victim's family and/or the site of the crime for saliva specimens. MtDNA screening yielded no match. Several telogen hairs from this case, thought not to be capable of providing DNA sufficient for STR analysis, were stored. Later, after a new approach of analysing degraded DNA samples [12] had been published in 2004, the Bundeskriminalamt (BKA—German Federal Office of Criminal Investigation) resumed investigation using the stored hairs. By means of primers, which produced very short amplicons, nuclear DNA profiles were obtained from six hairs. While five hairs clearly showed the victim's profile, one hair could be identified as being of male origin. Thus, about 10 years after the crime, the result obtained by the BKA was reason enough to start a mass screening action in a sample of 2,335 men, of the appropriate age and living near to the scene of the crime. Both STR and mtDNA patterns were used as search criteria.

Materials and methods

DNA was extracted from hairs as published [12]. The pubic hair was analysed immediately after the crime (Table 1). The other six hairs were stored and investigated by the BKA team later. DNA extraction from buccal swabs was performed using Nucleo Spin Tissue Kit according to manufacturer's recommendations (Macherey-Nagel, Düren, Germany).

Table 1 MtDNA profiles of the Cambridge Reference Sequence [1], victim, hair trace and perpetrator

Polymorphism	CRS	Victim	Hair trace	Perpetrator
L00073	A	A	G[a]	G
L00152	T	T	C[a]	C
L00263	A	G	G[a]	G
L315.1	–	C	C[a]	C
CA repeat	$(CA)_5$	$(CA)_5$	$(CA)_6$	$(CA)_6$
L16093	T	T	C	C
L16224	T	T	C[a]	C
L16311	T	T	C[a]	C

CRS Cambridge Reference Sequence
[a]The position detected by conventional sequencing and confirmed by sequencing with stain selective primers

Nuclear DNA analysis

In 1995, our attempts to establish an STR profile from the trace hair were based on relevant publications using singleplex amplification methods [23, 31, 37]. The BKA team obtained results for TH01, VWA, FGA, D3S1358, TPOX and FES from six hairs, as published [12]. For the dragnet screening on the sample of 2,335 buccal swabs, the test kit Nonaplex I (Biotype AG, Dresden, Germany) was applied. In one sample a homozygosity of FGA was analysed using two further kits, i. e. Nonaplex II (Biotype AG) and Powerplex SE (Promega, Madison, WI).

MtDNA sequencing

The mt(CA) repeat and the HV1 and HV2 mtDNA sequences of the pubic hair and seven buccal swabs were analysed as described before [33, 34]. But as the hair trace was contaminated with the victim's blood, the extracted DNA contained the victim's DNA as a minor component. To verify our first interpretation of the perpetrator's mtDNA profile, we carried out a second sequencing procedure using primers mismatching the victim's sequence. This approach, described in detail earlier [32], yielded the proper perpetrator's mtDNA haplotype. The following sequences show the stain-specific selective primers with their mismatching positions underlined:

1. L16224(16222^{mis}/16224^{sel}):GCAAGTACAGCAATC AACTCC
2. L00073(00071^{mis}/00073^{sel}):TATTTTCGTCTGGGGG ATG

MtDNA (CA) repeat and SNP analysis

Based on the trace mtDNA haplotype, we started five individual assays suitable to analyse each of the trace-relevant d-loop SNP loci. Using a binary exclusion system, we tested the buccal swab DNA specimens for the following loci: $(CA)_n$, nt00152, nt16223, nt16311 and nt00073. The loci were tested in the above order. Hence, we were able to exclude, in a stepwise manner, groups of specimens from further investigations. Finally, all but seven out of the 2,335 specimens were eliminated.

