Identification of spermatozoa on sexual assault swabs: a comparative analysis of traditional tube extraction and direct slide elution methods

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Thesis

IDENTIFICATION OF SPERMATOZOA ON SEXUAL ASSAULT SWABS: A COMPARATIVE ANALYSIS OF TRADITIONAL TUBE EXTRACTION AND DIRECT SLIDE ELUTION METHODS

by

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B.S., Southern Illinois University – Edwardsville, 2012

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ABSTRACT

The purpose of this study was to compare the efficiency of three sperm elution methods on sexual assault swabs; factors such as solvent type, solvent volume, sperm concentration, and duration of extraction and elution method were evaluated with respect to observed sperm recovery. Swabs containing dilutions of semen ranging from 1:10 to 1:1,000 and simulated post-coital swabs were extracted via the traditional tube extraction, as well as two direct slide elution techniques, tapping and swirling. For the slide elution techniques, a swab cutting was placed directly onto a microscope slide, a small volume of water or buffer was added, and sperm were eluted by either tapping the sample with a stirring stick or swirling it around the slide with metal forceps. The tube method requires a minimum of one and one half hours for extraction, while the slide elution techniques require only ten seconds for extraction. The average sperm counts from 1:10 dilutions processed with the tapping elution method were statistically
higher than the 1:10 dilutions samples processed with tube and swirling methods. Elution by tapping also recovered a significantly higher amount of sperm cells from the 1:1,000 dilution compared to the tube extraction of the same dilution. The tapping elution method consistently resulted in the greatest number of spermatozoa observed, followed by the swirling method and then tube extraction; additionally, incidents of false negatives (no sperm observed) were observed with the tube and swirling methods. Simulated post-coital samples produced similar results to the semen samples; however, vaginal swabs from one donor resulted in an extremely high ratio of exfoliated epithelial cells that obscured the spermatozoa, especially with the direct slide elution methods. The slide elution methods resulted in similar and consistent relative standard deviations between dilutions in samples, while the tube extraction results suggest an increase in variance as the dilution increases. Overall, slide elution methods yielded the most observed sperm cells in a significantly shorter amount of time.
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LIST OF ABBREVIATIONS

AP  acid phosphatase
C   Celsius
DNA deoxyribonucleic acid
KPIC Kernechtrot-Picroindigocarmine
ml  milliliter
mm  millimeter
mtDNA mitochondrial deoxyribonucleic acid
ng  nanogram
PBS phosphate buffered saline
PCR polymerase chain reaction
pg  picogram
PSA prostate-specific antigen
RCMP Royal Canadian Mounted Police
rsd relative standard deviation
rpm revolutions per minute
SAECK Sexual Assault Evidence Collection Kit
Sg  semenogelin
STR short tandem repeat
µL  microliter
µm micrometer
1. Introduction

In 2012, an estimated 84,376 forcible rape occurrences were reported to United States law enforcement agencies – a 0.2% uptick from 2011 [1]. In reported incidents of forcible rape, defined by the FBI’s Uniform Crime Reporting Program as the carnal knowledge of a female forcibly and against her will, evidence is collected by medical personnel using a Sexual Assault Evidence Collection Kit (SAECK) [1-3]. The physical contact and nature of sexual assaults between perpetrator(s) and victim consequently transfers biological material; the extent and circumstances of this exchange, as well as other case-specific details, determine the extent of evidence collection and type of analysis performed [2, 4]. The purpose of SAECKs, introduced in the 1960’s, is to collect biological and trace evidence; these kits may contain collected items such as anal, oral, and vaginal swabs, pubic hair combings, fingernail scrapings, and other miscellaneous items [3].

Often, semen is the most probative evidence in cases of alleged sexual assaults; the most conclusive method of identifying semen is to microscopically confirm the presence of spermatozoa [5]. Prior to confirming the presence of semen, forensic analysts utilize less specific techniques to preliminarily indicate its presence.

Semen consists of a liquid portion, termed seminal fluid, and a cellular portion, or spermatozoa. The seminal fluid component is a mixture of various
gland secretions including acid phosphatase (AP) and prostate specific antigen (PSA)—originating from the prostate gland—and semenogelin (Sg), which originates from the seminal vesicle [6-10]. Preliminary analyses for the identification of semen most often target the presence or activity of AP and PSA.

Colorimetric tests, which target the presence of AP, are the most common presumptive tests for semen. Acid phosphatase, a water-soluble enzyme, is produced in high concentrations in seminal fluids [4, 6, 11]. The AP Spot test, a one-step catalytic color test, is a routinely employed screening analysis for semen in forensic laboratories [4, 6, 11]. Since AP is located in other body fluids relating to sexual assault analyses, e.g. vaginal secretions and breast milk, the AP spot test is only a preliminary tool utilized by analysts to identify potential semen stains suitable for further testing [4, 10, 11]. Although AP Spot test is fast and easy to use, there are other reagents available for preliminary semen analysis such as Fast Blue B or Diazo Red; once semen is presumptively identified, microscopic examination of the sample is performed for confirmation [6].

