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TECHNICAL NOTE

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mtDNA Investigations After Differential Lysis*

ABSTRACT: In order to gain information regarding the ethnic origin of an unknown offender in a murder case it was necessary to sequence the HV1 and 2 regions of the mitochondrial DNA (mtDNA). The only evidentiary material that could be linked to the suspect was DNA, extracted from spermatozoa after differential lysis. The observed mtDNA sequences were identical to the sequences of the victim. Therefore, we had to check if this was a coincidence or the result of a technical limitation of the testing procedure. Subsequently, we performed a systematic study. In cases with complete separation of sperm and female cells it wasn't possible to obtain a mtDNA sequence for the sperm fraction. This phenomena is based on the loss of the sperm's flagellum and mid-piece during the first lysis step and a concomitant loss of the sperm's mitochondria. In our murder case, a minor carry-over of female cells to the sperm fraction was responsible for the sequencing result.

KEYWORDS: forensic science, mtDNA, differential lysis, sperm

During investigations in a sexually motivated murder case, we were asked to gain information regarding the ethnic origin of the unknown offender. The only stain, which could be attributed to the offender, were sperm found on the vaginal swab of the victim. DNA from the sperm was extracted by differential lysis and organic extraction. A DNA profiling with eight autosomale STRs (genRES® MPX-2 amplification kit, Serac GmbH, Bad Homburg, Germany, (1)) as well as eight Y-chromosomal STRs (genRES® DYSplex-1 and 2) was carried out, which resulted in complete autosomale profile for the male and female fraction and a single source Y-chromosomal haplotype. Furthermore, we sequenced the HV1 and HV2 regions of the mtDNA from the sperm fraction, in order to obtain more information about the ethnic origin (2–4). The mtDNA from the vaginal cell fraction and a blood sample from the victim were sequenced in parallel. Surprisingly the sequences from all three samples were identical. A search in the mtDNA Population Database of the U.S. Department of Justice, FBI, revealed that this sequence showed a frequency of 0,0066 in a Caucasian population sample of 304 individuals (www.fbi.gov/hq/lab/fsc/april2002/mtDNA.htm).

The aim of this study was to check if this match was a coincidence or the result of a technical limitation of the testing procedure. In order to distinguish between these two possibilities, we investigated vaginal swabs, taken from test persons 24 h post coitum, which showed numerous sperm in a Hematoxylin-Eosin (HE) stained slide. The swabs were treated like the casework sample except that the sperm pellets were washed four times instead of only three times to make sure that there was no carry-over with vaginal cells. The completeness of the separation was checked by autosomal STR profiling. Additionally, some cases of incomplete differential lysis were tested.

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Materials and methods

Samples

Four vaginal swabs, taken from test persons 24 h post coitum, 4 vaginal swabs from routine case work showing an incomplete lysis and a neat semen sample were investigated. Saliva samples from the involved persons were tested in parallel.

DNA Extraction

From the sperm sample, DNA was extracted using 200 μL 5% Chelex solution including 0.25 mg proteinase K and 0.05 M DTT (5). For differential lysis of the vaginal swabs 1 mL lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 2% SDS, 10 mM EDTA) containing 0.5 mg proteinase K was added to the vaginal swabs in the first step and incubated by 56°C for 2 h. A small hole was punched into the bottom of this tube and the tube placed into a larger tube. The lysis buffer was collected in the larger tube by centrifugation for 8 min by 4000 g. The supernatant in the larger tube (vaginal cell fraction) was removed and additionally 1.25 mg proteinase K was added and incubated for 2 h at 56°C. The pellet was washed three or four times with 1 mL lysis buffer and then 1 mL lysis buffer containing 1.25 mg proteinase K and 0.05 M DTT was added and also incubated by 56°C for 2 h. After lysis the samples were extracted with phenol/chloroform, DNA was precipitated with 2.5 vols ethanol and the pellets were resuspended in 50 μL H_2O bidest.

Microscopic Examination

From all vaginal swabs and the corresponding pellets obtained after the first lysis step, smears on slides were made and stained

with Hematoxylin-Eosin (HE). Also samples of the pure seminal fluid and the pellet, obtained after the first incubation step during the chelex extraction, were investigated likewise.

