THE DETECTION OF SPERMATOZOA ON WASHED FORENSIC EXHIBITS

By

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Thesis submitted in fulfillment of the requirements for the degree of

Master of Forensic Science (Professional Practice)

in

The School of Veterinary and Life Sciences

Murdoch University

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Semester 2, 2016
DECLARATION

I declare that this manuscript does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due references has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed: A. Nolan

Dated: 11.11.2016
Acknowledgements

I would like to thank Mr B Chapman and Mrs J Murakami for their advice and suggestions in regards to the study design. I would also like to thank my study partner Mr S Beckwith for sharing research papers and for his support over the last 6 months.
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Part One

Literature Review

Detection of Spermatozoa on Washed Forensic Exhibits
ABSTRACT

Occasionally, items of interest have been washed following an alleged sexual assault and analysts may be requested to comment on the effects of laundering on seminal fluid and the persistence of spermatozoa. It is scientifically accepted that when items have been washed resulting in the dilution of semen, Acid Phosphatase activity may be reduced to the point in which it cannot be detected by the Seminal Acid Phosphatase test or by alterative light sources such as the Polilight®. Results documented by Spector (1971), Kafarowski (1996), Farmen (2008) and Crowe (2000) all recorded negative results for Acid Phosphatase following the first wash cycle. However, these results are dependent on different variables such as washing procedures, fabric type and fabric absorbency. Other studies, however, have indicated that spermatozoa from seminal stains on cotton fabric persist even after the fabric has been washed; Spector (1971), Kafarowski (1996), Farmen (2008) and Crowe (2000) all documented a positive result for spermatozoa following the first wash cycle. All of these studies have failed to continue on with the washing process to examine the effects of washing and wash duration on the ability to detect seminal fluid and spermatozoa on different fabric types. The study proposal is to examine the effects of washing and wash duration on the ability to detect seminal fluid and spermatozoa on six different fabric types (cotton, nylon, satin, lace, polar fleece and towel) using an alternative light source (the Polilight-Flare®), the acid phosphatase test and microscopically.
INTRODUCTION

Detecting and identifying body fluids at crime scenes plays a vital role in the provision of intelligence and evidentiary information in forensic science. Determining whether a specific body fluid is present and subsequently identifying that body fluid can assist in obtaining a viable DNA profile, which can aid in the identification of a suspect or victim and exonerate innocent individuals. Body fluid identification can be used as a tool by forensic analysts to facilitate decision making regarding sampling and extraction methodologies and provides source level data to inform DNA result interpretation. Since many body fluids are either invisible to the naked eye or appear similar to other substances, it can be difficult to identify the cellular source and their exact location\(^1\). In 2012 the rate of sexual assault victimisation increased to 80 per 100,000, from 78 per 100,000, whereas other violent crimes such as robbery, homicide and kidnapping/abduction recorded victimisation statistics of 58, 2 and 3 per 100,000 respectively\(^3\). As sexual assault cases account for a substantial proportion of the casework reported by forensic analysts, the detection of seminal stains on items such as bedding and clothing can be of great significance\(^1,2\).

Seminal fluid is a complex mixture of secretions from four urogenital glands in the male; the seminal vesical gland contributes 60%, the prostate gland approximately 30% and the remaining 10% is generated from the epidermis and bulbourethral glands\(^4\). The average male ejaculates approximately 3.5 millilitres of sperm with each millilitre containing between 10-50 million sperm cells. The quality of male sperm and the volume ejaculated is age dependent and can be negatively impacted by genetic predisposition, diet, smoking and drug use\(^5\). There are documented conditions that adversely affect male spermatozoa count; these include
oligospermia which is defined as an abnormally low sperm count and aspermia which refers to a male that produces no sperm. Conversely, a vasectomy can surgically prevent the release of sperm during ejaculation. Importantly, vasectomized, oligospermic or aspermic males are still able to produce normal amounts of seminal fluid containing secretions from both the prostate gland and seminal vesicle and can be detected by forensic examinations despite the absence of spermatozoa.

In 2007, 19,781 cases were reported by forensic biologists as sexual assault cases in Australia (9.2% of the total violent crimes reported). Sexual assault is a crime of violence and victims are typically women and children. Many cases of alleged sexual assault, involve penile penetration of the vagina, rectum or oral cavity and natural drainage from these cavities can lead to seminal fluid being found on fabric items. Examining items of clothing and bedding for seminal fluid is an important aspect in sexual assault cases. The detection of semen on these items and subsequent DNA profiling provides investigators and the courts with information of high evidentiary value, which, when coupled with additional evidence, is vital in assisting the triers of fact to reach a decision. The presence of sperm cannot be used to determine the activity, i.e. sperm does not necessarily equal sexual assault.

