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TECHNICAL NOTE

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Filtration Based DNA Preparation for Sexual Assault Cases

ABSTRACT: Police departments in the United States currently have as many as 500,000 unprocessed swabs taken from rape victims. The standard method for purifying sperm from these swabs is to resuspend first all cells and to digest selectively the excess of the victim's epithelial cells. The intact sperm are then separated from the contaminating solubilized DNA by centrifugation, careful removal of supernatant, and extensive washing of the sperm pellet, all steps that are difficult to automate. Vacuum driven filtration is an alternative method for separating sperm from digested epithelial cells that requires only pipetting steps and can be readily automated in a 96 well format. Sperm DNA is enriched 45-fold using this process and the yield of PCR ready DNA is roughly 20% of the amount originally present on the swab.

KEYWORDS: forensic science, sexual assault evidence, automation, filtration, epithelial cells, spermatozoa, DNA typing, D21S1435

Police departments in the United States currently have as many as 500,000 unprocessed swabs taken from rape victims (1). Most are from no suspect cases and sperm DNA profiles from these swabs can be used to query the 1.3 million entries in the FBI Laboratory's Combined DNA Index System database and to identify rape suspects whose DNA profiles are in the database or will be in the future (2). In September, 2002 the U.S. Senate approved the "DNA Sexual Assault Justice Act" which would allocate $250 million in funding to eliminate the backlog of rape kits, while the House of Representatives is currently debating a similar bill, "The Rape Kit DNA Analysis Backlog Elimination Act." The sexual assault backlog problem is due in part to the labor intensive process used to isolate sperm DNA from casework samples and a reliable automated process is urgently needed.

Sperm are normally obtained from a rape victim by rubbing a swab against a mucous membrane, resulting in large numbers of the victim's epithelial cells being collected with the rapist's sperm. The standard method for purifying sperm from swabs is to resuspend first all cells from the swab and to digest selectively the victim's epithelial cells with Proteinase K. The intact sperm are separated from the solubilized, contaminating DNA by centrifugation, careful removal of supernatant, and extensive washing of the sperm pellet (3–6). The processes of centrifugation and careful removal of supernatant are difficult to automate. In this paper, the alternative and easily automated approach of using vacuum driven filtration to separate sperm from digested epithelial cells is presented.

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Methods

Samples

Photomicrographs of filters were reproduced courtesy of Millipore Corporation, © 2001. Sperm-free vaginal swabs and semen were taken from healthy volunteers and the cells were counted using a hemocytometer. Vaginal swab cuttings (one half of a swab) each having approximately 1.3 million epithelial cells were spiked with 2 μL, 4 μL, and 10 μL of a 10% semen solution having 5000 sperm per μL, and the spiked cuttings were left at room temperature for two weeks. The cuttings were placed in a 96 deep-well microtiter plate with 600 μL of Qiagen ATL buffer (which contains SDS) and shaken for 30 min at room temperature at 500 rpm on a rotating shaker. The cotton material from the swab was removed with tweezers and pressed against the side of the well to reduce liquid loss, leaving about 500 μL of ATL buffer in the well. Twenty-five μL of a 500 μg/mL Proteinase K solution was added, mixed by pipetting, and incubated at 56 degrees for 1 h.

Filtration

Sperm were collected on a filter stack consisting of a 11 micron nylon net (Millipore) placed on top of a 2 micron ISOPORE™ track-etch filter (Millipore). The filters were treated with 10x BackGround Quencher (MRC, Inc) for 30 min at room temperature to reduce the binding of free DNA. Seven millimeter filter disks were made using a hammer and a disk punch and placed in the wells of a Qiafilter 96 plate whose provided filters had been removed. Vacuum at 200 absolute Torr was applied using a QIAvac 96 manifold (Qiagen) and the epithelial cell DNA was collected in the filtrate. Filters were washed with 500 μL of 1xBackground Quencher in ATL buffer followed by 3 mL of distilled water. Sperm DNA was then solubilized by treating the filters with 50 μL of 10% solution of beta-mercaptoethanol (BME) in ATL buffer for

30 min at room temperature. The filters were then washed with 150 μL ATL and 200 μL AL buffer (Qiagen) and the filtrate containing sperm DNA was collected in micro tubes in the manifold.