Sperm cells, typically identified under 400x magnification using a compound microscope, consist of three main components: a head, mid-piece, and flagellum (Figure 1). The head, approximately 4-5 micrometers (µm) in length, is a source for nuclear deoxyribonucleic acid (DNA) and also contains the acrosomal cap. The acrosomal cap, a structure that produces enzymes critical for fertilization, aids in microscopic sperm identification as it stains a lighter shade
compared to the nuclear portion of the sperm head [6]. The mid-piece, a source of mitochondrial deoxyribonucleic acid (mtDNA), and fragile flagellum may not always be microscopically observed in extracts prepared from sexual assault evidence. Several types of structural abnormalities such as double heads, double flagella, narrow heads, etc. occur in approximately 40% of sperm cells [6]. Typically, a differential staining technique is performed to visually enhance the morphological indicators of sperm – the ovoid-shaped head, acrosomal cap, and flagellum, if present.

![Figure 1. Structure of a sperm cell](image)

The purpose of analyzing SAECKs is most often to corroborate the victim’s account of the alleged sexual assault through detection of semen and to potentially identify a suspect through subsequent DNA analysis [14, 15]. Since
spermatozoa may persist inside the human vagina for up to seven days in living individuals, microscopic examination is a vital and highly informative step in the analysis of SAECKs [4, 16, 17]. Approximately 28% of victims report that the perpetrator(s) fully ejaculated at the time of the assault, which is substantiated by a spermatozoa recovery rate of about 30% from tested SAECKs [2, 18]. Detectives also use collection and analysis of SAECKs as leverage throughout the investigative process; although 90% of adjudicated sexual assault cases are resolved through plea bargains before analysis of collected biological evidence, collection of SAECKs result in longer detention times [2, 19].

With detectives, juries, and the public realizing the importance of sexual assault evidence—specifically DNA evidence—there is now a significant backlog of untested SAECKs in many jurisdictions. At the 2009 yearend, nearly 400,000 biological casework samples nationwide were considered backlogged, or not processed within 30 days of submission, an issue that 28% of publicly funded forensic crime laboratories have combatted by outsourcing casework to private labs [20, 21]. An estimated 57% increase in full-time analysts would be required to surmount the backlog without outsourcing cases, according to the Bureau of Justice Statistics [20, 21]. With the significance of SAECKs and the abundance of backlogged, untested kits, a rapid spermatozoa identification technique is crucial.

Since the discovery of spermatozoa in semen by Leewenhoek in 1677, laboratory methods for its identification have greatly progressed [22]. A crucial aspect of the identification process is the elution efficiency of sperm cells from
swabs. Many studies have been conducted to determine the best elution process based on amount of sperm recovered; studies range in focus from technique, solvents, substrate, etc. [5, 23-26]. Many sperm elution techniques currently employed in forensic laboratories require approximately two or more hours to complete and recover less than 15% of spermatozoa depending on the interval between swab collection and analysis [26].

In order to combat the growing number of untested Sexual Assault Evidence Collection Kits in a deft and economical manner, the development and utilization of a more efficient sperm elution technique would prove beneficial. The purpose of this study is to compare the efficiency of three sperm elution methods: traditional tube extraction, microscope slide elution via tapping and microscope slide elution via swirling. Factors such as solvent type, solvent volume, sperm concentration, and duration of extraction and elution method were evaluated with respect to observed sperm recovery.

2. Materials and Methods

This study was conducted in compliance with ethical standards set forth by the Institutional Review Board of Boston University School of Medicine (Protocol H-26187).
2.1. Sample Preparation

2.1.1. Preparation of Semen Dilutions

Liquid semen obtained from an anonymous, individual donor was stored at -20°C prior to use. A 1:10 working dilution was prepared by introducing 75 microliters (µL) of semen into a labeled 1.5 milliliters (mL) microcentrifuge tube that contained 675µL of water and mixing. The serial dilution method was utilized to attain additional dilutions of 1:100 and 1:1,000.

2.1.2. Preparation of Semen Sample Swabs

The swabs utilized in these experiments were 6-inch cotton tipped wooden applicators (Covidien, Mansfield, MA) similar to those supplied in SAECKs. To prepare the samples, 10µL of prepared semen dilution was pipetted onto a spot plate; the swab tip was then placed into the liquid and allowed to absorb the entirety of the semen sample. The swab was allowed to air dry and then stored at 4°C until use. A total of 100 swabs per dilution was prepared.

2.1.3. Preparation of Simulated Post-Coital Samples

Three semen-free vaginal swabs were obtained from each of four anonymous donors and stored at -20°C prior to use. The vaginal swabs were submerged into microcentrifuge tubes containing 250µL of diluted semen and continuously rotated for ten seconds in order to ensure consistent treatment between samples. Three swabs from each donor were individually prepared.
using either the 1:10, 1:100, or 1:1,000 semen dilution. The simulated post-coital samples were allowed to air dry and then stored at 4°C.

2.2. Sperm Extraction and Elution

2.2.1. Tube Extraction Methods

The tube extraction method was followed according to the sexual assault processing protocol utilized by the Boston Police Department Crime Laboratory [27]. Approximately one fourth of one swab was cut lengthwise and placed into a 1.5mL microcentrifuge tube and 250µL of distilled water was added. The microcentrifuge tube was placed onto an orbital shaker for a minimum of 60 minutes. The swab was then transferred into a Spin-X® Insert spin basket (Costar, Corning, NY) which was placed in the microcentrifuge tube and spun at 14,000 revolutions per minute (rpm) for five minutes. All but 20µL of the supernatant was removed in a manner such that the pellet was not disturbed. The spin basket containing the retained swab was then placed back into the microcentrifuge tube and 500µL of distilled water was added into the basket. The microcentrifuge tube was spun for an additional five minutes at 14,000rpm. All but 20µL of the supernatant was carefully removed. The pellet was then re-suspended by briefly vortexing, and 3µL was pipetted onto a 10x10 millimeter (mm) designated area on a glass microscope slide.
2.2.2 Direct Slide Elution Methods

Two direct slide elution methods were utilized in this experiment: tapping and swirling.