The (CA) repeat analysis procedure was published earlier [33]. All other loci were tested using a restriction site approach as described [35] for forensic casework. Some of the SNPs yielded natural restriction fragment polymorphisms (RFLP). In such cases amplicons were prepared, treated with the appropriate restriction enzyme and electrophorised. Initially, the SNP at the loci nt16223 and nt00152 could not be detected by restriction tests. Hence, we inserted artificial restriction sites along with the PCR in both cases by applying mismatch primers. Thus, we were able to investigate the SNPs in the same manner as natural RFLPs by treating the amplicons with the appropriate restriction enzyme. The primers and restriction

enzymes used in typing the SNPs and the $(CA)_n$ repeat are shown in Table 2. Primer synthesis was carried out by Eurogentec (Seraing, Belgium). All restriction enzymes were supplied by New England Biolabs (Ipswich, MA, USA).

Amplifications were carried out in a uniform manner for all loci using a special 25-μl singleplex mixture, which contained 1–2 ng DNA, 0.4 μM each primer, 15 mM Tris/HCl, 50 mM KCl, 100 μM each deoxyribonucleotide triphosphate, 1.5 mM $MgCl_2$ and 1 U Goldstar polymerase (Eurogentec, Seraing, Belgium). Amplification procedures carried out in a thermocycler (Biometra, Göttingen, Germany) were started with 12-min soak and enzyme activation at 95°C, followed by 28 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for a 10-min final extension.

Successful amplification was controlled by running a 5-μl aliquot in PAGE. A 5-μl aliquot of the PCR product was subjected to enzymatic digestion using 5 U of the appropriate enzyme overnight. All conditions were in strict compliance with the enzyme manufacturer's recommendations. Electrophoretical runs and the silver staining of amplicons and the digested products were carried out as published in the literature [5, 37]. Inclusion criteria of the wanted profile are described in Table 2.

Results

The analysis of the pubic hair, executed immediately after the autopsy, yielded the mtDNA haplotype (Table 1), but a clear STR profile was not be obtained. However, the nuclear DNA typing of the six stored hairs carried out by the BKA team was successful. Five hairs turned out to belong to the victim, whereas one hair's DNA profile was different and of male origin. This result gave reason to start the mass screening project on the basis of TH01, VWA, FGA, D3S1358, D18S51, TPOX and FES results (data not shown). The matching probability of this DNA profile was calculated to be 1 in 87.0 billion. One sample profile was nearly identical and differed only in one FGA allele. Whilst the hair pattern was typed as FGA 21–23, the buccal swab showed 21–21. The latter result could be reproduced with three different forensic kits. Summarising the above, we could not find any complete match between the assumed perpetrator pattern and the buccal swabs during nuclear DNA screening. However, except for FGA, one buccal DNA specimen matched the trace hair.

Concurrently, an mtDNA screening was carried out. The procedure of stepwise exclusion of samples is demonstrated in Table 3. The selected sequence of the SNP typing tests was based on the power of discrimination of each single SNP, as obtained from an mtDNA database search. Using the $(CA)_6$ allele as the inclusion criterion in a first step, we excluded approximately 92% of all buccal swabs from further investigations. Following the analysis of the nt73 locus, which was executed as the last step of the SNP typing tests, only seven buccal swab DNAs remained to be subjected to a full sequencing procedure. Finally, three buccal DNA patterns perfectly matched the pubic hair mtDNA profile. One of the donors was identical, with the suspect established in the nuclear DNA mass screening procedure. Thus, the perpetrator was identified and confessed the crime immediately during the first interrogation.

Discussion

In the actual case reported here, the cause for the FGA divergence between the trace DNA and that provided by the perpetrator during the screening procedure remains unclear. It is known that in low-copy DNA amplification, stochastic effects may produce artefacts [15]. This problem can be reduced, though not fully overcome, by using primers producing very short amplicons. Our screening procedure to type the d-loop SNPs of interest was carried out using a very conventional approach. Restriction site analyses of amplicons generated by PCR have been used as a mutation typing method since the introduction of clinical molecular genetics [9] and mtDNA typing [2, 7, 14].