DNA Purification, Quantitation, and PCR

DNA was purified from both the epithelial and sperm fractions using Qiamp mini-columns with a final elution volume of 50μL (Qiagen). Quantitation was done by ethidium bromide staining on a 1% agarose gel. Five μL of DNA was used as template for 30 cycles of PCR using primers for locus D21S1435, a locus for which the sperm and epithelial cell DNA samples used in this study have no alleles in common (unlike amelogenin, for which both fractions share the X chromosome allele). The 5′ primer was labeled with the Hex dye. Fragments were analyzed on an ABI 310 single capillary automated sequencer using GS500 size standards and peak areas were determined using Genescan software (Applied Biosystems).

Results

Intact sperm are about 5 microns in diameter while the particulate matter from digested epithelial cells has predominately submicron dimensions. Sperm should therefore be selectively trapped on a filter having an intermediate pore size of 2 microns. The large excess of the victim's cells, however, requires that the filter be extremely selective with minimal trapping of the digested material. Most filters have ill defined pores that consist of a tortuous path through randomly distributed fibers (Fig. 1*a*) and a filter of this type with a nominal pore size of 2 microns has many pores significantly smaller than 2 microns that trap much the victim's DNA as well as the sperm. ISOPORE™ track-etch filters, however, have precisely defined pores and are ideally suited for this application. These filters are made by subjecting a polycarbonate membrane to high-energy radiation and then etching away the impact points in an acid bath to produce circular pores of well defined size that go straight through the membrane (Fig. 1*b*).

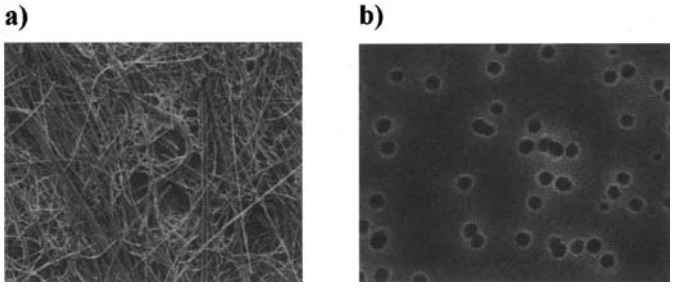

FIG. 1—*Photomicrographs of filters: a) A polypropylene depth filter. b) A 2 micron ISOPORE™ track-etch filter.*

Two micron track-etch filters should retain all sperm and very little of the digested epithelial cells as long as the filters do not clog. Filter clogging is a problem, however, due to cotton fibers and other debris eluted from the swab. Clogging can be prevented by placing an 11 micron nylon net pre-filter directly above the 2 micron track-etch filter. The filters are placed in a 96 well holder and sperm are separated from digested epithelial cells by vacuum driven filtration. The victim's DNA is collected in the filtrate, the filters are washed thoroughly, and the sperm are treated *in situ* with a reducing agent to break the extensive disulphide bonds in the sperm head. The solubilized sperm DNA is then washed through the filter by vacuum and collected. Figure 2 shows a flow diagram for this separation process that involves only pipetting steps.

Vaginal swab cuttings each having approximately 1.3 million epithelial cells were spiked with known amounts of sperm. The epithelial cell DNA generates a 170 basepair amplification product (Fig. 3*a*) while the sperm DNA generates 186 and 190 basepair products (Fig. 3*e*). When 10,000 sperm are present on the swab cutting the signal sperm fraction is predominantly epithelial (Fig. 3*b*) but when 20,000 sperm are initially present the combined sperm peaks represent 61% of the total signal (Fig. 3*c*). When 50,000 sperm are added to the swab cutting, the sperm signal is dominant with 86% of the total signal (Fig. 3*d*). Profiles of the epithelial cell DNA fractions showed only the 170 basepair peak (data not

