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CASE REPORT

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A Rape Case Solved by Mitochondrial DNA Mixture Analysis

ABSTRACT: In cases of stains that contain mixed DNA from different contributors, analyzing mitochondrial DNA (mtDNA) requires the use of cloning techniques. We developed an efficient cloning technique that was applied in a rape case. After a differential lysis-based DNA extraction from vaginal swabs, hypervariable region I and II (HVI, HVII) amplicons obtained from the male fraction were cloned. Although we mainly found the victim's haplotype, we were able to detect the suspect's haplotype in two clones for HVI and in one clone for HVII. As the midpiece of the flagellum, which contains mitochondria, can be lost during the differential lysis, we also investigated the female fraction by cloning to evaluate the proportion of victim/suspect mtDNA. Unfortunately, only clones presenting the victim's haplotype were found. This case highlights the need for an optimal differential lysis protocol to enrich the male fraction not only with nuclear but also mitochondrial DNA

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When faced with mixed DNA samples, forensic scientists commonly use short tandem repeats (STR) analyses to distinguish the different contributors. However, such a study cannot be performed completely unless biological material compatible with STR investigations is available. For instance, when only shed hairs from suspected contributors are available, the resolution of a case relies mainly on mitochondrial DNA (mtDNA) analysis.

Mitochondrial DNA displays two important advantages over nuclear DNA. Firstly, mtDNA is present in thousands of copies in one cell, and thus analyses based on mtDNA are highly sensitive even when working with degraded samples or minute amounts of biological material. Secondly, it is maternally inherited, which enlarges comparison possibilities to distant maternal relatives. It is therefore widely used for forensic investigations (1).

Direct sequencing of amplicons of the hypervariable regions I and II (HVI, HVII) is generally performed to retrieve the haplotype of a sample. Confronted with DNA mixture, such a procedure results in a haplotype combination of the different contributors. Thus, amplicons have to be cloned to distinguish the different haplotypes.

In this report, we present a rape case where DNA mixture analysis was performed by means of an in-house cloning technique applied to mtDNA fragments.

Case Report

A 39-year-old woman was raped at knifepoint. As vaginal swabs were found to contain spermatozoa, DNA was extracted following a differential lysis protocol leading to two DNA fractions corresponding to the separated male and female cells (2). STR analysis, performed on the female fraction, yielded the victim's profile, whereas those made on the male fraction resulted in a mix between the victim and the offender with an apparent mixture ratio of 1:1. Some weeks after these analyses, a suspect was found, but the local police only supplied hair samples. Unfortunately, the transport of the hairs took several weeks and nuclear DNA extraction failed because of the long and inappropriate storage. In the meantime, the suspect had fled in another country, leaving no possibility for obtaining easily new samples. As a consequence, for the comparison of the suspect's sample, the DNA mixture from the vaginal swab had to be investigated by means of mtDNA analysis.

Materials and Methods

Standard Molecular Techniques

Standard molecular biology procedures were performed (3). Unless otherwise stated, all enzymes used in this study came from MBI fermentas (Lithuania).

Vector Preparation

Vector preparation was performed by digesting 10 μg pUC 19 with 100 U HincII in 100 μL reaction volume at 37°C for 2 h. Digested plasmid was further purified using a Geneclean® Spin kit from Bio101 (Qbiogen, Illkirch, France). One base tailing was achieved using 3 μg of digested vector, 2 U of terminal deoxynucleotide transferase and 500 pmol of ddTTP in a final volume of 20 μL at 37°C for 1 h. The tailing product was purified by means of a Geneclean® Spin kit. Digestion and tailing efficiency was assayed by overnight self-ligation of 30 ng vector at room temperature.

Cloning of mtDNA Fragments

Mitochondrial DNA fragments were amplified as previously described (4) in 30 cycles and by adding to the PCR a final extension step of 30 min, ensuring complete A-tailing of all PCR products. A total of 100 ng of the PCR product was directly ligated in presence

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of 30 ng of pUC19-tailed vector with 1 U of T4-DNA ligase in 20 μL final volume at 20°C overnight. The ligation products were treated for 10 min at 70°C and 1 μL of each was transformed into *Escherichia coli* TOP10 (F-*mcr*A *Δ*(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZΔ*M15 *ΔlacX*74 *rec*A1 *ara*D139 *Δ*(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G). After transformation, bacteria were plated on Lennox Broth-agar plates containing Ampicillin and X-Gal. After overnight incubation at 37°C, white/blue screening allowed the selection of clones.

