Jian Chen,[1] M.D.; Lawrence Kobilinsky,[1] Ph.D.; Dora Wolosin,[2] Ph.D.; Robert Shaler,[2] Ph.D.; and Howard Baum,[2] Ph.D.

A Physical Method for Separating Spermatozoa from Epithelial Cells in Sexual Assault Evidence

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ABSTRACT: The analysis of genetic markers for the purpose of individualization of semen specimens is extremely important in cases of sexual abuse and assault. The serological analysis of sexual assault evidence can sometimes be complicated because stains are often composed of a mixture of spermatozoa, vaginal epithelial cells and white and red blood cells. A filtration method has been developed to cleanly separate spermatozoa from epithelial cells based upon differences in size and shape. Nylon mesh filters of the appropriate pore size can be used to separate the smaller oval shaped spermatozoal cells from the larger and flatter epithelial cells. The former pass freely through the membrane while the latter are retained on the filter.

In this study, cell separation was demonstrated by (a) microscopic observation of stained cells, (b) amplified fragment length polymorphism analysis of DNA obtained from separated cells. The results of these analyses indicate that: (1) Approximately 70% of spermatozoa in the mixed cell sample will penetrate the 10 μm pore size filter, (2) Only about 1–2% of intact epithelial cells will do so, and (3) A small number of nuclei from spontaneously lysed epithelial cells will cross the filter. Experimental results using mixtures of spermatozoa and vaginal epithelial cells prepared in different ratios support the conclusion that the filtration process is an efficient and reliable method to separate spermatozoa from epithelial cells in casework specimens for subsequent DNA analysis.

KEYWORDS: forensic science, sexual assault evidence, epithelial cells, spermatozoa, filtration, DNA typing, D1S80

The analysis of physical evidence in crimes of sexual assault can lead to information vital to the process of identifying the assailant (1). Although, at times, relatively clean semen stains are available for forensic analysis, it is far more common for rape kit evidence to be composed of a mixture of male and female components. Such mixed stains may be composed of a combination of cells (spermatozoa, vaginal and/or buccal epithelial cells, and white and red blood cells) and physiological fluids (seminal plasma, vaginal secretions, saliva). Classical methods of analysis in these cases have typically included antigen/antibody identification and isoenzyme phenotyping.

[1]John Jay College of Criminal Justice, New York, NY, and Graduate Center, City University of New York, New York, NY.

[2]Office of the Chief Medical Examiner, 520 First Avenue, New York, NY.

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With the development of methods to genotype DNA (2–4), a nucleic acid which is both highly polymorphic and very stable, the forensic biologist has available individualizing techniques with high discriminatory potential and high sensitivity (4,5). These methods have become very useful especially in the analysis of older stains and stains that had been subjected to various environmental insults. Regardless of which typing method is utilized, evidence consisting of a mixture of cells must be handled appropriately so as to identify the origin of each of the cell donors.

Currently, the only practical method available for isolation of DNA from mixtures of spermatozoa and vaginal epithelial cells is based on differential (preferential) lysis (6,7). This method is based upon differences in packaging of DNA in the sperm head and in other cellular material. The DNA in the former is packaged in tightly disulfide linked cross-linked protamines (8) whereas the DNA in epithelial cells is not. This method is somewhat inefficient and often does not produce complete separations. Subsequent typing of genetic markers often reveals crossover contamination and the finding of 3 or 4 alleles rather than the expected 1 or 2 that would have resulted from complete separation of cells within the mixture. The development of a method capable of more completely separating spermatozoa from epithelial cells prior to DNA analysis would result in more easily interpretable typing patters and would improve the chances for a successful individualization.