STR Profiling and Electrophoresis Conditions

A multiplex PCR was performed using the genRES MPX-2 amplification kit (Serac GmbH, Bad Homburg, Germany). 1–3 ng DNA sample (in a total reaction volume of 25 μL) was used for amplification in a PE 9600 thermal cycler according to the manufacturer's instructions, using a 30 cycle program. The PCR products were analysed on a ABI PRISM 310 (Applied Biosystems, PE Corporation, Foster City).

Amplification and Sequencing of mtDNA

The amplification of the HV1 region was carried out according to Holland and Vigilant (6,7), except that AmpliTaq Gold (Applied Biosystems, PE Corporation, Foster City) was used. Cycle conditions were 95°C for 12 min, followed by 32 cycles of 95°C for 30 s, 60°C for 40s, 72°C for 20 s and a final extension step at 72°C for 10 min. The yield of the amplification was checked by agarose gel electrophoresis and ethidium bromide staining. Subsequently, the PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequence analysis was performed with the ABI PRISM BigDye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City) in both directions. The amplification and sequencing primers were as fellows (6,7):

Ampl. HV1H: 5′-TTA ACT CCA CCA TTA GCA AA-3′ (F15971)
Ampl. HV1R: 5′-TGA TTT CAC GGA GGA TGG TG- 3′ (H164019
Seq. HV1H: 5′-CAC CAT TAG CAC CCA AAG CT-3′ (L15997)
Seq. HV1R: 5′-GAG GAT GGT GGT CAA GGG AC-3′ (R16410)

Excess dye terminators were removed with Centri-Sep columns after sequencing and the products were analysed on a ABI PRISM 310 (Applied Biosystems, PE Corporation, Foster City) using POP6.

Results

As shown in Fig. 1, we did not obtain any HV1 amplification product for the four sperm fractions derived from the vaginal swabs after differential lysis. Successful amplification of the mtDNA could only be seen for the vaginal cell fractions. The resulting sequences matched the sequences of the female test persons. Even at sequence positions where the female and the male partner differed, no minor peak could be detected for the male sequence in the electropherogramm. The subsequent autosomal STR profiling revealed that the differential lysis resulted in complete separation of the female cells from the sperm fraction (data not shown).

All sperm fractions, showing a mixed autosomal STR profile (which indicates incomplete separation) led to mtDNA sequencing results. The observed sequence matched the corresponding female profile (data not shown). Further swabs from the same cases were investigated a second time, but now with four instead of three pellet washing steps during the differential lysis step. This treatment resulted in a complete separation of the female cells and the sperm fraction (see MPX-2 profiles, Fig. 2) and again no HV1 amplification products could be shown for the sperm fraction (see Fig. 3).

The HE-stained slides, made from the native vaginal swabs and the sperm pellets after the first lysis step, were examined microscopically. In the native samples 10 to 20% of the sperm possessed a flagellum, whereas after the first lysis and centrifugation no flagellum could be seen at all (see Fig. 4*a,b*). The slides made during DNA extraction from the seminal fluid with chelex (before and

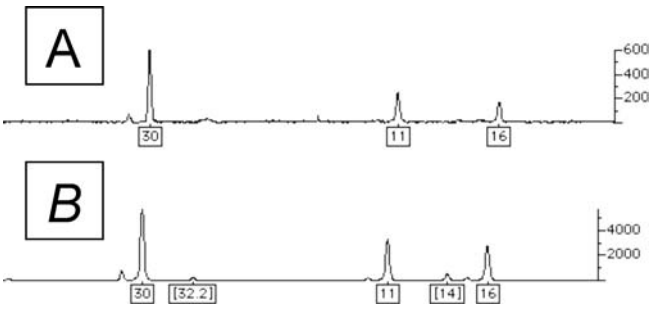

FIG. 2—*STR profiles of the sperm fractions from two vaginal swabs taken at the same time from the same victim. A: Complete separation after four washing steps; B: Three washing steps, resulted in a incomplete separation of female and sperm cells (female alleles in brackets).*

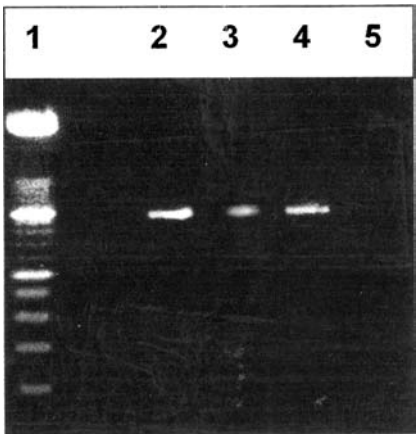

FIG. 3—*Agarose gel electrophoreses after amplification of the HV1 region: Lane 1 size standard, lane 2 vaginal cell fraction incomplete lysis, lane 3 sperm cell fraction incomplete lysis, lane 4 vaginal cell fraction complete lysis and lane 5 sperm cell fraction complete lysis.*