Plasmid Preparation

Individual clones were picked and cultivated in duplicated 2 mL 96-well blocks for 24 h at 37°C under 220 rpm shaking. Bacteria were spun down by a 5 min centrifugation at 1500 *g*. The pellets obtained from duplicated plates were resuspended in 150 μL of S1 (50 mM glucose, 25 mM Tris pH8, 10 mM EDTA and 20 μg/mL DNase free RNase) and pulled together. Five hundred microliters of S2 9 [0.2 M NaOH and 1% sodium dodecyl sulfate (SDS)] and 350 μL S3 (3 M potassium acetate, 10% acetic acid) were added with vortexing following each pipetting. This lysate was centrifuged for 10 min at 3500 *g* at 4°C to precipitate cell debris. The supernatant was transferred into a new 96-well block and 1 mL of isopropanol was added to each well before centrifugation at 2500 *g* for 15 min. The precipitate was resuspended in 100 μL of water and mixed with 100 μL of 5 M LiCl and further incubated at −20°C for 15 min before centrifugation at 2500 *g* for 15 min at 4°C. The supernatant was recovered and plasmid DNA was precipitated by a 15 min centrifugation at 2500 *g* after addition of 500 μL of ethanol. A final washing step in 70% ethanol was applied before resuspending the DNA in 40 μL of water.

Plasmid Verification and Quantitation

Plasmids were diluted 20 times and 5 μL of the dilution was used as PCR template under the following conditions: 500 nM of each primer (PU + 5′-GTG AAT TCG AGC TCG GTA CC-3′ and RP + 5′-CAG CTA TGA CCA TGA TTA CG-3′), 2 mM $MgCl_2$, 200 μM dNTP and 1 U of *Taq* polymerase in 25 μL final volume. After a first 5 min denaturizing at 96°C, 15 cycles of 10 sec at 96°C, 30 sec at 60°C and 50 sec at 72°C and a final extension step of 2 min at 72°C were carried out. A plasmid containing a 400 bp HVI mtDNA fragment was used at 5, 10, 15, 20, 25, 35 and 50 ng per reaction as a positive control and for quantitation of prepared plasmids. The subsequent PCR products were visualized on a 2% agarose gel.

Plasmid Sequencing

Sequencing was performed with 4 μL of BigDye® terminator kit v1.1 mix (Applied Biosystems, Foster City, CA), 1.6 pmol PU (5′-GTT TTC CCA GTC ACG ACG TTG-3′) or RP+ primer and 5 μL of plasmid in a final 10 μL volume. Sequencing reactions were precipitated with 40 μL 80% ethanol and further desalted by a 70% ethanol washing step. The purified sequencing products were separated by capillary electrophoresis and detected on an ABI Prism® 3100 genetic analyzer (Applied Biosystems).

Restriction Analysis of Amplified Insert

The PCR reaction resulting from the insert check step was submitted again to 10 PCR cycles. Ten microlitters of the PCR product were digested by 20 U *Bcc*I (New England Bioloabs, Ipswich, MA) in a final volume of 20 μL at 37°C for 1 h.

Results and Discussion

Amplification of the HVI and HVII regions was performed for the suspect and victim reference samples and sequenced to determine their respective haplotypes (Table 1). Amplification of the same regions from the male fraction extracted from the vaginal swab and further cloning were performed in different PCR-dedicated laboratory rooms to avoid contamination. As all negative controls were clean, the HVI and HVII fragments were cloned using our homemade T/A cloning vector. Vector preparation yields enough material for 50 cloning experiments. The prepared vector can be stored up to 6 months at −20°C as a ready-to-use stock. The cloning presented a very high efficiency, as almost 99% of the clones contained an insert. Plasmid preparation was performed in 96-well blocks following the adapted alkaline-lysis protocol described in Materials and Methods. The quality, quantity and insert size of the plasmids were checked (Fig. 1). Only plasmids presenting amplification with intensities corresponding to quantities higher than 30 ng μL were further sequenced. This procedure allows not only the quantitation of the plasmid DNA, but also quality assay by detection of DNA preparation containing PCR-inhibitors which could make the sequencing, the most expensive step, fail. Starting from the clone cultures, the whole preparation, quantitation, and quality assay of the plasmid DNA of 192–384 clones can be achieved within a working day, which is very convenient knowing that cloning methods are generally labor-intensive and low-thoughput.

A sequencing strategy was applied to discover all the different mtDNA molecules present in the mixture rather than predigest the DNA mixture searching after the suspect's haplotype. After the sequencing of 48 HVI clones, only the victim's haplotype was recovered. On the contrary, after sequencing of 32 HVII clones, the haplotypes of the suspect and the victim were found. Therefore, the mtDNA mixture ratio seemed to be <1:10.

Thus, the mixture ratio in the HVI region amplification product was investigated. We searched for differential restriction sites in the

TABLE 1—*Reference haplotypes for the suspect and the victim.*

	HVI						HVII						
	Start	1	1	1	1	Stop	Start				3	3	Stop
		6	6	6	6			0	0	2	0	1	
		1	2	2	3			7	9	6	9	5	
		2	9	9	0			3	3	3	.	.	
		6	4	6	4						1	1	
Suspect	16025					16380	55		G	G	C	C	380
Victim	16025	C	T	T		16380	55	G		G	C	C	380

HVI, hypervariable region; HVII, hypervariable region II.