Material and Methods

Samples

Fresh buccal epithelial cells and semen were obtained from several donors. Spermatozoa and epithelial cells were counted microscopically using a hemocytometer. Spermatozoa were prepared in concentrations varying form $0.2 - 1.0 \times 10^6$ and epithelial cells in concentrations varying from $0.1 - 0.3 \times 10^6$ cells per mL. A fixed volume (10 μL) was applied to a hemocytometer and cells in the four corner squares were counted. The total number of cells was determined as: N = Total Cells Counted/4 × 10,000 = cells/mL. Cell concentrations were adjusted with normal phosphate buffered saline (PBS), pH 7.0, for the filtration experiments described below.

The stain specimens used in this study were prepared by mixing spermatozoa and buccal epithelial cells of known cell count in different ratios, then applying a fixed volume of the mixture to sterile cotton swabs. The swabs were air dried and maintained at room temperature for up to two weeks.

Filtration

Semen and fresh saliva containing known amounts of spermatozoa and buccal epithelial cells, respectively, as well as prepared cell suspensions and mixtures of semen and saliva were applied to nylon mesh membranes with pore sizes of 5, 8, 10, 12, 25, or 35 μm (TETKO Inc.), which had been placed on top of 15 mL centrifuge tubes. Cells were allowed to filter across the membranes by gravity. Dried swabs which had been prepared as described above, were extracted in PBS, and then filtered through the nylon mesh membranes. Cell preparations were brought to a volume of 1 mL and applied to the filtration units. The process of filtration is completed in as little as 15 s depending on cell density and that the filter remains unclogged. The filters were washed one time with PBS to push through any residual sperm that may have adhered to the membrane or have gotten hung up on the epithelial cell mass. Low speed (approximately 50 × g) centrifugation or mild vacuum application to the filtration unit can replace gravity filtration but unless carefully regulated they tend to increase the number of epithelial cells which cross the filter, and therefore were not used. Cells which penetrated the filter (filtrate) and those that were retained on the upper membrane surface were collected as separate fractions. The filtrate and retained cell fractions and the original, unfiltered mixture of both cell types were examined microscopically. DNA was extracted from each fraction for PCR amplification and subsequent analysis.

Sample Staining—Chemical staining was performed using the "Christmas tree stain" described by Oppitz (9). Stained cells were examined using transmitted light microscopy at magnifications of 100, 200, and 400×.

DNA Extraction

Inorganic Extraction—Cells were washed once with protein lysis buffer consisting of 0.01M tris, 0.01M Na_2 EDTA · $2H_2O$, 0.1M NaCl, 2% sodium dodecyl sulfate (PLB), then resuspended in 200 μL PLB. To the suspension, 25 μL proteinase K (10 mg/mL) and 4 μL dithiothreitol (1 mM) (DTT) in 200 uL PLB were added, and the mixture was incubated at 65°C for 2 hours. The suspension was centrifuged at 1000 × g. An aliquot of the supernatant (10 μL) was quantitated using an agarose yield gel (0.8%) followed by ethidium bromide staining and observation using a transilluminator.

Chelex Extraction—DNA extracted by the inorganic method (50 μL) was added to 150 μL of 5% Chelex, boiled for 8 min, and centrifuged at 1000 × g (10). Chelex extracted DNA in the supernatant was then used for PCR amplification.

PCR Analysis of D1S80

Amplification Conditions—The D1S80 locus (11) was analyzed using the amplification protocol established by Perkin-Elmer. A TC-1 thermal-cycler (Perkin-Elmer) was used for the PCR amplification reaction.

Analysis of D1S80 Amplification Products—Acrylamide gels (5%) were prepared containing 0.5 M ribose, and 47 mM tris-formate buffer, pH 9.0, and cross linked with 0.17% piperazine diacrylamide (12). A discontinuous system was established using tris-borate soaked wicks. Bromophenol blue was used to monitor the progress of the electrophoretic run. Samples (8 μL) were loaded in formate buffer. A D1S80 allelic ladder and a phi-X 174 marker ladder were included on each analytical gel.