2.2.2.1. Tapping Method

For the tapping method (Figure 2), approximately one fourth of one swab was cut lengthwise and metal forceps were used to place the swab cutting onto a 10x10 mm designated area on a glass microscope slide. Forty-five microliters of distilled water was carefully pipetted onto the sample. Using a wooden stirring stick (Eco-products®, Boulder, CO), the swab cutting was tapped for ten seconds in an effort to loosen/release the cells. The swab was retrieved using metal forceps and retained for further analyses; during removal from the microscope slide, the remaining liquid in the swab was squeezed out onto the designated sample area.
2.2.2.2. Swirling Method

For the swirling method (Figure 3), approximately one fourth of one swab was cut lengthwise and metal forceps were used to place the swab cutting onto a 10x10 mm designated area on a glass microscope slide. Forty microliters of distilled water were pipetted onto the sample. A sterile wooden stick was used to tap the swab cutting for ten seconds. Metal forceps were then used to squeeze out any extra liquid while removing the swab cutting from the microscope slide.

*Figure 2. Tapping method diagram*
water was carefully pipetted onto the sample. Using metal forceps, the swab cutting was continuously and gently pressed against the glass slide and swirled around the sample box for ten seconds. The excess liquid remaining in the swab cutting was squeezed out onto the sample area as it was removed from the microscope slide and retained for further analysis.

*Figure 3. Swirling method diagram*
2.2.3 KPIC Staining

Once samples were pipetted/eluted onto a glass microscope slide, a Bunsen burner was used to heat-fix the samples onto the slide. Samples were visually enhanced by using a double staining technique known as Kernechtrot-Picroindigocarmine (KPIC); this method consisted of Christmas Stain A and Christmas Stain B (SERI, Richmond CA). One drop of Christmas Stain A was placed into each sample area for approximately 15 minutes. The slide was then gently rinsed with distilled water to remove excess stain and allowed to air dry. Next, one drop of Christmas Stain B was placed into each sample area for approximately 1 minute. The slide was then gently rinsed with ethanol to remove excess stain and allowed to air dry. To secure the sample, a drop of Cytoseal XYL mounting media (Richard Allen Scientific, Kalamazoo, MI) was placed onto a cover slip (Thermo Fisher Scientific, Inc., Waltham, MA), which was then placed onto the slide (Figure 4).
Figure 4. KPIC staining procedure

2. Added Christmas Stain A for 15 minutes.
3. Gently rinsed with distilled water and air dried.
4. Added Christmas Stain B for 1 minute.
5. Gently rinsed with ethanol and air dried.
6. Mounted with coverslip and Cytoseal XYL.

Figure 4. KPIC staining procedure
2.3. Preliminary Analysis

2.3.1. Determination of Sperm Concentration

In order to determine the concentration of sperm, 6µL of a 1:100 semen sample was spotted onto a glass microscope slide and heat-fixed with a Bunsen burner flame. The slide was stained with KPIC and secured with a cover slip as previously described. This process was replicated 10 times.

Samples were observed at 400X magnification. The number of sperm cells per 6µL of 1:100 dilution of semen was determined to be 2,585 ± 53. To calculate the number of spermatozoa per 1mL of neat semen, the following formula was used:

\[(\text{# of sperm cells/6}\mu\text{L}) \times (1,000\mu\text{L}/1\text{mL}) \times (\text{dilution factor})\]

The number of sperm is 2,584 ± 53, six microliters is the amount of semen spotted onto the microscopic slide, and 100 is the dilution factor. The final concentration of sperm was determined to be in the approximate range of 42.2 to 44 million spermatozoa per milliliter of neat semen. The results of this preliminary trial also acted as a measure to analyze consistency between dilution mixture and collection processes.

2.3.2. Determination of Optimal Protocols

Trials were performed to determine the optimal protocol for each sperm elution/extraction protocol; variables such as volume and type of buffer, as well as time of elution were analyzed. For direct slide elution methods, volumes
between 20 and 50 microliters of phosphate buffered saline (PBS) and distilled water were used, along with elution times of 10 seconds and 60 seconds; the use of PBS and distilled water was compared within the tube extraction protocol only. For each unique variable and dilution, samples were analyzed in triplicate.

2.4. Comparison of Methods

Once optimal protocols were determined for tube extraction and the two direct slide elution methods, samples were extracted/eluted in quadruplicate. In this trial, each protocol was tested using the three different dilutions of sample swabs. Samples from the preliminary analyses representing the final protocols of each method were combined with results from this trial, for a total sample number of seven per dilution and protocol.

2.5. Tube Extraction of Post-Slide Eluted Samples

Exhaustion of samples following slide elution was analyzed by retaining the previously eluted swab cuttings and further extracting them via tube extraction; the tube extraction protocol was followed, however the entire pellet was pipetted onto a microscope slide for staining and visualization.

2.6. Analysis of Simulated Post-Coital Samples

In this trial, prepared simulated post-coital samples were analyzed in order to determine the effect of vaginal fluid and cellular material on the elution of
sperm from swabs. Samples were extracted/eluted in triplicate and compared based on dilution, protocol, and donor swab.

