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Application of Deoxyribonucleic Acid (DNA) Polymorphisms to the Analysis of DNA Recovered from Sperm

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ABSTRACT: Sperms, collected following sexual activity of volunteers, were processed to isolate high-molecular weight deoxyribonucleic acid (DNA). These DNA samples were digested with particular restriction endonucleases and analyzed with probes that recognize polymorphic DNA regions within the human genome. The pattern of restriction fragment length polymorphisms (RFLP) detected by this test is identical to that observed with DNA prepared from blood of the male sexual partner. Therefore, RFLP analysis can be used to exclude or to determine the probable identity of an assailant in rape cases.

KEYWORDS: pathology and biology, deoxyribonucleic acid (DNA), spermatozoa

Biological materials recovered as criminal evidence can provide the means by which the phenotypic makeup of an assailant may be determined. In the case of sexual assault, the presence of spermatozoa or prostatic acid phosphatase indicates that sexual activity has occurred [*1-3*], but yields no information concerning identity. The comparison of pubic hairs retrieved from the victim and an accused individual is one procedure that may be used to exclude or to determine the likelihood that a particular suspect might have been the assailant [*3*]. In addition, the analysis of polymorphic proteins from semen recovered intravaginally or as a dried stain can be used to increase the probability of making a correct identification.

However, a thorough protein analysis is difficult. Only the ABO blood group and a select number of human leukocyte antigens (HLA) antigens have been typed on sperm [*4-6*] while the polymorphic red cell enzymes, found in semen, are limited in their phenotypic diversity [*7*]. As a result, the likelihood of excluding a falsely accused individual is low, or, alternatively, there is a high probability that a random individual from the population might have the same markers as the assailant.

It has been shown that differences in deoxyribonucleic acid (DNA) sequence between individuals can be visualized as size polymorphisms in restriction endonuclease digested DNA. These polymorphisms are inherited as Mendelian traits, therefore they can be used as mark-

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ers for genetic studies [*8,9*]. Certain restriction fragment length polymorphisms (RFLPs) display a large number of discrete DNA fragments [*9*],[3] thus greatly decreasing the probability that two individuals will have the same set of alleles.

This article describes the recovery of sperm from various sources postcoitus, the purification of male-specific DNA from these samples, and comparison of size polymorphisms with DNA isolated from the blood of the male sexual partner as a demonstration of identity assignment using RFLPs.

Materials and Methods

Collection of Blood and Semen Samples

A total of 120 volunteers donated blood and semen samples for comparison studies. In addition, ten volunteer couples participated in the semen sample studies. Blood was collected in Vacutainer® tubes containing potassium ethylenediaminetetraacetate (EDTA) to prevent coagulation, while semen was left untreated and stored at 4°C until processed.

Three approaches towards the recovery of spermatozoa following sexual activity were investigated. Five samples were generated by swabbing the vagina, followed by a lavage with physiological saline. Five samples consisted of an agglutinated semen plug removed from the cervix, two of which were followed by a lavage with saline. These ten samples were kept refrigerated for one to three days before processing. Ten samples consisted of semen stains deposited on the women's undergarments or sanitary napkins following intercourse and dried at room temperature. These samples were processed at a later date to extract the spermatozoa from the dried stain.

Recovery of Sperm from Vaginal Swabs and Lavage

Cotton swabs were soaked with physiological saline and the solution recovered was mixed with that obtained from the lavage. Sperms from this solution were recovered by centrifugation at 3600 rpm, 4°C.

Recovery of Sperm from Dried Stains

Fabric containing dried semen was cut into small pieces and scrubbed with a small brush in 30 mL of phosphate-buffered saline (PBS = 136m*M* sodium chloride, 8m*M* sodium phosphate, dibasic, 17m*M* sodium phosphate, monobasic, pH 7.0), while sanitary napkins were processed by removing the plastic and cutting the absorbant material into small pieces. The materials were then soaked with mild agitation for 24 h in 100 mL of PBS containing 2% Sarkosyl® at 4°C. Solutions were compressed through nylon mesh to prevent fabric contamination, then centrifuged for 10 min at 3600 rpm, 4°C, to pellet the suspended spermatozoa. The pellet was resuspended in 1.0 mL of PBS and a sperm count of sample was performed using a hemocytometer.

Isolation of DNA

DNA was isolated from human peripheral blood as described by Kunkel et al. [*10*].