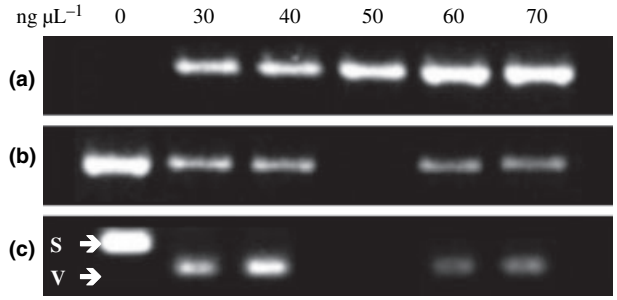

FIG. 1—*Plasmid insert check by semiquantitative PCR. Standard of known concentration (a). Amplification of the plasmid insert for six different clones (b). Digestion of the amplification of the plasmid insert to detect clones containing a haplotype corresponding to the suspect (S, 330 bp) and the one of the victim (V, 223 bp) (c). Gel electrophoresis was performed in a TBE-4% agarose gel.*

TABLE 2—*Digestion products of the HVI region amplification for the victim and the suspect.*

	Size in bp
Suspect	330, 76
Victim	223, 107, 76

HVI, hypervariable region I.

victim's and suspect's HVI sequences. The restriction enzyme *Bcc*I gives a differential restriction product of the two different amplicons (Table 2) because the restriction site includes the position 16294, which is mutated (T) in the victim but not in the suspect. Thus, the HVI amplification product from the male fraction was digested with *Bcc*I. The band of 330 bp corresponding to a part of the suspect HVI sequence was barely visible on agarose gel, but was excised and cloned. Because of low insert concentration, only eight clones were obtained. The plasmids were prepared and sequenced but only haplotypes presenting mutations T16126C, C16294A or G, and C16296T were found. These molecules derive from the victim's mtDNA by *Taq* errors and were selected because of the lack of thymine at position 16294. In a first approach, these results could point on a high *Taq* error rate because the same site seems to be hit several times by *Taq* point mutations. However this is not the case, but a result of the RFLP procedure which specifically selects molecules lacking the C16294T, putting this background noise to the foreground. Finding only haplotypes derived from the victim by *Taq* caused point mutations leads us to conclude that the mtDNA mixture ratio is extremely low.

Therefore, to avoid sequencing hundreds of complete HVI clones, we selected them by means of restriction analysis. The bias of this approach is that the suspect was targeted for the match, whereas additional variants, from alternative suspects for example, will not be detected. The plasmids of 192 clones containing the complete HVI region were prepared and the PCR amplification used to check the quality and quantity of the preparation was submitted to 10 additional PCR cycles and further digested with *Bcc*I. Out of the 192 clones, four presented a restriction profile corresponding to the lack of the C16294T mutation as illustrated in Fig. 1. Sequencing these candidates revealed two mutated haplotypes as previously found belonging to the background noise of the procedure and two haplotypes corresponding to the suspect. This points to a mtDNA mixture ratio of roughly 1:100.

The mtDNA profiles found in the mixture correspond to the victim's and suspect's haplotypes. The latter is not found in the downloadable FBI forensic mtDNA population database (http://www.fbi.gov/hq/lab/fsc/backissu/april2002/miller1.htm). We considered both HVI and HVII regions in all populations of the forensic database set (2008 records), because the French police never provides the ethnical origin of a suspect. A frequency estimator of 1.49×10^{-3} was calculated, corresponding to the upper bound of a 95% confidence interval taking into account database size and distribution bias (5). These biological evidences allowed the prosecution to require further biological samples from the police of the country in which the suspect fled.

It has been pointed out that the spermatozoa lose their flagellum and mid-piece, containing the mitochondria, during the first differential lysis step and therefore the male fraction was found to be exempt of sperm mtDNA (6). As these findings may explain our results, we decided to investigate the female fraction resulting from the differential lysis. The HVI region was amplified using the female fraction as a template and further cloned. The plasmid DNA from 96 clones was prepared and the PCR check amplification of the insert was digested with *Bcc*I to detect clones containing the suspect's sequence. Six clones, presenting a digestion profile indicating the absence of C16294T mutation, were sequenced but were identified as haplotype deriving from the victim's haplotype by a punctual mutation at position 16294 caused by the *Taq*. Thus, we did not find higher male mtDNA content in the female fraction. Such results could be explained by the difference in the mtDNA copy number between the female epithelial cells and the spermatozoa. Indeed, 200–1700 mtDNA copies can be found per somatic cell (1). In contrast, the spermatozoa, which have only 70 to 80 mitochondria with only one mtDNA copy per mitochondrion, present only roughly 100 mtDNA copies per cell (7). Therefore a ratio of 1:10 can be expected with an equal amount of each cell type, however in forensic samples, dominance of the female cells lowers significantly this ratio.

Although the spermatozoa tails were supposed to be retained in the female fraction of the differential lysis extraction, in this case we observed male mtDNA in the male fraction only. The development of new protocols allowing a conservative separation of the two cell types would improve mtDNA mixture analysis from rape cases.

Mitochondrail DNA was used as an investigational tool pointing out a preliminarily match with the suspect. Indeed, the obtained mtDNA haplotype match presented as an evidence for obtaining a sample adapted for STR typing. Fortunately, we were able to complete the match with STR profiling. Therefore, this investigation turned out to be a powerful application of the cloning approach.

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