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The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen

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Abstract

This investigation was undertaken as an efficient method for isolating sperm DNA from a mixed fluid sample which contains vaginal epithelial cells in a greater amount. The modified method of the two-step differential extraction procedure was found to be suitable for separating sperm DNA and vaginal epithelial cell DNA from the mixed stains. As the first step of digestion, vaginal epithelial cells in the mixed stains were lysed with Proteinase K and SDS, and sperm heads remaining in the lysed solution were collected by centrifugation. As the second step digestion, the sperm heads were lysed with the buffer containing Proteinase K, SDS and DTT as reducing agent. DNA fractions extracted from the two lysed solutions were enriched, one with sperm DNA and the other with vaginal epithelial cell DNA. MCT118(D1S80), ApoB VNTR and HLADQα types of sperm DNA were detected and were confirmed by matching with corresponding male blood DNA. In the case of vaginal secretion mixed with semen of two males, the mixture of MCT118 types of the two males was detected in sperm DNA fraction.

Keywords: Vaginal fluid mixed with semen; The modified method of the two-step differential extraction procedure; DNA typing

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1. Introduction

In the identification of a suspect (rapist) in a sexual offence case, it was important to isolate sperm DNA from vaginal swab mixed with semen in order to ensure the DNA typing of the sperm DNA.

Gill et al. [1] and Guisti et al. [2] reported the preferential lysis method to isolated sperm DNA from the mixed stain. The preferential lysis method was devised for DNA typing using the Southern blotting method [3]. There were some cases in which the mixed fluid contained a greater amount of the vaginal epithelial cells than the sperm cells. In such cases, a considerable number of vaginal epithelial cells remained undigested in the sperm DNA fraction even after the preferential lysis procedure, yielding both male and female bands on the electrophoresis gel.

Therefore, we devised a modified method of the two-step differential extraction procedure in order to confirm both male and female bands separated in the case of mixed fluid sample which contains vaginal epithelial cells in a greater amount. In this paper the modified method was used for the extraction of DNA from the mixed fluids followed by PCR based DNA typing.

2. Method

Vaginal fluid stains mixed with semen were dried overnight at room temperature. The density of sperm and vaginal epithelial cell in the mixed stains was roughly estimated by examining the smear from each sample prepared on a glass slide and stained with acid fuchsin and methylene blue.

As the first step of digestion, the mixed stain sample of 3 mm^2 was cut off and soaked into 0.5 ml of the lysis solution 1 (TNE buffer: 10 mM Tris–HCl (pH 8.0), 10 mM ethylenediamine tetraacetate (EDTA), 100 mM sodium chloride with 1% sodium dodecyl sulfate (SDS) and 100 μg/ml Proteinase K) in reaction tubes. The incubation time in the lysis solution 1 was 3 h at 70°C in a shaking water bath.

After incubation the sample was transferred to a new reaction tube in order to avoid contamination of fiber and undigested cellar materials and centrifuged for 5 min at 15 000 rev./min (18 500 × g). The supernatant was enriched with vaginal epithelial cell DNA and the pellet was washed with 0.1 ml of TNE buffer in order to remove remaining vaginal epithelial cell DNA. The presence of sperm heads in the pellet was examined by microscope. If the vaginal epithelial cells still remained, the pellet was lysed in lysis solution 1 at 70°C for about 1 h.

As the second step of digestion, the sperm heads were lysed in 0.5 ml of lysis solution 2 (TNE buffer with 1% SDS, 100 μg/ml Proteinase K and 0.04 M dithiothreitol (DTT)) for more than 8 h at 56°C in a shaking water bath [4]. The second digestion solution contained principally sperm DNA.

Each solution that contained vaginal epithelial cell DNA and sperm DNA was purified with phenol extraction. Precipitation of DNA was carried out with 0.1 volume of 3 M sodium acetate (pH 5.3) and 2.5 volumes of absolute ethanol and was collected by centrifugation for 15 min at 15 000 rev./min (18 500 × g). The DNA precipitation was dried and resuspended in 60 μl TE buffer. The amount of DNA recovered was quantified at 260 nm using a UV spectrophotometer.

