TECHNICAL NOTE

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ABO Genotyping of Suspects from Sperm DNA Isolated from Postcoital Samples in Sex Crimes

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ABSTRACT: In sexual assaults against women, one key to identifying the suspect is ABO phenotyping or the typing of other polymorphic markers of the seminal fluid in the victim's vagina. However, ABO phenotyping is frequently unsuccessful, since mixtures of fluids cannot be separated to be subjected to conventional methods for the detection of antibody or antigen material.

We therefore studied ABO blood group genotyping of sperm DNA isolated from contaminating vaginal fluid by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Seminal samples of genotypes OO, AO, BO and AB were experimentally mixed with vaginal fluid (OO, AO, BO and AB), and were successfully separated and genotyped by this method.

In practice, we also separated and genotyped the seminal DNA of suspects from contaminated postcoital vaginal fluid obtained in 4 sexual assaults. These forensic samples were easily separated and completely genotyped. This reliable ABO genotyping method by PCR-RFLP, using separated sperm DNA, should be of value in forensic identification in sexual assaults.

KEYWORDS: forensic science, DNA, ABO blood group system, polymerase chain reaction, human identification, semen, vaginal fluid, sexual assault

After sexual assaults against women, samples of vaginal fluid contaminated with semen are analyzed to aid in identification of the suspect (1). Previously, genetic marker typing of seminal fluid was limited to the analysis of a few types of blood group markers and soluble polymorphic protein markers (2). Determination of ABO blood group and of various polymorphic markers, for example, phosphoglucomutase 1, glyoxalase 1, peptidase A or Lewis, can be used for identifying suspects from semen (3). However, since these markers have no clinical significance, they are not usually tested at public hospitals and, consequently, there is generally no public record of types of these markers. The ABO blood group system has been used extensively as a marker in clinical medicine (3,4). It is the best known and most venerable of the genetic markers, quite stable and can be detected in most types of biological evidence (4). Since the majority of the population has already been tested and phenotyped for this marker, and the records on the phenotype are readily available. ABO phenotyping of sperm remaining at the crime scene has particular significance in the initial investigation. However, the semen samples to be analyzed in sexual assault are often contaminated, and such mixtures cannot be separated for subjection to conventional immunological methods, since the vaginal fluid contains has enzymes and blood group activities (1,5) (Table 1), and semen contains proteolytic enzymes that further reduce the amount of the protein recovered (6). Furthermore, bacterial activity in the vagina may be responsible for producing erroneous results in the identification of ABO blood groups (7). For these reasons, a suspect frequently cannot be identified by the fluid left in the victim's vagina (8). Therefore, mixed samples should be separated into male and female components and the purified male-origin materials from the sperm donor should be typed independently to help identify the suspect (9,10).

Here, we describe the recovery of sperm from vaginal secretion experimentally contaminated with seminal fluid, the extraction of suspected male-specific DNA from these specimens, and the genotyping of ABO blood groups by PCR-RFLP (11–13). We also describe the employment of these methods in 4 sexual assaults.

Materials and Methods

The semen and vaginal fluid used in these experiments were collected from 8 Japanese volunteers (4 males and 4 females, one male and one female of each ABO genotype were OO, AO, BO and AB). Five μL of seminal fluid was experimentally mixed with the same amount of vaginal fluid. 10 μL of post-coital vaginal fluid in 4 sexual assaults were collected from the victim's vaginas with syringes within 24 h after the crimes. Generally, there are few sperm in the vaginal fluid in sexual assaults. Therefore, we extracted the DNA from all samples by the Nal extraction method, since the quantity of DNA isolated by this procedure is greater

TABLE 1—*Possible ABO phenotypes of suspects in sexual assaults.*

ABO phenotypes of semen-contaminated vaginal fluid	ABO phenotypes of victim	Possible ABO phenotypes of suspect
AB	AB	AB, A, B, O
AB	A	AB, B
AB	B	AB, A
A	A	A, O
B	B	B, O

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than that obtained by the conventional procedure, especially when the samples are very small (14). We have established a two-step extraction procedure for the purification of sperm DNA and vaginal epithelial DNA in contaminated specimens by modifying this extraction method.