Purified sperm DNA was prepared using a procedure communicated by Dr. Brian Seed, with modifications to eliminate the female cells present in the samples collected postcoitus. Samples were first suspended in 20 mL of PBS + 2% Sarkosyl, mixed briefly and centrifuged at 3600 rpm at 4°C. This process was repeated two more times, for a total of three washes and centrifugations. Postcoital samples were then suspended in 2 mL of PBS, and

[3]M. Baird et al, unpublished manuscript, 1985.

proteinase K and sodium dodecyl sulfate (SDS) were added to final concentrations of 100 μg/mL and 1%, respectively. These samples were incubated for 4 to 6 h at 65°C with mild agitation. Sperm heads were pelleted by centrifugation, as above. Sperm heads were lysed by resuspending the sperm samples in 2 mL of PBS + 2% Sarkosyl containing 100 μg/mL proteinase K, 10m*M* dithiothreitol (DTT), 25m*M* EDTA, and incubating overnight at 37°C on a rocking platform. Then, DNA was purified by extraction and dialysis as described for blood samples. The amount of human DNA recovered was quantitated at 260 nm in an ultraviolet (UV) spectrophotometer.

Recombinant DNA Probes and RFLP Analysis

The two recombinant clones used in this study were pAW101 [*9*] and pLM0.8 (a 879 base pair Cla 1-Sph 1 fragment from the flanking region of the H-Ras-1 oncogene [*11*]). Labeling of DNA was done with a mixture of all four [$-^{32}$P]deoxyribonucleotide triphosphates (Amersham Corp.) by the nick-translation procedure [*12*]. DNA samples were digested with restriction endonucleases (Eco R1 and Taq 1), size fractionated by agarose gel electrophoresis, transferred to nylon membrane (Zetabind, AMF Cuno) as described by the manufacturer, and hybridized to denatured radioactive DNA probes (pAW101 or pLM0.8). The hybridization buffer was composed of 50% formamide, 6 × SSC (SSC = 0.015*M* sodium citrate, 0.15*M* sodium chloride, pH 7.0), 0.05*M* sodium phosphate, 0.5% sodium lauryl sulfate (SLS), 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll, at 37°C for 48 to 72 h. Excess counts were removed by multiple washings with 2 × SSC, 0.05*M* sodium phosphate, 0.1% SLS, followed by 0.4 × SSC, 0.01*M* sodium phosphate at 65°C. A detailed description of the techniques required for gel electrophoresis, transfer of DNA to membrane filters, and hybridization can be found in the manual by Maniatis et al [*13*].

Results

Comparison of DNA Size Polymorphisms

Two probes, each specific for a polymorphic region of DNA, were used to analyze the DNA prepared from matched samples of whole blood and semen to determine the presence of any size variations in the DNA from different cell types. Taq 1-digested DNA from 106 matched samples were hybridized to pLM0.8. Also, 40 Eco R1-digested DNA samples were hybridized to pAW101. Comparison of the position of the radioactive bands in the adjacent lanes, containing the matched samples, was done by calculating the size of the DNA fragments relative to known DNA size standards. No size difference was seen, within the polymorphic region, between the DNA from the two tissues in the matched samples. An example of the results observed in this type of comparison, with DNA from five individuals, is shown in Fig. 1.

Postcoital Sperm Recovery and DNA Yields

The number of sperm recovered from semen at various steps of the purification procedure and the final amount of DNA purified from each sample is presented in Table 1. The theoretical amount of DNA expected from a given sperm count (Potential DNA Recovery column in Table 1) was calculated by multiplying the number of sperms in the sample by the amount of DNA/sperm (2.5×10^{-12} g). DNA was not purified from samples of sperms if blood samples were not available or were of poor quality, for example, coagulated, or if the sperm count was considered too low to isolate a sufficient amount of DNA (that is, <5 μg of DNA).

The number of sperm recovered from the various samples varied considerably. This is probably a result of the different sample sources, the method of collection, storage conditions, and natural variations in the number of sperm present in semen from different indi-