Silver Staining Method (13)—Following electrophoresis, the analytical gel was soaked in 250 mL of a 1% nitric acid solution for 3 min, washed once with deionized water, then placed in a 1% silver solution for 20 min. After staining, the gel was washed three times with deionized water and bathed in a reducing solution (280 mM $NaCO_3$ and 0.019% formaldehyde) for 5–9 min. Chemical reduction was terminated by placing the gel in 200 mL of 10% acetic acid for 5 min.

Results

Filtration of Spermatozoa and Epithelial Cells

Filters of varying pore size (5–35 μm) were studied to evaluate their ability to separate spermatozoa from epithelial cells. Initial experiments focused on pure semen and buccal cell suspensions. Microscope observation indicated that 5–10 μm nylon mesh membranes retained epithelial cells but allowed spermatozoa to pass through. Use of membranes with average pore sizes larger than 10 μm (12 μm, 25 μm and 35 μm) resulted in an increase in the number of epithelial cells found in the filtrate.

Table 1 illustrates the efficiency of filtration using an 8 μm nylon mesh. Ten experiments were conducted in which a solution containing 1.30×10^6 spermatozoa was allowed to pass through the filter. On average, 91% of the applied sperm cells passed through the membrane and were recovered in the filtrate. The prefiltration mixture appeared microscopically as shown in Fig. 1. The 8 μm pore filter is shown in Fig. 2. After filtration fewer epithelial cells are seen in the filtrate, demonstrating that intact spermatozoa pass easily through the filter while the larger epithelial cells do not (Fig. 3).

Table 2 shows the efficiency by which the filter retains epithelial cells but not spermatozoa. Ten separate experiments were conducted in which a suspension containing 0.38×10^6 spermatozoa and 0.40×10^6 epithelial cells was applied to the filter. The results show that approximately 74% of the spermatozoa had penetrated the filter while more than 99% of the epithelial cells had been retained on the filter surface.

Mock casework-like swabs were prepared from semen-free postmortem vaginal epithelial cells. Spermatozoa were added to the swab, air dried, then extracted in PBS. The extracted cell suspension was filtered. The results of four separate experiments are

TABLE 1—*Ability of spermatozoa to pass through nylon 8 μm mesh filters.*

No. Sperm* in Filtrate ($\times 10^6$)	Percent of Sperm in Filtrate
1.40	107
1.25	96
1.00	92
1.00	77
1.00	77
1.05	81
1.25	96
1.15	88
1.20	92
1.35	104

*1.3×10^6 spermatozoa and 1.3×10^6 buccal epithelial cells were mixed and applied to an 8 μm pore size mesh nylon membrane.

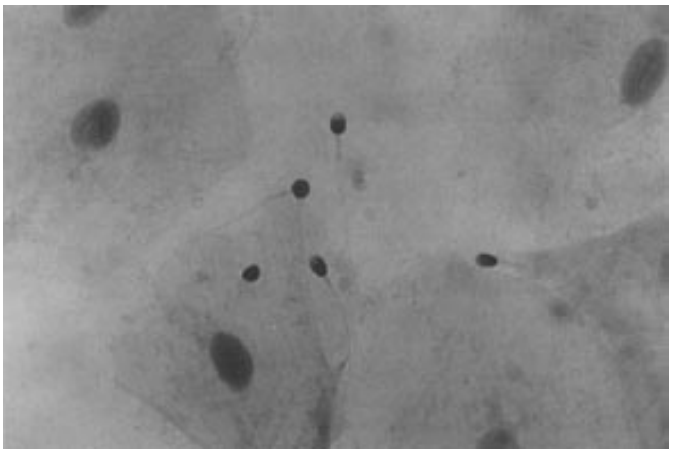

FIG. 1—*The pre-filtration mixture of spermatozoa and buccal epithelial cells.*

TABLE 2—*Filtration of spermatozoa/epithelial cell mixtures through nylon 8 μm mesh filters.*