The protocol for the first step of digestion is as follows:

1) Dispense 10 μL of sperm-contaminated vaginal fluid into a 1.2 mL microtube.
2) Add 300 μL of the digesting solution (6 M Nal/13 mM EDTA/0.5% sodium N-lauroylsarcosine/10 μg glycogen/26 mM Tris-HCl, pH 8) to the tube, mix and incubate at 55 °C for 4 h.
3) Centrifuge this mixture at 12,000 rpm for 10 min to precipitate the sperm heads. The pellets of sperm heads will be resuspended in the second step of digestion.
4) Remove supernatant from the tube into another tube and add an equal volume of isopropanol. Shake gently for 15 min.
5) Centrifuge the tube at 12,000 rpm for 10 min to precipitate non-sperm DNA.
6) Add 1 mL of 40% isopropanol and vortex. Centrifuge at 12,000 rpm for 10 min to recover the non-sperm DNA, vacuum and dry the pellet, and dissolved it in 20 μL of distilled water.

The protocol for the second step of digestion is as follows;

1) Wash the pellet described in 3) of the first step of digestion with washing buffer (10 mM Tris-HCl pH 7.6, 13 mM EDTA) three times to remove the remaining non-sperm DNA.
2) Add 300 μL of the digesting solution (described in the first step of the digestion) with 50 mM DTT, mix and incubate at 55 °C for 1 h.
3) Add an equal volume of isopropanol. Shake gently for 15 min.
4) Centrifuge the tube at 12,000 rpm for 10 min to precipitate sperm-origin DNA.
5) Add 1 mL of 40% isopropanol and vortex. Centrifuge at 12,000 rpm for 10 min to recover the sperm DNA. Vacuum and dry the pellet, and dissolved it in 20 μL of distilled water.

The amount of isolated sperm or non-sperm DNA is then determined with a spectrophotometer.

The first step of digestion was to solubilize the non-sperm cells in the mixed fluids. Since sperm nuclei are protected by cross-linked thiol-rich proteins, they remained intact in the first step of digestion without DTT (15–17). Thus, the mixed fluids were separated into the sperm heads and the female components by centrifugation. The precipitates of the first step (sperm heads) should be thoroughly washed at least three times to remove the contaminating female components. In the second step of digestion, the precipitated sperm heads were solubilized with DTT. Sperm DNA and vaginal epithelial cell DNA were isolated by this protocol.

Two PCR fragments, spanning positions 261 and 703 of the cDNA sequence, are amplified (18–20). The site at position 261 allows us to differentiate the O allele from the A and B alleles. The position of nucleotide 703 of cDNA from A transferase was used to distinguish A, B, and O alleles by restriction enzyme digestion (18–20).

PCR amplification was performed with 1 μL of DNA solution with 1.5 mM MgCl, 0.8 mM dNTP and 0.5 unit Taq polymerase (Cetus), using the following primers, in a total volume of 50 μL;

primer 1f: CAC CGT GGA AGG ATG TCC TC;
primer 1r: AAT GTC CAC AGT CAC TCG CC;
primer 3f: TGG AGA TCC TGA CTC CGC TG;
primer 3r: GTA GAA ATC GCC CTC GTC CTT.

A total of 30 cycles of amplification were carried out with the following reaction cycles; denaturation for 24 s at 96 °C,annealing for 30 s at 60°C, and extension for 90 s at 72°. The amplified PCR product was centrifuged in Ultrafree-C3 (Millipore Limited) sample cap to remove the PCR buffer solution (21), and was dissolved in 20 μL of distilled water. The restriction digestion was performed on 2 μL of PCR products, using 10 units of Kpn I or Msp I (Boehringer-Ingelheim), according to the manufacturer's instructions. To identify the 261th nucleotide, a 95 or 96 bp DNA fragment was amplified by PCR with primer 1f and 1r and digested with Kpn I. For the 703 th nucleotide, a 181 bp PCR amplified fragment with primer 3f and 3r was digested with Msp I. The amplified products before and after each restriction digest were then analyzed by electrophoresis on 10% polyacrylamide gels. The bands on gels were visualized by silver staining (22,23). All specimens were genotyped as shown in Table 2.