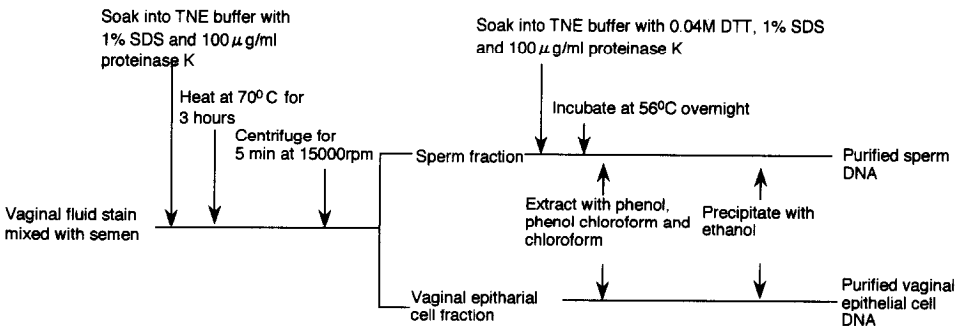

Fig. 1. The modified method of the two-step differential extraction procedure for isolated sperm DNA and vaginal epithelial cell DNA from vaginal secretion mixed with semen.

The presence modified method of the two-step differential procedure is shown in Fig. 1.

Control DNAs were isolated from human peripheral blood as described by Sakai et al. [5].

The isolated DNAs were used for PCR amplification with primers for MCT118, ApoB VNTR and HLADQα [6–8].

A 10-ng quantity of each template DNA sample was added in a reaction mixture:

MCT118; 2.5 μM of primer 1 5′GAAACTGGCCTCCAAACACTGCCCGCCG 3′
2.5 μM of primer 2 5′GTCTTGTTGGAGATGCACGTGCCCCTTGC 3′
2.5 units of *Taq* polymerase,
Reaction buffer (67 mM Tris–HCl (pH 8.3), magnesium chloride, 16.6 mM ammonium sulfate, 10 mM 2-mercaptoethanol, 170 μg/ml bovine serum albumin, 10% dimethyl sulfoxide (DMSO), 1 mM each of the deoxy forms of nucleotide triphosphate).

ApoB VNTR; 1.6 μM of primer 1 5′ATGGAAACGGAGAAATTATG 3′
1.6 μM of primer 2 5′CCTTCTCACTTGGCAAATAC 3′
2 units of *Taq* polymerase,
Reaction buffer (100 mM Tris–HCl (pH 8.3), 15 mM magnesium chloride, 50 mM potassium chloride, 0.2 mg/ml acetylated bovine serum albumin)

PCR amplification was performed with Thermal Cycler 2000 of Cetus Corporation. The conditions of PCR amplification were as follows:

MCT118: denature 95°C 1 min,
annealing 65°C 1 min,

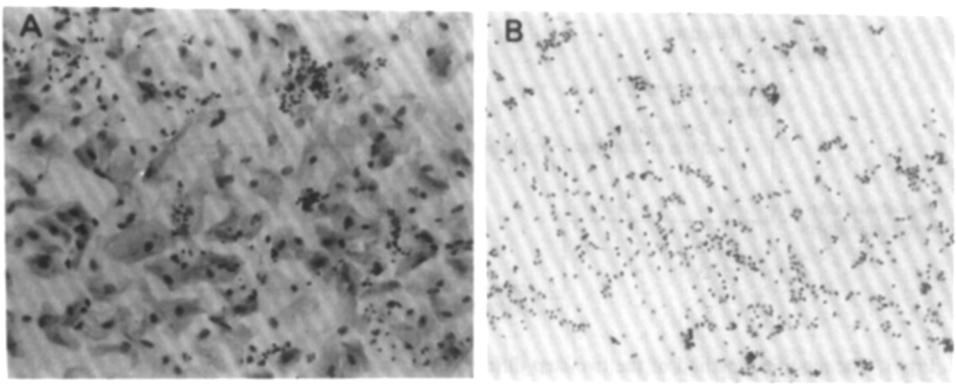

Fig. 2. The cellular materials collected from a mixed stain before and after the first digestion step. (A) Sperm and vaginal epithelial cells were observed before the first digestion step (×200). (B) Sperm alone were observed in the pellet after the first digestion step (×200).