Today, more sophisticated techniques, such as SNaPshoot minisequencing [3, 8, 22, 30], are available. For

Table 2 Overview of primers and the testing regime for five mtDNA loci used for the mass screening

D-loop SNP	Primer 1	Primer 2	Analysis method		
			Restriction site	Enzyme	Wanted
16224	L16038 TCTGTTCTTT CATGGGGAAG	H16225 (mis16227) GCAGTTGATGTGTGATAGC^{m}TG	CTNAG	*Dde*I	Uncut
16311	L15996 CTCCACCATT AGCACCCAAAGC	H16498 CCTGAAGTAGGAACCAGATG	GCN_7GC	*Mwo*I	Cut
00073	L00029 TCTATCACCC TATTAACCAC	H00408 CTGTTAAAAGTGCATACCGCCA	GTGCAC	*Apa*L1	Cut
00152	L00029 TCTATCACCC TATTAACCAC	H00153 ACGTAGGTGCGATAAATC^{m}AT	CATG	*Nla*III	Cut
$(CA)_6$	L00484 CTCCCATACT ACTAATCTCA	H537 TGGTTGGTTCGGGGTATG	PAGE		

Restriction sites induced by mismatch primers are marked with an m at the primer's mismatch position

Table 3 Procedure of stepwise exclusion of specimens from further investigations

Locus	Number of specimens investigated	Restriction enzyme	Criterion for inclusion	Number of exclusions (%)	Number of inclusions (%)
$(CA)_n$	2,335		$(CA)_6$	2,148 (92)	187 (8)
nt00152	187	*Nla*III	+[a]	131 (70)	56 (30)
nt16223	56	*Dde*I	Uncleaved	31 (55)	25 (45)
nt16311	25	*Mwo*I	+[a]	12 (48)	13 (52)
00073	13	*Apa*LI	+	6 (46)	7 (54)
d-loop (cycle sequencing)	7	–	Full frequency match	4 (57)	3 (43)

Plus signs (+) indicate cleavage of a polymorphic restriction site in the amplicon
[a]The existence of a non-polymorphic restriction site

certain patterns and high-throughput applications, the DNA chip technology can be used [10, 11]. We followed a low-tech approach adapted to our specific requirements, which was suitable as a dragnet method for the screening of several thousand specimens.

In our screening procedure the majority of samples was excluded on the basis of the $(CA)_6$ repeat motif. Thereafter, we used the SNPs nt00152, nt16223, nt16311 and nt00073 in the given order, under due consideration of their exclusion power and other practical aspects. Although this approach was successful, we have learned in retrospective discussions that the chosen order was suboptimal. Apart from nt16093, nt00152 exhibits the highest mutation rate within the control region, and is therefore a candidate prone to show mutation between hairs and other tissues. Hence, there is a considerable risk of erroneously excluding a perpetrator by using this site as the screening SNP. Another risk is heteroplasmy, which often occurs at this point. In contrast, nt16223 and nt16311 also exhibit a good power of discrimination (PD), but do not involve the risks described. As we had to do with a mixed stain, the point of heteroplasmy was not an issue. However, SNP mutability should be considered when using the above screening procedures in future investigations [30].

Apart from describing the procedure, we also want to pose the question whether the mtDNA dragnet is a suitable and permissible tool. The case reported here indicates that matches between mtDNA of trace and of buccal swabs of a population can support police investigations in that the police can focus their attention on a few individuals, or even on one person only. On the other hand, mtDNA patterns are never truly individual. Hence, mtDNA mass screenings involve the potential risk of not excluding all innocent people, who may finally suffer inquiries by the police. Initially, this was also the reason why the public prosecution refrained from starting an mtDNA mass screening immediately after the crime. However, we feel, with respect to some unspecific results, that the mtDNA dragnet method does not significantly differ from other manhunt methods, such as the publication of photofits. Of course, an mtDNA mass screening should not be performed for a more common mtDNA haplotype. Although our database did not contain any relevant entry for our specific sequence, it could have been more frequent on a regional scale. Population studies published in the literature [18, 25, 29] and databases [4, 21, 26, 27, 38] may be helpful to obtain information on whether a specific haplotype is rare enough to perform a screening. Mention should also be made of the opportunity to subtype mtDNA of a more frequent haplotype with regard to HV1 and HV2, using further d-loop polymorphisms [17, 19, 24, 36] and even SNPs in the coding area [6, 8, 20, 30].

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