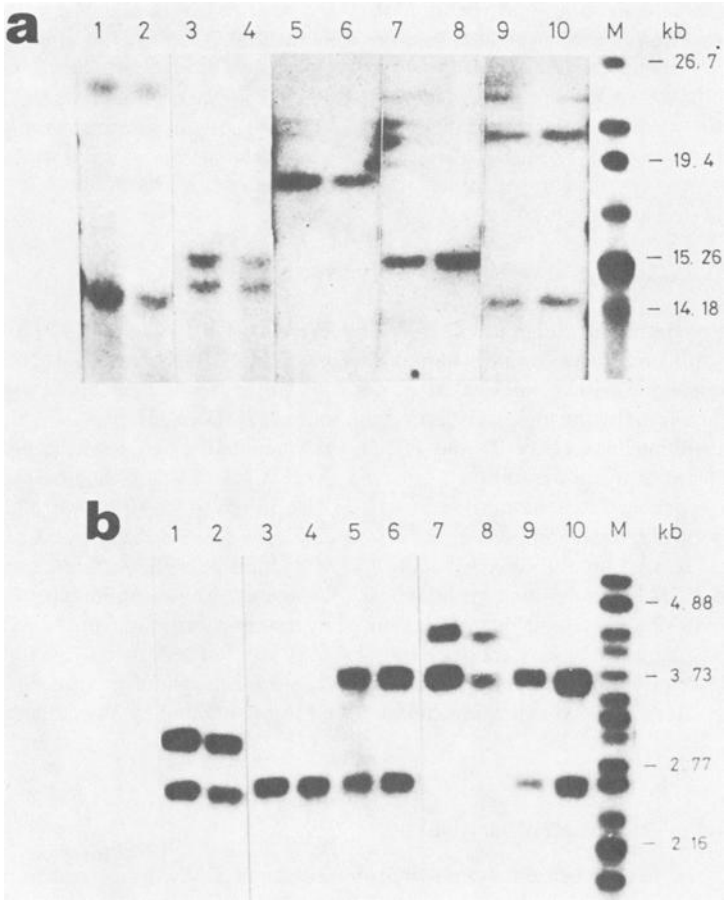

FIG. 1—*Pattern of hybridization of P^{32}-labeled probes to DNA isolated from matched blood and sperm samples.* (a) *Eco-R1 digested DNA hybridized to pAW101.* (b) *Taq-1 digested DNA hybridized to pLM0.8. Odd numbered lanes contain DNA from blood. Even numbered lanes contain DNA from sperm. DNA size markers (Lane M).*

viduals. The average recovery of DNA from the 13 samples shown in Table 1 was approximately 88%.

Elimination of Female Cells from Semen Recovered Postcoitus

The female cells contaminating the sample were removed by a two-step, or differential, lysis (see Materials and Methods). Figure 2*a* is a photograph of a semen sample before processing, Figure 2*b* shows the same sample following the three washes in PBS + 2% Sarkosyl, and Fig. 2*c* the sample following the first lysis in 100 μg/mL of proteinase K and 1% SDS. The results presented in this figure show that the contaminating cells are specifically removed by this lysis protocol.

Comparison of DNA Between Known Male and Female Sexual Partners and DNA from Recovered Spermatozoa

Electrophoresis of undigested DNA samples, purified from sperm, show that this material is high molecular weight (that is, >40 kilobases (Kb), results not shown). This property is

TABLE 1—*Sperm number and DNA recovered from semen samples.*

Sample	Collection Time Postcoitus	Process Time Postcollection, Days	Total Sperm Count in Millions	Potential DNA Recovery, μg	Actual DNA Recovery, μg	Method of Collection[a]
1	45 min	2	11	28.0	NP	VSL
2	11 h	2	0.88	2.2	NP	VSL
3	9 h	2	0.22	0.6	NP	VSL
4	2 h	2-3	5.4	13.5	18.8	VSL
5	3 h	1	3.4	8.5	NP	VSL
6	<30 min	2-3	79	197.5	179.6	DSS
7	6 h	1	0.05	0.1	NP	ASP
8	5 h	1-2	0.2	0.5	NP	ASP
9	10 h	1-2	2.3	5.8	4.0	ASPL
10	3 h	3	3	7.5	5.0	ASPL
11	<30 min	2	11	27.5	30.3	DSU
12	<30 min	1	67	167.5	NP	DSU
13	<30 min	7	6.7	16.8	12.0	DSU
14	<30 min	6	14	34.8	18.0	DSU
15	<30 min	2	10	26.0	23.3	DSU
16	<30 min	2	21	52.2	38.5	DSU
17	<30 min	3-4	65	163.5	197.0	DSS
18	<30 min	3	2.4	6.0	7.9	DSU
19	<30 min	2-3	12	29.8	25.1	DSS
20	<30 min	2-3	18	45.8	22.4	DSU

[a]NP = not processed, VSL = vaginal swabbing and lavage, DSS = dried stain on sanitary napkin, ASP = agglutinated semen plug, ASPL = agglutinated semen plug and lavage, and DSU = dried stain on underwear.

important since degraded DNA consists of randomly cleaved DNA fragments that are too small and variable in size to generate, upon restriction enzyme digestion and size fractionation, the specific DNA fragments representing the alleles of that particular DNA polymorphism. As a result, samples of degraded DNA suffer a loss of signal in the radioactive bands normally observed after hybridization.