2.7. Data Collection and Analysis

All samples were viewed under 400x magnification using a Nikon Eclipse E200 compound microscope (Nikon, Tokyo, Japan) and examined for spermatozoa. Sperm cells were counted manually. Data were stored using Microsoft Excel®.

Average observed sperm cell counts and standard deviations were calculated; in some trials, relative standard deviations (RSD) were also calculated. Statistical significance was also calculated for particular comparisons addressed in section 3.3. Microsoft Excel® and StatPlus® were utilized for statistical analyses and graphical representations.

3. Results and Discussion

3.1. Preliminary Analysis and Determination of Sperm Concentration

Trials were performed to determine the optimal amount of buffer for the slide elution techniques. The volume of buffer utilized needed to be almost fully absorbed by the swab sample, and after implementation of each technique, a small volume of liquid had to remain within the sample area on the microscope slide for optimal cellular recovery; too much buffer would result in release of
liquid and cellular material outside of the prepared sample area and too little buffer would fail to release enough spermatozoa from the cotton matrix of the swab. Amounts of buffer ranging from 20µL to 50µL were tested. The optimal volume of buffer for the tapping method was determined to be 45µL, while the optimal volume for the swirling method was determined to be 40µL. With these buffer volumes, the swab cuttings were fully saturated with buffer and released a small amount of liquid onto the slide during and after elution.

A second preliminary experiment was performed to determine sperm concentration and to ensure that the preparation and data collection processes were consistent between replicates.

Table 1. Sperm Concentration Results (6 µL of 1:100 Semen Dilution)

<table>
<thead>
<tr>
<th>Sperm Concentration Trial</th>
<th>Sperm Counts</th>
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<tbody>
<tr>
<td></td>
<td>2489</td>
</tr>
<tr>
<td></td>
<td>2633</td>
</tr>
<tr>
<td></td>
<td>2608</td>
</tr>
<tr>
<td></td>
<td>2567</td>
</tr>
<tr>
<td></td>
<td>2499</td>
</tr>
<tr>
<td></td>
<td>2598</td>
</tr>
<tr>
<td></td>
<td>2604</td>
</tr>
<tr>
<td></td>
<td>2655</td>
</tr>
<tr>
<td></td>
<td>2589</td>
</tr>
<tr>
<td></td>
<td>2603</td>
</tr>
<tr>
<td>Average</td>
<td>2584.5</td>
</tr>
<tr>
<td>2 Standard Deviations (SD)</td>
<td>+/-101.07</td>
</tr>
<tr>
<td>Relative Standard Deviation (RSD)</td>
<td>2.06</td>
</tr>
</tbody>
</table>
Table 1 shows that the average number of observed spermatozoa in ten replicates was 2,584.5 ± 101.07. The relative standard deviation is equal to 2.06% and suggests adequate consistency between the preparation protocol and data collection process. The variability between sperm counts of the replicates could be explained by variables such as failure to pipette identical volumes of sample onto the slide, inability to produce a truly homogenous semen dilution, or washing and rinsing of the slide during KPIC staining. The relevance of this result is that it allows the analyst confidence when comparing samples between different extraction/elution methods. This result also confirms that the analyst's counting method was consistent.

3.2. Determination of Optimal Protocols

Optimal protocols were determined for the slide elution methods by testing variables such as elution technique, solvent (distilled water versus PBS), and time (ten seconds versus sixty seconds). The results are as follows:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dilution</th>
<th>Sperm Count on Slide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 Sec. Elution Time</td>
</tr>
<tr>
<td>Water</td>
<td>1:10</td>
<td>519.7 ± 773.3</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>29 ± 44.5</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>10.3 ± 18.9</td>
</tr>
<tr>
<td>PBS</td>
<td>1:10</td>
<td>353.7 ± 465.6</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>24.3 ± 21.0</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>3 ± 6.9</td>
</tr>
</tbody>
</table>
Table 3. Swirling Elution Method – Average of Triplicate Samples

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dilution</th>
<th>Sperm Count on Slide</th>
<th>Elution Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 Sec. Elution Time</td>
</tr>
<tr>
<td>Water</td>
<td>1:10</td>
<td>206.7 ± 327.0</td>
<td>744.7 ± 730.0</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>77.3 ± 295.5</td>
<td>175.7 ± 295.5</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>3.7 ± 6.4</td>
<td>11.3 ± 7.0</td>
</tr>
<tr>
<td>PBS</td>
<td>1:10</td>
<td>450.7 ± 793.6</td>
<td>515.7 ± 196.7</td>
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<tr>
<td></td>
<td>1:100</td>
<td>72.3 ± 65.7</td>
<td>79.7 ± 36.3</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>2 ± 2</td>
<td>5 ± 11.1</td>
</tr>
</tbody>
</table>

Table 4. Tube Extraction Method – Average of Triplicate Samples

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dilution</th>
<th>Sperm count in 3µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1:10</td>
<td>84.3 ± 147.3</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>54 ± 71.2</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>PBS</td>
<td>1:10</td>
<td>32.7 ± 19.7</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>19.3 ± 23.4</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>3 ± 8.7</td>
</tr>
</tbody>
</table>

As demonstrated in Tables 2-4, spermatozoa were visualized using all extraction methods, regardless of the solvent or elution method used. Since the main focus of this research is to compare efficiency of several sperm extraction techniques, elution times of ten seconds were chosen for the remaining slide elution experiments. The results show that when 10-second elution times were
utilized, sufficient amounts of spermatozoa for observation were released. Elution times of sixty seconds consistently yielded more cellular material than the shorter elution time, with the exception of samples that were eluted with PBS using the tapping method; a 60-second elution time may be preferred in cases of dilute samples to ensure adequate cellular recovery.