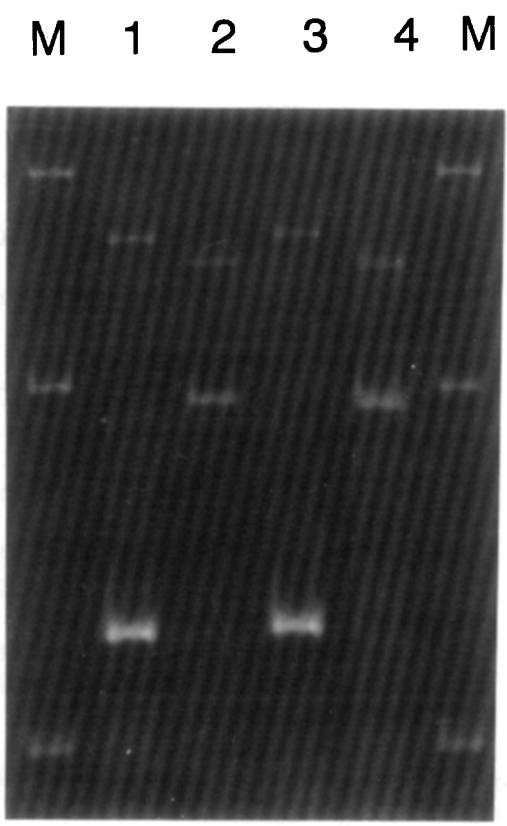

Fig. 3. MCT118 profile of DNAs isolated from vaginal secretion mixed with semen by modified method of the two-step differential extraction procedure. Lane 1, Male blood control DNA; Lane 2, Female blood control DNA; Lane 3, Sperm DNA; Lane 4, Vaginal epithelial cell DNA; M, 123-bp ladder marker.

extension 70°C 8 min,
repeated for 30 cycles.

ApoB VNTR: denature 94°C 2 min,
annealing and extension 70°C 6 min,
repeated for 26 cycles.

The amplified DNA products were detected by ethidium bromide staining after electrophoresis on 5% polyacrylamide gels.

For HLADQα types detection, the extracted DNA were amplified and typed using the reverse dot blot format of the Amplitype HLADQα Kit (Perkin Elmer Corporation).

3. Results

Fig. 2 shows the cellular materials of the mixed stain before and after the first digestion step. Before the digestion, many cellular components, containing many sperm, were observed in the smear from the mixed stain. After the first step of diges-

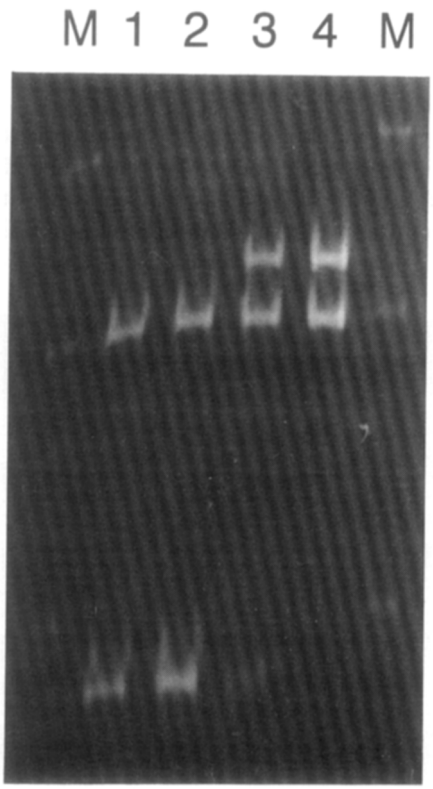

Fig. 4. ApoB VNTR profile of sperm DNA and vaginal epithelial cell DNA isolated from the mixed stain. Lane 1, Male blood control DNA; Lane 2, Sperm DNA; Lane 3, Vaginal epithelial cell DNA; Lane 4, Female blood control DNA; M, 123-bp ladder marker.

tion, all cellular materials, except for sperm heads, were lysed by the Proteinase K. The sperm heads were obtained as a pellet from the centrifugation.

DNA typing of MCT118 locus could be detected from the sperm DNA and vaginal epithelial cell DNA, separately. In Fig. 3, the MCT118 type of sperm DNA matched that of corresponding blood DNA, indicating a good result of complete separation of sperm DNA from vaginal epithelial cell DNA. The MCT118 type of the vaginal epithelial cell DNA was identical to that from the corresponding blood DNA.

The same result as for MCT118 was obtained in ApoB VNTR typing (Fig. 4). The ApoB VNTR type of sperm DNA matched the ApoB VNTR type of corresponding DNA, but the vaginal epithelial cell DNA gave a weak band coincident with the band of sperm DNA.