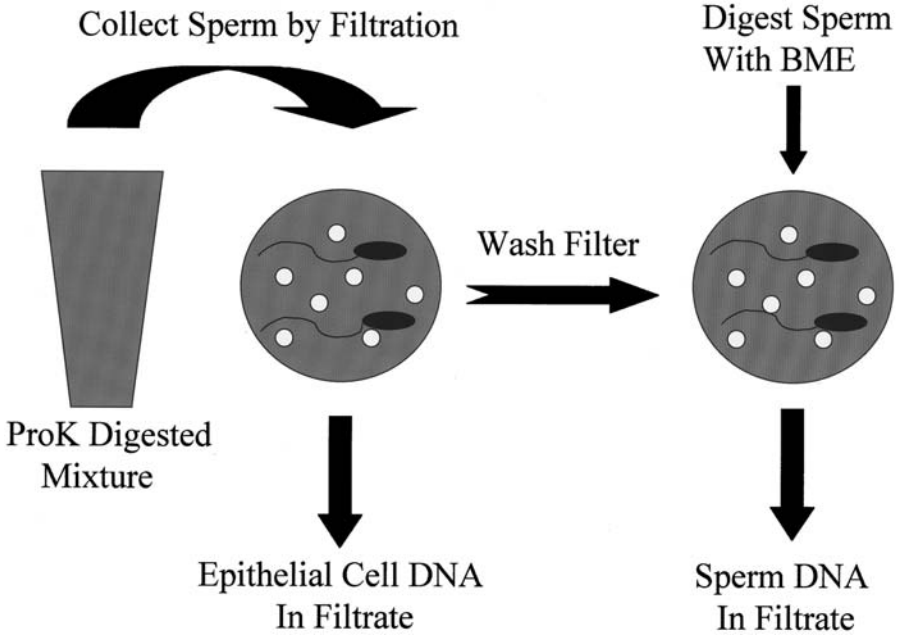

FIG. 2—*Flow chart showing steps required to separate sperm from digested epithelial cells by filtration.*

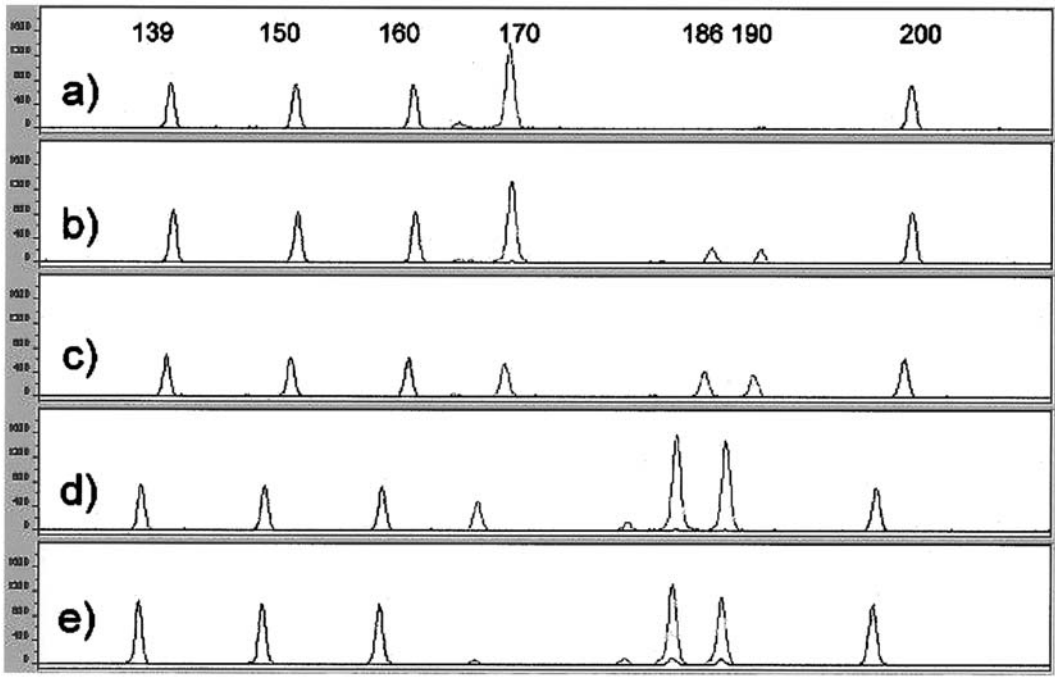

FIG. 3—*STR profiles of sperm DNA fractions. The sperm DNA fractions from swab cuttings containing epithelial cells and a) 0, b) 10 000, c) 20 000, d) 50 000 sperm or, e) only 50 000 sperm(no epithelial cells). GS500 size standards at 139, 150, 160, and 200 bp are indicated.*

shown). The apparent drift of the peaks to the left in each successive panel of Fig. 3 is due to the increased mobility of DNA in the capillary with each successive run on the same instrument. Thirty nanograms of DNA was present in the purified sperm fraction from the swab cutting that originally contained 50,000 sperm (data not shown) and all swab cuttings yielded enough DNA to perform PCR amplification.