The use of distilled water as a solvent typically released more cellular material than PBS. In some forensic laboratories, PBS, a non-toxic solution that does not negatively impact subsequent DNA profiling and prevents cell ruptures or shriveling, is used as a substitution for distilled water during biological screening extractions [29]. The literature also suggests that there is no statistical difference between the use of distilled water and PBS as an extraction medium for sperm cells from swabs [29, 30]. Since distilled water is a more readily available and cheaper alternative to PBS, distilled water was chosen to continue subsequent analyses.

3.3. Comparison of Methods

In this portion of the study, elution methods were directly compared based on average numbers of sperm cells observed, relative standard deviations of the average sperm recoveries of each method and average number of tube-extracted sperm cells from post slide-eluted samples. Results from the final elution procedures determined from the experiments outlined in section 3.2 were also included in this trial, resulting in 7 total samples per method.
Figure 5. Number of sperm cells observed in 1:10 samples (n=7) using the tube, tapping and swirling methods

Figure 6. Number of sperm cells observed in 1:100 samples (n=7) using the tube, tapping and swirling methods
Figure 7. Number of sperm cells observed in 1:1,000 (n=7) samples using the tube, tapping and swirling methods.

Table 5. T-Test Values

<table>
<thead>
<tr>
<th>T-Test Direct Comparison</th>
<th>Test Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 Tap vs. Tube</td>
<td>3.059*</td>
</tr>
<tr>
<td>1:10 Swirl vs. Tube</td>
<td>1.973</td>
</tr>
<tr>
<td>1:10 Tap vs. Swirl</td>
<td>2.197*</td>
</tr>
<tr>
<td>1:100 Tap vs. Tube</td>
<td>0.750</td>
</tr>
<tr>
<td>1:100 Swirl vs. Tube</td>
<td>0.704</td>
</tr>
<tr>
<td>1:100 Tap vs. Swirl</td>
<td>0.160</td>
</tr>
<tr>
<td>1:1,000 Tap vs. Tube</td>
<td>3.041*</td>
</tr>
<tr>
<td>1:1,000 Swirl vs. Tube</td>
<td>2.093</td>
</tr>
<tr>
<td>1:1,000 Tap vs. Swirl</td>
<td>1.892</td>
</tr>
</tbody>
</table>

*A result of 2.179 or more indicates statistical significance. The method in boldface outperformed the other method to which it is compared.
In Figures 5-7, the numbers of observed spermatozoa per dilution and elution method are shown. The tapping elution method released more sperm cells than the other elution methods, with the exception of the 1:100 dilutions, where all methods eluted similar amounts of spermatozoa. Table 5 shows the difference in the mean number of observed spermatozoa was statistically significant between the tapping and tube methods at the 1:10 and 1:1,000 dilutions and also between the tapping and swirling methods at the 1:10 dilution.

Occurrences of false negatives, where sperm failed to be visualized following extraction, were seen at the 1:1,000 dilution with both the tube method (two false negatives) and the swirling method (one false negative). Incidents where zero spermatozoa were observed in this experiment are considered false negatives since ten microliters of semen dilution, known to contain sperm cells, was previously added to the swab. During microscopic analysis for sperm cells, it is imperative that an extraction/elution method results in the visualization of one sperm cell, at minimum; these results show that the tapping method yielded visualized sperm cells at every dilution. The absence of false negatives with the tapping method is crucial because it indicates that the tapping method may yield probative results in situations where the swirling and tube method fail. The identification of sperm during this step in the process also saves time, as a subsequent semen screening test is not needed.

The combination of zero incidents of false negatives and a higher average of observed sperm cells suggests the tapping elution method is the superior
method as it is consistently eluting more cellular material than the other elution protocols and does so in a considerably shorter amount of time than the traditional tube extraction method. However, the sample size in this experiment is small and more samples must be tested to ensure reproducibility.

Figure 8. Relative standard deviation and trendline of average number of observed spermatozoa with tube extractions
Figure 9. Relative standard deviation and trendline of average numbers of observed spermatozoa with tapping elutions

Figure 10. Relative standard deviation and trendline of average numbers of observed spermatozoa with swirling elutions
Relative standard deviation (RSD) is a statistical measure of variance compared to the mean; it allows an analyst to compare the repeatability, or precision, of different methods. Figures 9-10 show that the RSD between mean numbers of observed spermatozoa with tapping and swirling methods are similar across each dilution. The tapping and swirling methods have consistent relative standard deviations within dilutions; the tapping method averages an approximate 75% relative standard deviation, while the swirling method averages an approximate 82% relative standard deviation. Figure 8 shows that tube extraction does not yield consistent relative standard deviations between dilutions, and preliminarily suggests an increasing trend of deviation from the mean as the dilution increases.

Relative standard deviations of 75% and 82% depict high variations between samples; low variation between cellular recovery of similar samples is optimal. However, the consistency of variation between dilutions when utilizing the tapping and swirling method suggest the methods are reproducible; the direct slide elution methods should be optimized to lower the variance and increase precision. The results from the tube extraction method illustrate a possible decreasing trend in precision as the dilution of semen deposited onto the sample is increased, and needs to be confirmed with the inclusion of more data points (dilutions).