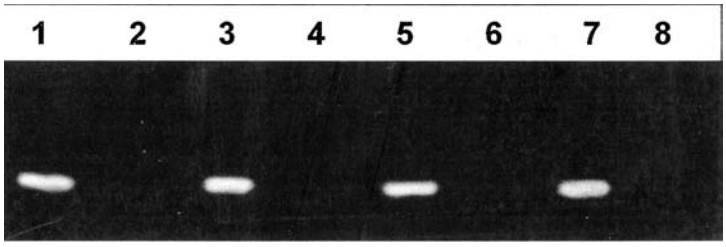

FIG. 1—*Detected HV1 amplicons (~400 bp) in an agarose gel electrophoreses after amplification: Lane 1, 3, 5, and 7 vaginal cell fractions, lane 2, 4, 6 and 8 sperm cell fractions derived from test person 1 to 4, respectively.*

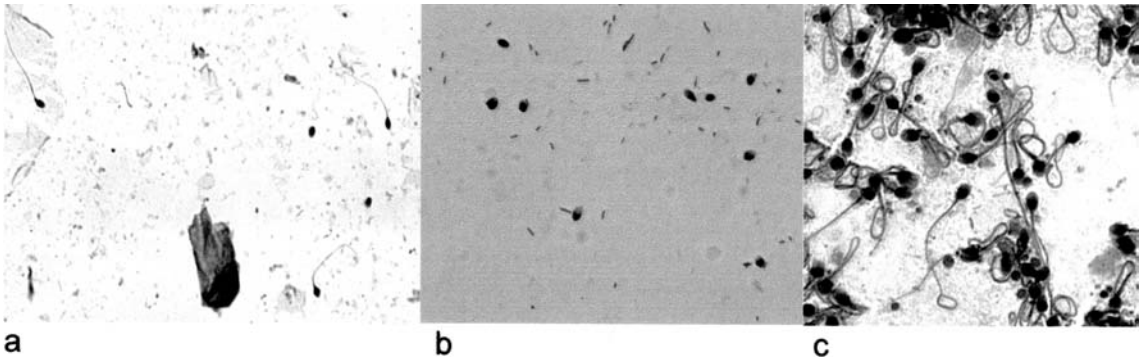

FIG. 4—*Images of the sperm from a vaginal swab before (a) and after (b) the first lysis step. Image of the chelex extracted neat semen after incubation with water (c).*

after the first incubation with water and the following centrifugation) showed that none of the sperm lost the flagellum (see Fig. 4 *c*). The extracted DNA yielded in a complete HV1 sequence.

Discussion

Considering the distribution of nuclear and mitochondrial DNA in sperm it is obvious that the nuclear DNA is located in the sperm head, whereas the mitochondria are found in the mid-piece between the acrosome and the flagellum (8). When comparing the results of this investigation and the location of mitochondria in sperm, it's likely that the loss of the flagellum and mid-piece, during the first lysis and centrifugation step, is responsible for the failure of sperm mtDNA amplification in the sperm fraction. Although is possible to get an autosomal STR profile, no mtDNA sequence could be obtained. As shown in the microscopic investigations, the loss of the flagellum and mid-piece will be due to the treatment with lysis buffer, because, as the chelex extracted sample showed, incubation in water doesn't lead to detachment of the flagellum.

Sequencing results for sperm fractions could only be obtained in cases where the corresponding autosomal STR profiles showed mixtures of male and female alleles. The fact that no sperm mtDNA could be detected in the non sperm fraction will be due to the fact that the amount of sperm mtDNA relative to the female mtDNA is to under the threshold for mixture detection.

Obviously, the sequencing results in our example sample must have been caused by a carry-over of female cells or female mtDNA to the sperm fraction. During autosomal STR profiling the minor component was simply not detected, because the female alleles were below the detection threshold. Nevertheless with the help of Y chromosomal STRs, which were carried out in parallel, information regarding the ethnic origin of an unknown offender could be obtained.

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