DNA prepared from peripheral blood from the male and female sexual partners and from the sample recovered postcoitus, was used to prepare filters containing Eco R1 or Taq 1 digested DNA (see Materials and Methods). DNA in these filters was hybridized to radioactively labeled DNA probe, pAW101 or pLM0.8. Results from two such cases are shown in Fig. 3. In each trio, the DNA from the peripheral blood of the female and the male are located on the left and the right, as indicated, of the DNA prepared from the recovered sample. It is clear from these autoradiograms that the alleles present in the center lane exactly match those alleles of the male sexual partner and that there is no indication of female specific bands. Therefore, DNA purified from these samples are not contaminated with detectable amounts of female DNA.

Discussion

We have presented evidence indicating that spermatozoa can be recovered from several sources following sexual activity and that a sufficient quantity of DNA can generally be isolated from them to perform RFLP analysis. As the above results indicate, it is possible to treat the samples stringently to extract the spermatozoa, and that the DNA remains stable under these extraction conditions. Therefore, in addition to material recovered from the victim, large number of sperms can be obtained from fresh or dried stains. This should simplify the accumulation of sufficient material to perform RFLP analysis. The persistence

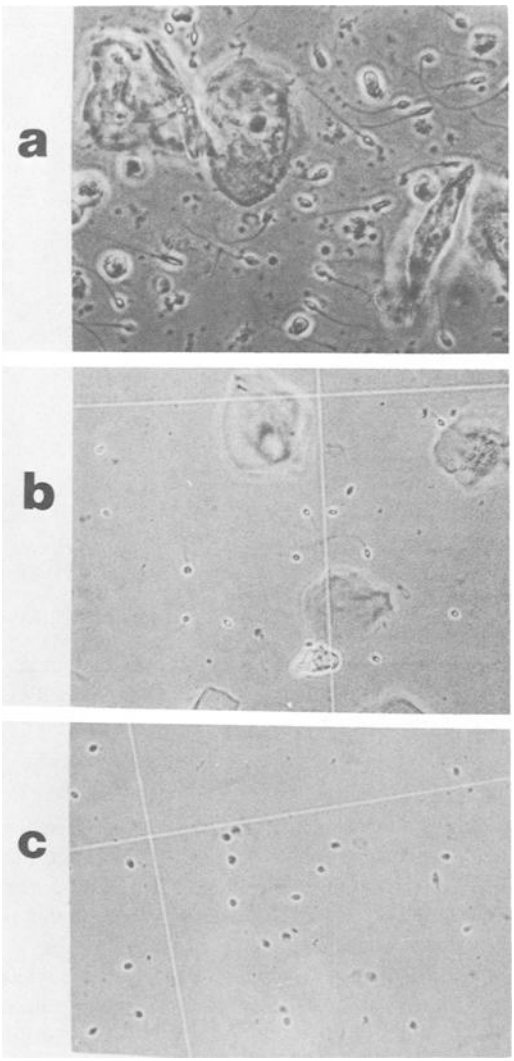

FIG. 2—*Photomicrographs of sperm at different stages of purification from a postcoitus sample.* (a) *Recovered material, by lavage, before processing.* (b) *Sample after three washes with PBS and 2.0% sarkosyl.* (c) *Sample following digestion with proteinase K and SDS, showing lysis of female cells.*

of DNA in a dried semen stain is unknown, but so far our studies indicate that DNA remains intact for at least one month. Note that high molecular weight DNA has been extracted and RFLP analysis performed in three-year-old bloodstains [*14*], and it is possible that DNA in semen stains will demonstrate a similar degree of stability. Thus, the use of DNA polymorphisms for identification purposes could complement or substitute some of the current procedures for identification. However, there are circumstances where the analysis of soluble enzymes or proteins still remains the only means for identity and that is the case for semen samples derived from vasectomized individuals or aspermic stains.