Relating these results to actual casework, implementation of an elution/extraction method with consistent results is imperative. Unlike analysis of
laboratory-controlled samples, the concentrations of semen samples being analyzed in a crime laboratory are unknown. The decreasing consistency as the dilution of semen increases that was observed with the tube extraction method is disconcerting since forensic analysts typically encounter dilute samples when examining sexual assault evidence.

3.4. Tube Extraction of Post-Slide Eluted Samples

Once samples were eluted via tapping or swirling, the swab cuttings were retained and then extracted using the traditional tube extraction method in order to determine if any more spermatozoa could be eluted from the swab and used for subsequent analysis. Figure 11 shows that spermatozoa were observed in every dilution of post-slide eluted samples, with more spermatozoa being observed after extraction of the post-tap samples. There is no correlation between numbers of sperm initially eluted using the direct slide elution methods compared to the number of sperm cells released from subsequent tube extraction. The higher number of sperm cells released from post-tap samples could be from the force of tapping; sperm cells might become detached from the cotton matrix, but not fully eluted until subsequent tube extraction.
In the cases of very dilute semen samples, it is important to take into account how much cellular material remains on the swab following direct slide elution for subsequent DNA analysis. Since approximately one fourth of a sexual assault swab may be consumed for presumptive semen analysis, the amount of the sample remaining for subsequent analysis can become an issue in forensic laboratories when low numbers of sperm cells are present [31].

With traditional tube extraction, only a portion of the pelleted extract is transferred to the slide for observation (3µL of an approximate 20µL extract in this experiment); the remaining pellet/extract is available for subsequent analyses. Figure 11 shows that tube extraction of post-tap eluted samples...
resulted in a substantial number of eluted spermatozoa. If direct slide elution methods are utilized in the laboratory, analysts should retain and submit the swab cutting for further analysis, as it is shown to still contain substantial cellular material.

Standard protocols for DNA examination suggest performing analysis on one nanogram (ng) of template DNA, which translates into approximately 330 haploid cells; however, recent advances in short tandem repeat (STR) profiling have enabled analysts to obtain STR profiles with 50-100 picograms (pg) of DNA template, or 17-33 sperm cells [32 – 36]. Both the post-tap and post-swirl eluted samples yielded an adequate amount of sperm cells from the 1:1,000 samples, 21 and 17 sperm cells respectively, to perform polymerase chain reaction (PCR) analysis. Thus, sufficient numbers of sperm cells remained in the swabs following slide elution to be detectable in subsequent analyses. These results further support that forensic laboratories should retain post-eluted swabs, especially in cases where samples are presumed to be degraded or when a low number of sperm cells was visualized.

3.5. Analysis of Post-Coital Samples

Simulated post-coital samples were processed via tube extraction, tapping, and swirling elution methods using semen dilutions of 1:10, 1:100, and 1:1,000. Samples of four female donors were prepared and examined, however
only results from three donors are depicted. The removal of one donor set will be subsequently discussed.

Figure 12. Average number of observed sperm cells in 1:10 simulated post-coital samples

![Graph](image)

Figure 13. Average number of sperm cells observed in 1:100 simulated post-coital samples

![Graph](image)
Figure 14. Average number of sperm cells observed in 1:1,000 simulated post-coital samples

The tapping method eluted the highest average number of sperm cells, followed by the swirling method; the tube extraction method resulted in the lowest number of observed spermatozoa (Figures 12-14). These results mimic those from the semen samples in the preceding trials, suggesting that there is no significant change in cellular recovery when vaginal epithelial cells and secretions are present. The higher numbers of sperm cells recovered from vaginal swabs for all dilutions are likely due to the total submersion of the swab into semen dilutions for complete saturation, whereas samples in previous trials contained only 10µL of semen dilution. The simulated post-coital samples are particularly susceptible to fluctuations in the numbers of sperm cells observed
due to variations in the amount of semen absorbed by the swabs and the sizes of the one-fourth swab cuttings.

**1:10 Simulated Post-Coital Samples**

![Graph showing observed number of spermatozoa on 1:10 simulated post-coital samples per vaginal swab donor.]

*Figure 15. Observed number of spermatozoa on 1:10 simulated post-coital samples per vaginal swab donor*

**1:100 Simulated Post-Coital Samples**

![Graph showing observed number of spermatozoa on 1:100 simulated post-coital samples per vaginal swab donor.]

*Figure 16. Observed number of spermatozoa on 1:100 simulated post-coital samples per vaginal swab donor*
Figures 15-17 depict the number of spermatozoa observed with each extraction technique and dilution from three different vaginal swab donors. The literature states that the cyclic nature of the menstrual cycle, as well as other factors including age, infections and sexual activity, produce variations in vaginal secretions [37-41]. When comparing results of similar samples between each donor, no readily visible trend was observed in amounts of cellular material recovered.
<table>
<thead>
<tr>
<th></th>
<th>Donor 4</th>
<th>Donor 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Tapping</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Swirling</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

*Figure 18. Comparison of 400X magnified fields of view between donors 1 and 4 using different extraction/elution methods*
While the results from three of the donors appeared similar with respect to mean number of observed sperm cells, one donor’s results were vastly different. Figure 18 illustrates the discrepancy in fields of view of each method viewed at 400x magnification between donor 1 and 4. Donor 4 consistently eluted a higher ratio of exfoliated epithelial cells, so much so that quantitation of observed sperm cells was impossible. The tapping method resulted in complete saturation of the field of view with epithelial cells, while the swirling and tube method results were not as extreme.