Number Cells* ($\times 10^6$): Sperm/Epithelial in Filtrate	Percent Isolated: Sperm/Epithelial Cells
.28/ND	74/ND
.33/1 (cell)	87/1 (cell)
.25/ND	66/ND
.27/ND	71/ND
.28/ND	74/ND
.30/ND	79/ND
.35/ND	92/ND
.24/ND	63/ND
.25/ND	66/ND
.26/ND	68/ND

*Initial cell count: spermatozoa 0.38 $\times$ 10^6, epithelial cells: 0.40 $\times$ 10^6. ND = not detected.

shown in Table 3. Two of these experiments did not utilize any spermatozoa. On average, approximately 73% of spermatozoa and only 1–2% of the vaginal epithelial cells were recovered in the filtrate. The percentage of spermatozoa recovered in the filtrate is consistent with the results shown in Table 2, where approximately 74% of the sperm passed through the filter.

TABLE 3—*Filtration of semen-free vaginal epithelial cell/spermatozoa mixtures.*

Starting Cell Count ($\times 10^6$)		Filtrate Cell Count ($\times 10^6$)		Percent Cells Recovered in Filtrate	
Sperm	VEC*	Sperm	VEC	Sperm	VEC
1	1	.77	.02	77	2.25
1.53	1	1.02	.03	69	2.5
0	.85	0	.01	0	1.2
0	.34	0	.007	0	2.2

*VEC = Vaginal epithelial cells.

D1S80 Assay of Filtered Specimens

In order to determine the extent of separation of spermatozoa from epithelial cells, the filtered fractions from a 1:1 mixture of spermatozoa and epithelial cells were analyzed at the D1S80 locus. The DNA was isolated from the filtrate (sperm fraction) and from the retentate (epithelial cell fraction) and amplified using primers specific for the D1S80 locus. Amplified products were separated

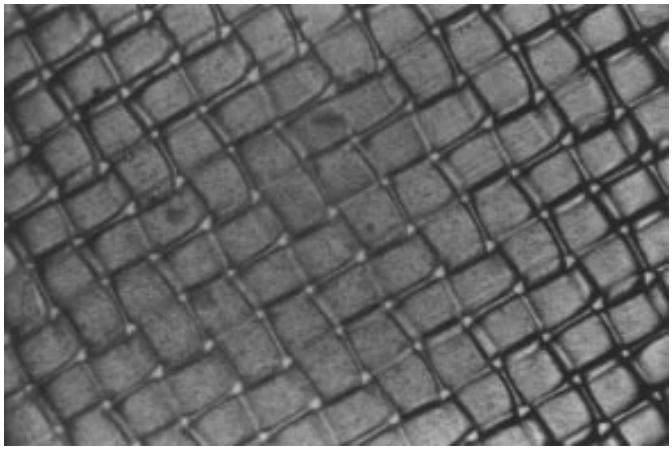

FIG. 2—*The 8 μm pore filter.*

FIG. 3—*Epithelial cells are too large to penetrate the 8 μm filter and are retained on the surface.*

using polyacrylamide gel electrophoresis then visualized by silver staining. The results are shown in Fig. 4. Lanes 1 and 9 contain the phi-X 174 ladder. Lanes 2 and 8 contain the amplified D1S80 allelic ladder; lane 3 contains amplified DNA from the epithelial cells; lane 4 contains amplified DNA from the spermatozoa; lane 5 contains amplified DNA from both the sperm and epithelial cells; lane 6 contains amplified DNA from the cells retained on the filter; and lane 7 contains amplified DNA obtained from cells in the filtrate. The results show that no epithelial cell alleles were observed in the sperm fraction, lane 7, and that no sperm cell alleles were observed in the epithelial cell fraction, lane 6.