Discussion

A number of attempts have been made to circumvent the selective lysis process. For example, Y chromosome polymorphic markers can be amplified from unfractionated swab DNA (7). However, this approach has the following disadvantages: the data provided cannot be used to probe the autosomal STR profiles in the FBI CODIS database, it won't work when the rape victim is male, and males of the same paternal lineage usually have identical Y chromosome STR patterns.

Another approach towards avoiding selective lysis is to separate physically sperm from intact epithelial cells. This has been done by flow cytometry (8); however, this technique is inherently slow due to the need to analyze and sort one cell at a time and is unlikely to be applied to casework. Attempts have also been made to use anti-sperm antibody coated magnetic beads (9). Epitope stability, however, will likely be a problem with this approach when applied to casework because detergents such as Sarkosyl or SDS are required to elute efficiently sperm from the swabs and these detergents destroy most of the epitopes recognized by the anti-sperm antibodies. Magnetic beads have been successfully used for many cell separation applications (10), but it remains to be seen if they can be used to separate human cells that have been dried onto an adsorbent substrate and then resuspended. Sperm can also be physically separated from the much larger intact epithelial cells by size using a 10 micron nylon weave filter (11). Unfortunately, the pores of these filters will expand under pressure requiring that only gravity be used as the driving force to minimize the unwanted passage of epithelial cells. In the absence of a strong driving force, capillary action on the filter surface competes with gravity flow through the filter and results in a large retention volume and difficulties with sample handling (personal observation). Furthermore, DNA from epithelial cells lysed by the harsh detergent required for efficient cell re-suspension will pass through the filter with intact sperm.

While sperm are smaller than intact epithelial cells, they are larger than the particulate material present after epithelial cells are digested with Proteinase K and this size difference can serve as a means for separation. A 2 micron track-etch filter has well defined pores that are stable under pressure and these filters trap very little solubilized epithelial cell DNA if treated with a blocking agent. Filter clogging is avoided by using an 11 micron nylon net above the track-etch filter. The swab cutting used for panel 3d had 1.3 million epithelial cells (7.8 μg of DNA) and 50,000 sperm (150 ng of DNA) for an initial sperm DNA content of 1.9%. The purified sperm DNA fraction gave an STR profile with sperm specific signals constituting 86% of the total signal for a 45-fold enrichment, which compares well with the 40-fold purification obtained using the standard selective lysis method and washing the centrifuged sperm pellet 3 times (data not shown). The amount of DNA recovered from the sperm fraction was calculated to be 20% of the input when 50,000 sperm (the amount found in less than 1 μL of a typical human semen sample) were present on the swab cutting. All swab cuttings yielded enough sperm fraction DNA to see a signal following PCR amplification, even when no sperm were initially present on the cutting (panel 3a), indicating that yields are a secondary consideration for sexual assault cases and that the primary concern is purification. The commercially available PCR kits now used for human identification recommend 1 ng of DNA as template, which is the amount present in 330 human sperm, or 3 nL of

a typical human ejaculate. Most swab cuttings have more than enough sperm DNA to perform PCR; the overriding problem in getting an unambiguous profile of the suspected rapist is the contaminating epithelial cell DNA.

The described procedure is identical to the robust and well accepted selective lysis method prior to the steps required for separating sperm from digested epithelial cells. The initial steps required to elute cells from the swab cutting and to digest the epithelial cells involve only pipetting, shaking, and temperature controlled incubation, all of which can be readily done in a 96 deep well microtiter plate by a robotic workstation. Furthermore, the steps downstream of sperm purification of sexual assault cases have already been automated in a multi-array format (12) and the final steps of PCR amplification and fragment analysis by capillary electrophoresis are currently processed in high throughput formats for a variety of industrial genomic applications. At the present time, the rate limiting step in processing sexual assault cases is that required to separate digested epithelial cells from intact sperm. Vacuum driven filtration holds promise as a means to remove this bottleneck and to allow fully automated processing of the sexual assault case backlog as well as future sexual assault cases. Plans are underway to compare this vacuum based method directly with the standard protocol on non-probative casework samples.

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