These results concur with those reported by the Royal Canadian Mounted Police as part of an internal validation study of cellular material transfer—the tapping method resulted in a higher amount of epithelial cell deposition, but also produced the best results in deposition of sperm cells [28]. The excess amount of epithelial cells from extraction samples of donor 4 could be due to factors suggested previously, or could be a result of variations in the vaginal swab donor’s collection technique despite the fact that all donors were given the same collection instructions.

3.6. Crime Laboratory Application

The process of analyzing a SAECK can take several days to several months for completion, depending on the workflow and backlog of the laboratory. A typical laboratory protocol for the processing of semen samples is depicted in Figure 17. The implementation of presumptive analyses is imperative as
confirmation techniques for biological samples are more costly and time-consuming; samples yielding negative presumptive results are not processed further, thus saving analysts valuable time that can be devoted to more probative items of evidence. Microscopic examination is the most time-consuming process in the screening of semen, but may also be the most important since it unequivocally confirms the presence of semen and can give the analyst an idea of how much cellular material is available for DNA testing. Reducing any amount of time required for microscopic examination can have a significantly positive impact on reducing the rising number of backlogged and outsourced biological case samples [20-21].
Implementation of a new method into a laboratory’s protocols requires the method to be reliable and reproducible, as well as cost and time efficient; high sensitivity and specificity are also important factors to be considered. Introducing the tapping or swirling method into laboratory protocol could significantly cut down time spent on preliminary semen analysis. With tapping and swirling methods, the elution time requires ten seconds, while the tube method typically requires a minimum of one hour; however, tube extraction does offer advantages.

Figure 19. Flowchart of a typical semen screening protocol for SAECK swabs
over the slide extraction techniques such as overnight (unattended) and simultaneous sample extractions. Even so, the hands-on time required for microcentrifuging and pipetting offsets these benefits, making tube extractions less efficient.

One factor to be considered when implementing the tapping method into a laboratory’s protocol is optimizing deposition of sperm cells within the sample area on the microscope slide. When microscopically viewing tap-eluted samples, spermatozoa were visualized outside of the designated sample area; this could be a result of the swab moving outside of the sample area during elution or from the force of the tapping itself with too much buffer. During tapping elution, the swab did not remain stationary on the microscope slide; sometimes, the swab would adhere to the wooden stick, while other times the swab would slightly move laterally with each tap.

Using a larger sample area than the 10x10 mm area used in these experiments could alleviate the issue of sperm cells depositing outside of the sample area, however this would increase the time required to microscopically scan the entire sample area.

Another factor to be considered with tapping elution using a wooden stick is the possibility of the tool absorbing some of the buffer and cellular material; implementation of a plastic or metal stir rod would address this issue. A metal stir rod may also improve the tapping method overall as it can be sterilized and reused, becoming a more cost-effective tool.
In a validation study conducted by the Royal Canadian Mounted Police (RCMP), 10µL of buffer was utilized for similar elution experiments [28]. Results from the swirling and tapping methods utilized in the RCMP validation study were similar to results in experiments performed with 40 and 45µL, respectively, in the present study. Approximately one fourth of a swab was utilized in both experiments. This suggests that the volume of buffer may be decreased while achieving similar results; less liquid during the elution process could also combat the problem of deposition of sperm cells outside of the designated sample area.

Crime laboratories take into consideration many factors when choosing techniques to implement in their protocols; tube extraction of sexual assault evidence is only one method employed in some labs. Along with the tube extraction method and the direct slide elution methods, other alternative sperm identification methods exist. One example is Sperm Elution© (Cellmark, Gothenburg, Sweden), a two-phase recovery method that utilizes multiple detergent and buffer washing steps in order to separate epithelial cells and sperm cells for improved visualization in low dilutions of semen [24-26]. The Sperm Elution© method is more costly as it requires special buffers and detergents, results in greater physical manipulation of the sample, and is more time consuming with approximately three hours required extraction time.

Other attempts to improve sperm identification from SAECKs include automated KPIC staining techniques and fluorescent-based staining for easier and quicker identification [5, 42, 43]. Automated KPIC methods utilize existing
microscope hardware and specialized software in order to locate potential sperm cells, while fluorescent-based staining such as Sperm Hy-Liter™ (Independent Forensics, Lombard IL) relies on highly specific fluorescently dyed antibodies to tag human sperm heads [5, 42, 43]. Additionally, recent research has suggested that different types of swabs, such as nylon, rayon, foam, polyester, etc., be utilized for sample collection as certain swab types yield higher amounts of cellular material during extraction [44].

4. Future Research

Limited data has been published regarding the tapping and swirling direct slide elution methods [5, 28]. Implementation of direct slide elution methods into laboratories’ SAECK protocols should occur only after additional research is performed to address several key issues. One area of future research includes the effects of vaginal secretions on the elution of cellular material using the direct slide elution techniques. Results of this experiment showed that direct slide elution methods resulted in a high ratio of exfoliated epithelial cells in the sample area in the case of one donor; tube extraction also resulted in a higher ratio of observed epithelial cells, but at a lower amount compared to the direct slide eluted samples, allowing the sperm cells to be more easily visualized.

Relating to tapping elution, the technique should be optimized to improve elution of sperm cells onto the designated sample area. In this experiment,
sperm cells spread beyond the sample box perimeters, which could cause sperm to be missed if microscopic examination is restricted to the designated area; a different tapping tool, lower volume of solvent or larger sample area may alleviate this concern.