Results

As described in Materials and Methods, the semen and the vaginal fluid of volunteers of each phenotype of the ABO blood group were experimentally mixed. The sperm was separated from the epithelial cells of the vaginal walls by the differential extraction method described. By micrographic examination, we confirmed that abundant quantities of sperm heads were recovered by the first extraction and centrifugation. In the second extraction, the resulting sperm pellets were examined. These specimens were subjected to DNA extraction, PCR amplification and enzyme digestion, using the procedures described in Materials and Methods. Genotyping of the ABO blood groups of separated sperm DNA is shown in Table 1. The genotypes of the ABO blood group of all separated sperm-DNA examined were successfully determined (Table 3), showing that the recovered sperm DNA separated by the method did not contain female DNA as detected by

TABLE 2—*ABO genotyping by PCR-RFLP.*

ABO genotype	Primer 1f and 1r, Kpn-1	Primer 3f and 3r, Msp-1
OO	69	140
AA	96	140
AO	96,69	140
BB	96	159
BO	96,69	159, 140
AB	96	159, 140

TABLE 3—*ABO genotyping of recovered sperm DNA mixed with vaginal fluid.*

ABO genotypes of vaginal fluid	ABO genotypes of sperm			
	OO	AO	BO	AB
OO	OO	AO	BO	AB
AO	OO	AO	BO	AB
BO	OO	AO	BO	AB
AB	OO	AO	BO	AB

PCR methods. These findings clearly showed that sperm DNA of any ABO phenotype could be completely determined by these methods, even when mixed with the vaginal fluid of any ABO phenotype.

For the application of these methods in crime scene in practice, the contaminated postcoital vaginal fluids in 4 sexual assaults were genotyped by the same methods (Table 4, Fig. 1).

The phenotype of the female victim and of the mixed vaginal fluid collected after the sexual assault in case 1 was AB. Thus, the phenotype of the male suspect was not clearly identified by the conventional techniques that detect antibody or antigen material. There were four possible phenotypes of the suspect, AB, A, B and O. We attempted to separate the sperm and vaginal cells from the sperm-contaminated vaginal fluid and to separately genotype these specimens. Only the 69 bp DNA fragment of locus 1 and the 140 bp DNA fragment of locus 3 were found in the digested PCR products of the recovered sperm DNA (lane 2 of Fig. 1*a, b*), whereas the 96 bp DNA fragment of locus 1 and the 140 bp and 159 bp DNA fragments of locus 3 were found in the digested PCR products of the recovered vaginal cell DNA (lane 3 of Fig. 1*a, b*). These findings clearly showed that the genotype of the male suspect was OO and the genotype of the female victim was AB. Since the band of the 96 bp DNA fragment of locus 1 and the 159 bp DNA fragment of locus 3 were not found in lane 2 shown in Fig. 1*a, b*, we concluded that overlapping bands from female DNA were not present in the recovered sperm DNA. These findings show that sperm DNA extracted by these methods was not contaminated by any female components. As expected, the bands of the PCR-products from the sperm-epithelial mixture showed a mixed band pattern of male and female samples (the 69 and 96 bp in locus 1, the 140 and 159 bp in locus 3).