An additional property of RFLP analysis is its high degree of reproducibility. Conditions that destroy or degrade DNA do not produce an abnormal pattern of bands, instead no

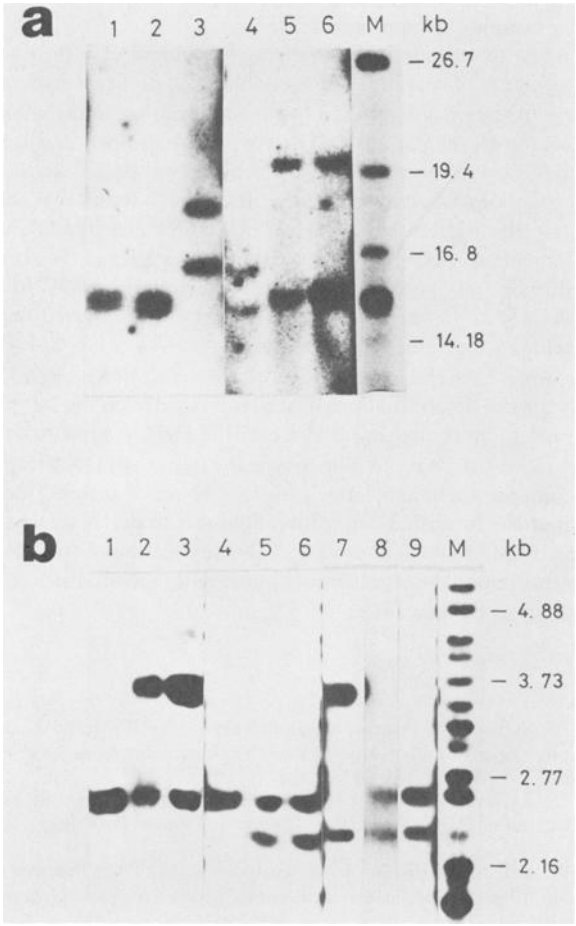

FIG. 3—*Pattern of hybridization of P^{32}-labeled probes to DNA isolated from male and female blood and postcoitus sperm samples.* (a) *Eco RI digested DNA hybirdized to pAW101. Lanes 3,4 contain DNA from blood of the female (Samples 17 and 6, respectively, from Table 1). Lanes 1,6 contain DNA from blood of the male. Lanes 2,5 contain DNA from recovered sperm.* (b) *Taq 1 digested DNA hybridized to pLM0.8. Lanes 1,4,7 contain DNA from blood of the female (Samples 14, 6, and 15, respectively, from Table 1). Lanes 3,6,9 contain DNA from blood of the male. Lanes 2,5,8 contain DNA from recovered sperm.*

bands are observed. For a particular sequence to be detectable, the DNA has to be cleaved at specific sites along the genome so that the resulting fragments can migrate to discrete regions of the gel. In this way there will be enough DNA in that region to produce, after hybridization to a particular probe, a radioactive band. Nucleases or other factors that randomly break DNA produce, for a specific DNA sequence, a multitude of fragments of variable sizes which migrate at all positions in a gel. In this last case, the concentration of a specific sequence, at any point in the gel, is too low to be detectable and no bands are observed. However, to insure the accuracy of the results it is important that the digestion of DNA with the restriction enzyme is brought to completion by using a large excess of enzyme.

The use of the differential lysis procedure described here results in the recovery of sperm without detectable contamination with female cells or DNA. This is indicated by the fact

that the bands in the adjacent lanes, containing the woman's DNA and DNA from sperms purified from semen samples, are different.

The capacity of a test to exclude a suspect from a criminal case is important, especially when only limited amounts of material are available. The ability to type a blood or semen stain for the ABO group system is countered by the relatively low information content of such a discrimination. Of course, the power of a DNA-based identification will be determined by the number and frequency of the alleles detected by the probes. Extensive work has been done with the two probes described in this study.[3] It has been found that the probe pAW101 detects approximately 30 different sized alleles, in Eco R1 digested DNA, ranging in size from 14 to 28 Kb. The other probe, pLM0.8, displays 18 alleles in the size range of 1.85 to 4.45 Kb. The distribution frequencies of the alleles detected with pAW101 and pLM0.8 will be published elsewhere.[3] As more polymorphic probes are discovered and applied to this procedure, the discrimination value will increase.

In the results presented here the sperm DNA and the male DNA samples are analyzed side by side. This allows for the determination of similarities between the samples by simple visual inspection. However, by measuring the size of the DNA fragments, relative to that of DNA fragments of known size, it is possible to type the pattern of DNA fragments in a sperm DNA sample and compare them at a latter time with those of accused individual(s).

The primary goal of the forensic science investigator is to derive as much information as possible from the recovered biological samples. The application of the DNA-based technology as described in this paper can significantly improve the possibilities of recovering material useful for the analysis of rape cases.

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