HLADQα types were determined by the Amplitype HLADQα Kit from the DNA samples. Fig. 5 shows HLADQα typing of male and female blood. They are of types 2–4 and 1.3–1.3, respectively. The mixed stain of semen and vaginal secretion was prepared from the male and female subjects as described above. The type of sperm DNA matched the corresponding male type, whereas the type of vaginal epithelial cell DNA yielded multiple results with mixed type originating from semen and vaginal epithelial cell, that is, alleles of 1.3, 2 and 4.

Fig. 6 shows the MCT118 types from the vaginal secretion mixed with the semen of two males. The MCT118 type of the sperm DNA fraction gave a mixture of types, of corresponding male blood DNAs.

Table 1 shows that the two-step differential extraction procedure made it possible to detect the DNA type of the sperm DNA from aged mixed stain. Both MCT118 and HLADQα types of sperm DNA were obtained from the mixed stain sample stored for 4 years at room temperature. From the mixed stain sample stored for 5

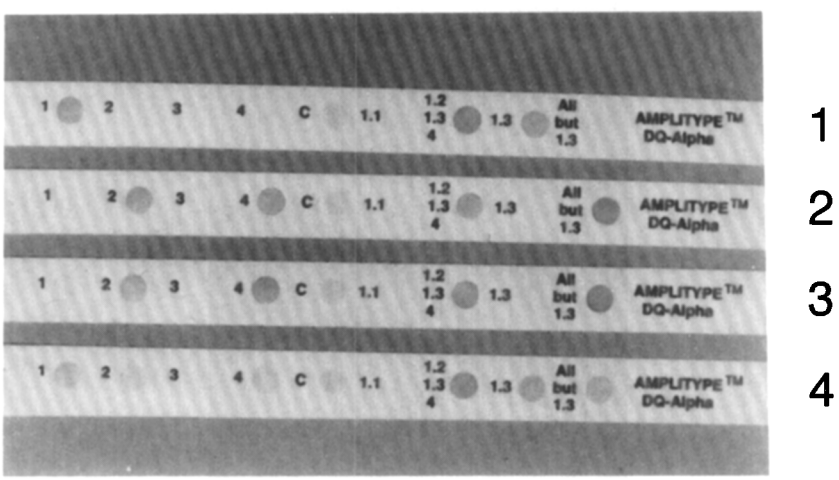

Fig. 5. HLADQα typing of sperm DNA and vaginal epithelial cell DNA from a mixed stain. 1, Female blood control DNA; 2, Male blood control DNA; 3, Sperm DNA; 4, Vaginal epithelial cell DNA.

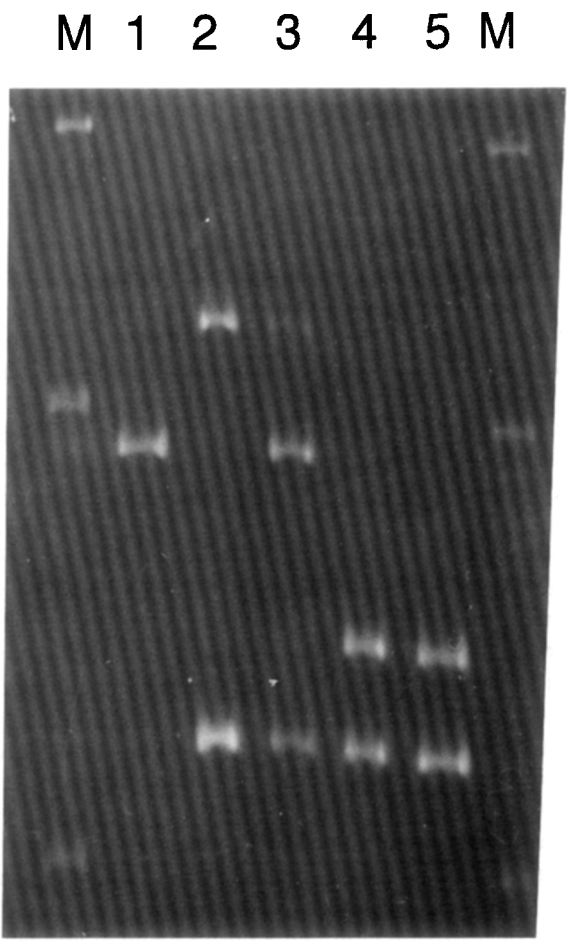

Fig. 6. MCT118 profile of DNAs isolated from the vaginal secretion mixed with the semen of two males. Lane 1, Male 1 blood control DNA; Lane 2, Male 2 blood control DNA; Lane 3, Sperm DNA; Lane 4, Vaginal epithelial cell DNA; Lane 5, Female blood control DNA; M, 123-bp ladder marker.