TABLE 4—*ABO genotypes of recovered sperm-DNA by PCR-RFLP from semen-contaminated vaginal fluid in 4 sexual assaults.*

Case	ABO phenotypes of semen-contaminated vaginal fluid	ABO phenotypes of victim	Possible ABO phenotypes of suspect	ABO genotypes of recovered sperm-DNA by PCR-RFLP
1	AB	AB	AB, A, B, O	OO
2	B	B	B, O	OO
3	AB	A	AB, B	AB
4	AB	A	AB, B	AB

The phenotype of the female victim and the semen-contaminated vaginal fluid in case 2 was B. The possible phenotype of the suspect was thus B or O. Separated sperm and vaginal cells in case 2 were genotyped, as in case 1. Only the 69 bp DNA fragment of locus 1 and the 140 bp DNA fragment of locus 3 were found in the recovered sperm DNA (lane 5 of Fig 1*a, b*), whereas the 69 and the 96 bp DNA fragments of locus 1 and the 140 bp and 159 bp DNA fragments of locus 3 were found in the recovered vaginal cell DNA (lane 6 of Fig. 1*a, b*). The genotype of the male suspect was OO and the genotype of the female victim was BO. There was no band of the 96 bp DNA fragment of locus 1 or the 159 bp DNA fragment of locus 3 in the recovered sperm DNA, demonstrating that the sperm DNA did not contain any female components.

The phenotypes of the semen-contaminated vaginal fluids of cases 3 and 4 were AB, and that of both female victim was A. The possible phenotypes of the suspects were thus AB or B. The 96 bp DNA fragments of locus 1 and the 140 bp and the 159 bp DNA fragments of locus 3 were found in the recovered sperm DNA (lanes 8 and 11 of Fig. 1*a, b*), whereas the 69 and 96 bp DNA fragments of locus 1 and the 140 bp DNA fragment of locus 3 were found in the recovered vaginal cell DNA (lanes 9 and 12 of Fig. 1*a, b*). These findings indicate that the genotype of the male suspect was AB and the genotype of the female victim was AO. There were no bands of the 69 bp DNA fragment of locus 1 in the recovered sperm DNA, demonstrating that the sperm DNA extracted by these methods did not contain any female components.

Sperm from the male suspects were successfully recovered and the isolated DNA was extracted from these samples by this method. By examining the patterns of the digested DNA fragments, we were easily able to determine ABO genotypes. With this differential lysis procedure described here, sperm DNA was recovered without detectable contamination with female DNA. The genotypes of the