Another area of research should investigate the effects of other biological material on cellular elution. Samples from SAECKs can include oral, vaginal, anal, and other miscellaneous swabs that could include biological material such as saliva, urine, blood, and fecal matter. The parameters of this experiment did not include biological material other than vaginal secretions.

Further experiments should also be performed to determine the lowest dilution of semen that is detectable with direct slide elution methods. In preliminary trials aimed at optimizing protocols, 60-second elution times resulted in higher amounts of sperm cells being observed, however ten seconds was chosen as the elution time for maximum efficiency. If further research concluded that 60-second elutions have a higher sensitivity than 10-second elutions, protocols may want to incorporate sixty seconds elution times. Also, longer elution times may be implemented in instances of backlogged SAECK analysis, since the longer period of time between collection and analysis of the swab may result in less recovery.

Research should also be conducted on samples collected as part of SAECKs. This experiment was performed on laboratory-prepared samples. Collection techniques employed by medical personnel, unknown amount of
semen deposited onto swabs, time since intercourse, other cellular material collected on swabs, etc. are all added factors to true casework samples that should be explored.

5. Conclusion

This study compared the efficiency of three separate sperm elution methods as a means of microscopically identifying sperm cells present on cotton swabs. Traditional tube extraction, direct slide elution - tapping, and direct slide elution - swirling methods all performed adequately as sperm was visualized in all 1:10 and 1:100 dilutions. The tapping method consistently eluted higher amounts of observed sperm cells, with the swirling method eluting slightly less. Statistical differences were observed between 1:10 tapping and 1:10 tube extractions, 1:10 tapping and 1:10 swirling extractions, and 1:1,000 tapping and 1:1,000 tube extractions. Zero incidents of false negatives were observed with implementation of the tapping method; however, two occurrences of false negatives were observed with the tube method and one incident of a false negative occurred with the swirling method, with analysis of the 1:1,000 diluted sample swabs.

The direct slide elution methods resulted in high levels of variance between samples; the variances were, however, consistently around 75% and 82% between samples from the mean in tapping and swirling, respectively. The
consistent level of variance between samples using the direct slide elution methods shows consistent levels of error, however these techniques should be optimized to increase precision. The tube extraction method also resulted in high levels of variance but unlike the direct slide elution methods, the tube extraction method was not consistent in its level of error suggesting a possible relationship between the decrease in precision as the semen dilution increased.

Trials were also performed to determine if implementation of direct slide elution methods resulted in an exhausted sample. When slide elution method was employed, results showed that after samples were processed using the tapping or swirling method, sperm cells still remained on those swabs, indicating that analysts should retain and submit these processed swab samples for DNA analysis as they contain crucial evidentiary material.

The effect of vaginal secretions on the elution of sperm cells from swabs was found to be minimal. Each elution method from vaginal swabs produced results in a similar trend compared to the semen-only samples. Simulated post-coital samples from one donor resulted in a higher ratio of exfoliated sperm cells, particularly with direct slide elution methods, where a significant increase of epithelial cells was visualized. Although sperm were visualized and counted with the samples from the other three donors, quantitation was impossible with donor 4 as saturation of the field of view with epithelial cells hindered the ability to visualize sperm cells. Although samples from donor 4 resulted in complete saturation of epithelial cells while using the direct slide elution methods, the tube
extraction method also resulted in a higher number of epithelial cells being observed, somewhat hindering visualization and quantitation of sperm cells observed.

The tapping and swirling methods eluted more sperm cells when compared to the tube extraction method, and the tapping method resulted in zero incidents of false negatives. The direct slide elution methods require only ten seconds for sample elution, compared to tube extraction which requires a typical incubation period and centrifugation step of more than one and one half hours after all steps are completed. Overall, these results preliminarily indicate that the tapping method provides reliable, reproducible, and time and cost effective results that could alleviate some of the issues associated with backlogged sexual assault cases.
BIBLIOGRAPHY


Curriculum Vitae

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• Case Report Writing
• Evidence Recognition, Collection, and Preservation
• Biological Screening Analyses
• Immunochromatographic Analyses
• DNA Analysis (Chelex®, QIAamp, Amicon, ABI 3130, PP®16, Identifiler®)

Relevant Professional Experience

LABORATORY TECHNICIAN Genetic Services, Inc. Cambridge, MA
January 2013 - Present
• Prepare media, such as agar plates and nutrition for Drosophila stocks.
• Sustain back-up stocks for over 200 strains of Drosophila organisms.
• Assist other technicians with Drosophila crosses, preparing Drosophila embryos for target DNA injection

49
INTERN (Unpaid) 

Madison County Coroner’s Office, Edwardsville, IL

January 2012 – May 2012

- Attended autopsies under multiple Pathologists and Forensic Pathologists
- Researched the current and upcoming practices under the Coroner’s Office and field of Forensic Science.
- Assisted Death Investigators at scenes, including consultation with family members, medical community, and law enforcement officials.
- Filed and organized death reports in conjunction with the Office Associate

Achievements, Certifications, and Memberships

- American Academy of Forensic Science (AAFS) Member (2014 – Present)
- Northeastern Association of Forensic Scientists (NEAFS) Member (2013 – Present)
- Boston University Forensic Science Society (BUFSS) (2012 – Present)
- FEMA Incident Command System (ICS) Level 100 Certified (2012)

Professional Conferences Attended

- AAFS Annual Scientific Meeting, Seattle, WA (2014)
- NEAFS Annual Meeting, Cromwell, CT (2013)