Table 1
DNA typings of MCT118 and HLADQα loci of isolated sperm DNA from aged vaginal swabs in cases

Forensic samples	Age of stain	Preserved condition	MCT118	HLADQα
No. 1	8 years	Room temperature	–	–
No. 2	5 years	Room temperature	+	–
No. 3	4 years	Room temperature	+	+
No. 4	2 years	−20°C	+	+
No. 5	6 months	−80°C	+	+
No. 6	3 months	Room temperature	+	+

Symbols: +, detectable; –, not detectable.

years, MCT118 types could be detected but HLADQα type was not detected. No DNA type could be determined from the mixed stain sample stored for 8 years at room temperature.

4. Discussion

The number of sperm in post-coital vaginal contents depended on the time lapse after intercourse and the viability of the victim. Before the two-step differential extraction, it was necessary to estimate initially the density of the sperm and vaginal epithelial cell.

Since the differential extraction of DNA from the mixed stain sample containing more vaginal epithelial cells than the sperm cells has been one of the great concerns in forensic DNA typing practice, we devised the modified method of the two-step differential extraction procedure to isolate sperm DNA from mixed stain samples. In the present investigation 70°C was adopted for the lysis temperature of vaginal epithelial cells under proteinase K and SDS instead of 37°C of the original report. Under the lysis condition, none of the sperm heads were lysed, giving no detectable sperm DNA production. Dithiothreitol (DTT) as a reducing agent has generally been used for collecting sperm head DNA [1]. Since the disulfide cross links in the protamine surrounding sperm DNA were not broken without treating the reducing agent [9], the sperm heads remained undecomposed in the first step of the two-step differential extraction procedure. As a result, in the modified method of the two-step differential extraction procedure, sperm DNA and vaginal epithelial cell DNA could be favorably isolated from mixed fluid sample which contains a greater amount of vaginal epithelial cells than sperm.

Wiegand et al. [10] reported 'mild preferential lysis', i.e. that the condition of lysis was less stringent to reduce the probability of sperm lysis during the first stage. In the present investigation, although the mixed stain contained a small amount of sperm, most of the sperm was resistant to the digestion of Proteinase K at 70°C. Under the condition of relatively high temperature, vaginal epithelial cells could be lysed and sperm DNA gave no traces of contamination by vaginal epithelial cells.

As shown in Fig. 4, in ApoB VNTR typing of vaginal epithelial cell DNA a band, although very weak, presumably originated from the sperm DNA was added to the native bands of vaginal epithelial cells. Moreover, also in HLADQα typing, two specific hybridization reactions were observed in the formation of a colored precipitate adding to the native reactions of vaginal epithelial cells, although at a lesser intensity. This result may suggest the possibility of slight disruption of sperm heads in the condition of the first step of digestion (70°C) of the vaginal epithelial cell in the mixed fluid [11,12]. In ApoB VNTR and HLADQα typing of sperm DNA, bands and reactions originating only from sperm were observed without any band or reaction originating from vaginal epithelial cells. This may suggest that the digestion of vaginal epithelial cells was completed at the first step of a two-step differential extraction procedure used in the present investigation.

Adoption of 70°C as a digestion temperature at the first step in the present two-step differential extraction is to aim at inhibiting the unfavorable DNAase activity of intrinsic and bacterial origin, and therefore the sperm DNA remains relatively in-

tact after the first step of digestion. In the present procedure the addition of male DNA to female DNA is indeed expected to be observed in the aged samples. The positive DNA typing of male origin from aged samples is, however, definitively performed without any participation of DNA of female origin in that of male origin because of complete digestion of female cells at the first step of digestion.

Mixed fluid samples consisting of vaginal secretions of one individual and semen of two individuals were examined as to MCT118 DNA typing under the present extraction procedure and three different alleles were clearly detected from the sperm fraction without giving any bands originating from the vaginal epithelial cell. These three bands were individually identified by reference to the types of corresponding individual bloods.

The modified two-step differential extraction procedure in the present investigation will be helpful for correctly isolating sperm and vaginal epithelial cell DNAs and for reasonably evaluating the male and/or individual origin of mixed fluid samples from DNA typing results.

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