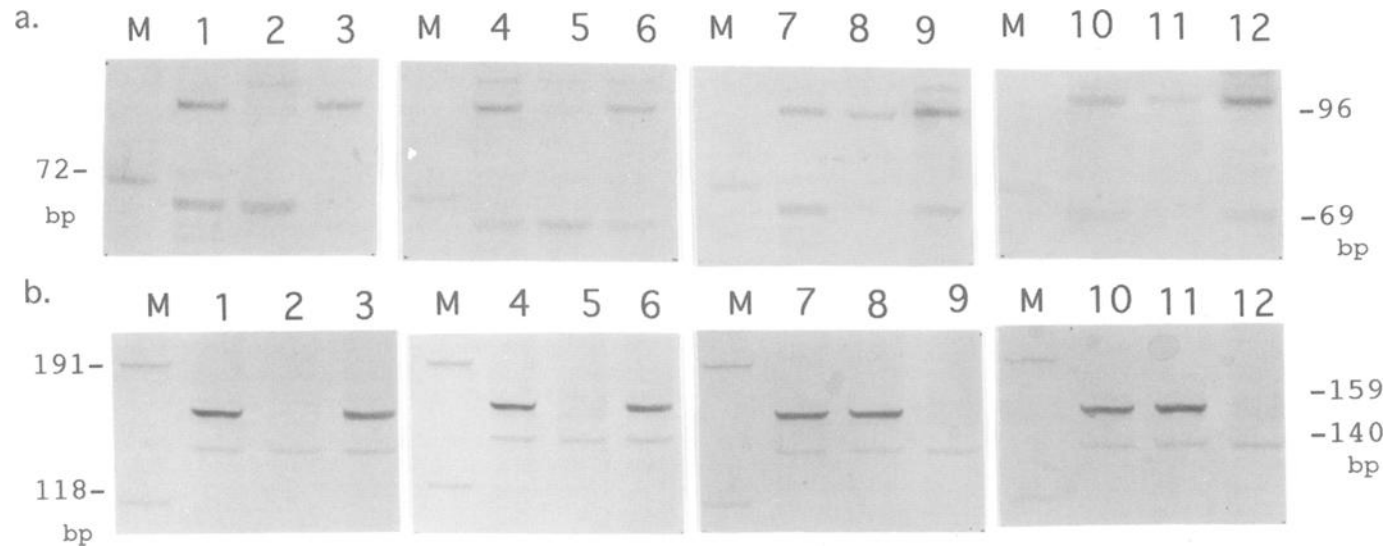

FIG. 1—*Electrophoresis in 10% polyacrylamide gels of each digested PCR product.* a; *PCR products of Locus 1 digested with Kpn-1.* b; *PCR products of Locus 3 digested with Msp-1. M: ϕ X174/Hae III digest, Lane 1–3; Case 1, Lane 4–6; Case 2, Lane 7–9; Case 3, Lane 10–12; Case 4. Lane 1, 4, 7, 10; contaminated vaginal fluid, Lane 2, 5, 8, 11; recovered sperm DNAs, Lane 3, 6, 9, 12; recovered vaginal epithelial DNAs.*

recovered vaginal cell DNA determined by the alleles present in lanes 3, 6, 9 and 12 in Fig. 1*a, b*, exactly matched the phenotypes of the female victims. With this method, mixed samples can be completely separated into the male and female components, and the genotype of the recovered sperm DNA or vaginal epithelial cells can easily be determined.

Discussion

The ABO blood group system, the first discovered genetic marker, has been widely employed clinically for blood transfusion and in forensic identification (3,4). ABO typing is conventionally determined by the detection of antibody or antigen material (6,7), and these methods have fairly high sensitivity. However, we must consider several problems inherent in these methods, since the activity of antigens and antibodies is influenced by their survival and by nonspecific reactions under various conditions (7,8). The typing of specimens in sexual assaults for forensic purpose entails more serious problems, since sperm samples are frequently contaminated with vaginal fluid (5,7,10). Such mixtures cannot be separated for the performance of conventional immunological methods, since vaginal fluid itself contains abundant antigen and antibody that affects these forensic tests (6–8). In sexual assaults, the key to identify the suspect is the ABO phenotyping of the material that was left, since the ABO phenotype of most people is already recorded. It is necessary for this phenotyping in sexual criminals that male components are separated from mixed samples and that only male-origin materials from the sperm donor are phenotyped or genotyped independently (10,15,16). Determining the ABO phenotype of the seminal fluid by the conventional methods designed to detect antibody or antigen does not yield sufficient information in the material available in sexual assaults. For accurate ABO typing, it is important to isolate sperm DNA from the mixed vaginal fluid. We therefore studied an efficient method for the isolation of sperm DNA and vaginal epithelial cell DNA, and examined the ABO blood group genotyping of separated specimens by the PCR-RFLP method. In this study, we developed a two-step cell digestion method for the isolation of sperm DNA from mixed fluid containing semen and vaginal secretions. This DNA extraction using NaI is more rapid than the conventional phenol extraction (14,20). Furthermore, because this method does not need confluent change of tubes, the DNA can be extracted from even small samples without loss (20). Both the sperm DNA and the vaginal epithelial cell DNA isolated by this method were very pure and caused no problems in ABO genotyping by PCR-RFLP. Although it has been reported that non-sperm male cells are occasionally present in post-coital vaginal fluids (24), no male-origin bands were shown in the recovered vaginal cell DNA that we examined by this method. Since the ABO genotyping of female victims can be done more easily from the peripheral blood cells, there may be little significance in genotyping from recovered vaginal cells.

In conclusion, this method, which does not rely on antigen or antibody activity, is fast and reliable, and should be widely applicable in sexual assaults.

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