Discussion

Gill developed a differential lysis method to isolate spermatozoal DNA from mixed sperm/epithelial stains (6,7). Wiegand modified this preferential lysis method so that in mixed cell specimens containing relatively small numbers of sperm, spermatozoal DNA can be preferentially extracted by reducing the amount of epithelial cell DNA in the mixture (14). The drawback of both of these methods is that the separation of spermatozoal DNA from epithelial cell DNA is less efficient than desired, that is, there is often cross contamination especially of the former with the latter.

The preferential lysis method requires a number of washes in order to obtain a relatively satisfactory separation. Extended washing tends to diminish the efficiency of sperm recovery, whereas insufficient washing tends to result in an excess of epithelial cells in the sperm fraction (14). Because PCR amplification produces millions of copies of target DNA, even a small amount of contaminating residual vaginal cell DNA may be detected.

The advantage of the filtration method for separating male from female cells over preferential lysis is that the former uses a static physical method to separate cells based on the large differences in their size and shape. Far better separation of sperm and epithelial cells can be achieved by filtration of mixed cell specimens through porous nylon filters. The effectiveness of the separation can easily be verified by light microscopic examination of stained or even

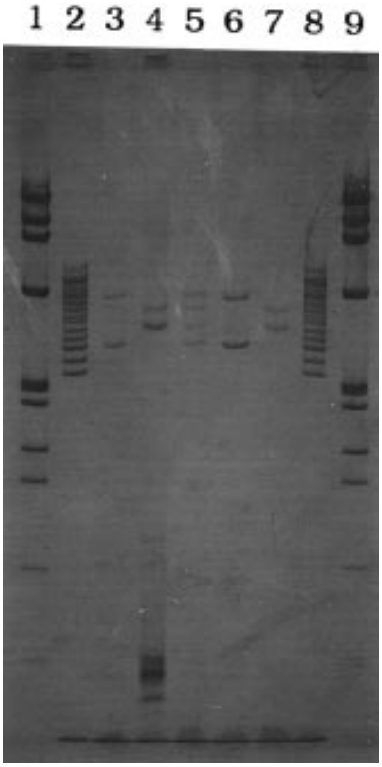

FIG. 4—*Autoradiograph illustrating the clean separation of spermatozoal alleles from epithelial cell alleles as a result of prior filtration of a sperm/epithelial cell mixture through an 8 μm porous membrane filter. 1 + 9: phi×174 HAEIII ladder, 2 + 8: D1S80 allelic ladder, 3: amplified DNA from epithelial cells, 4: amplified DNA from spermatozoa, 5: combined amplified DNA from spermatozoa and epithelial cell, 6: amplified DNA from cells retained on filter, 7: amplified DNA obtained from the filtrate.*

unstained cells. An additional benefit of using this filtration method is that because it is both rapid and easy to perform, criminalistics laboratories which routinely process a high volume of sexual assault evidence will find it especially useful.

The results of our study show that filtration is a rapid, effective technique for isolating spermatozoa from a sperm-epithelial cell mixture. The filtered sample is sufficiently pure so that PCR-based analytical methods can then be successfully employed with little apparent cross contamination.

We plan to continue this work by testing authentic casework specimen swabs that have been obtained from both recent and older cases. Table III indicates that the filtration method separates spermatozoa from vaginal epithelial cells with high efficiency. However, it is apparent that some epithelial cells do penetrate the filter. This may result from microscopic imperfections in the filter. It is also possible that some epithelial cells become folded or twisted and find a pathway through the membrane. Further studies are warranted to determine if vaginal epithelial cells obtained from dehydrated, older, authentic casework specimens react to filtration like our mock specimens or if they penetrate the membrane in relatively higher quantities as a result of the effect of aging on cell configuration. It should also be noted that because older epithelial cells easily lyse or may already be broken prior to swab extraction, it is possible that their nuclei pass through the filter resulting in some degree of cross contamination of spermatozoal DNA.

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Additional information and reprint requests to:
Dr. Lawrence Kobilinsky
Associate Provost and Professor
John Jay College of Criminal Justice
899 Tenth Avenue
New York, NY 10019