**DISCUSSION**

There are several presumptive tests to identify semen as well as confirmatory tests. The following section explains various techniques that have been used for semen detection throughout literature.
Screening, Alternative Light Sources and Confirmatory Tests for Semen Detection

Presumptive Tests for Semen

The most widely accepted presumptive test is the seminal acid phosphatase test (SAP). Acid Phosphatase (AP) is a water soluble enzyme that is generally found in high concentrations in seminal fluid; and has the ability to act as a catalyst in the hydrolysis of organic phosphates. This forms a product that reacts with diazonium salts resulting in a colour change. The identification of AP provides a fast, cost effective and straightforward way to pinpoint the locations of possible semen stains. The indirect (blot) screening method involves pressing damp blotting/filter paper onto the surface of the item of interest to transfer any seminal fluid present onto the paper. The paper is then subjected to the acid phosphatase reagent that changes colour from orange to purple in the presence of AP. A swab technique can also be used that involves dampening a cotton swab with deionized water, swabbing the area of interest lightly and then applying AP directly to the swab. There are a number of substrate/colour developer combinations, these include beta-naphthol with Fast Garnet B, alpha-naphthol with Fast Red AL, and the most popular, alpha-naphthyl phosphate with Brentamine Fast Blue. The colour reaction develops over time and the time taken to induce a colour change, in tandem with contextual information, can be used to inform whether or not the colour change is due to the presence of semen. There are a number of negative factors associated with using the direct/blot method for detection of AP. Large amounts of liquid are applied to the sample which leads to diffusion of the spermatozoa resulting in decreased recovery for microscopic evaluation and sampling errors can arise when using the direct/blot method if the test paper is not replaced precisely to identify the correct area for sampling.
direct/blot method also increases the chances of losing spermatozoa from the stain onto the blot paper.

Lewis (2013) studied the effects of directly applying AP reagent to the surface of an item as opposed to the indirect (blotting) method. A number of tests were undertaken on a series of semen dilutions (1 in 50 up to 1 in 3000) to assess the sensitivity and the effect of AP reagent on haematoxylin and eosin staining of spermatozoa. For each dilution series, one pair of semen stained knickers was tested using the indirect AP method and the other two pairs were tested using the direct method: two methods of application were used, a spray bottle and an aerosol. Areas that were AP positive were excised completely and slides were prepared for microscopic examination. It was documented that the aerosol method for the direct AP testing method was the most sensitive detecting AP in the dilution of 1 in 3000; this method was found to have used significantly less reagent which, apart from cost benefits, meant that it was possible to test only one side of a double layer of fabric. Testing only one side of the fabric assists in determining what side of the fabric the stain was originally deposited. The slides from both the direct and indirect methods showed no significant difference in the numbers of spermatozoa found. Lewis (2013) concluded that adopting the direct AP aerosol method for items such as clothing and bedding would eliminate the potential for sampling errors that arise from the indirect blot method; the indirect method requires the test paper to be carefully replaced to identify the correct area of the item for sampling. It was also stated that the direct aerosol method would be most effective on light coloured exhibits where the positive purple reaction can be easily seen. The study conducted by Lewis (2013) is a standalone study that
The effects of AP reagent on DNA from semen stains deposited on various fabric types was also studied by Lewis (2012). Lewis (2012) concluded that it could be influenced by the application method of the AP (i.e. the aerosol method uses significantly less reagent than if directly applied by spray bottle). This statement was supported by the results where, for semen samples tested, direct spray application produced no DNA profile above a 1 in 750 dilution whereas the direct aerosol method had no detrimental affect up to a 1 in 3000 dilution.

Initially, the cut off time for detecting AP had been set to two minutes despite there being no scientific basis for this; this meant that if no reaction was seen within two minutes then the test was recorded as being negative. In a study conducted by Lewis (2012) it was documented that despite a literature search there was no scientific basis for the two-minute cut off period; the study also determined whether the make and type of filter paper affected the likelihood of obtaining a positive result. It was documented that most brands of test paper were able to detect semen dilution of 1 in 40 within the two-minute cut off period. However, positive reactions were detected at greater dilutions when the cut off time was extended to 5-10 minutes, although these reactions did appear very faint and speckled. When left for 4 hours, the thickest filter paper tested (Whatman grade 3) detected a 1 in 1000 semen dilution. In cases where more dilute semen samples are expected, using a thicker grade filter paper and/or
increasing the cut off time would be expected to increase the likelihood of yielding a positive
AP test result; this is expected when garments have been washed\(^ \text{13} \).

In cases where no spermatozoa were detected on samples taken from sexual assault cases,
other methods such as the prostate specific antigen (PSA or the P30) can be employed to
detect the presence of semen; to screen for PSA forensic analysts use a test known as the
ABAcard\(^ \circledast \) or P30 test\(^ \text{7,14} \). PSA is a glycoprotein that is produced by the prostatic gland and
secreted into the seminal plasma. Consequently, PSA is a valid marker for semen detection in
sexual assault cases, including those samples that have been deposited by vasectomised or
aspermic males\(^ \text{7} \). The ABAcard\(^ \circledast \) and P30 tests are both commercial kit tests that rely on mobile
monoclonal anti-human PSA antibodies which bind to human PSA, this then migrates along a
strip to immobilize polyclonal anti-human PSA antibodies to form a visible line\(^ \text{7} \). PSA in semen
samples that have been diluted \(10^6\) times can still be detected by these tests and only male
urine has been found to give rise to a false positive; this could be due to small amounts of
prostatic fluid being present in the urine\(^ \text{14,15} \).

A presumptive test that has been around for a long time but no longer routinely used is the
choline test. There are several different methods testing for choline, the first involves placing
an extract of the stain in question on a microscope slide, washing it with a solution of iodine
and potassium iodide and observing any brown needle like crystals that form\(^ \text{16} \). The number of
false negatives is high due to low sensitivity but the test still gives a negative result for other
body fluids such as vaginal fluid; cross reactivity with vaginal secretions would lead to false
positives even if semen was not present within the sample. Other methods used to detect the presence of choline are dependent on a reaction with choline oxidase, including a chemiluminescent test involving a choline/luminol solution. Isotachophoresis is a much more complicated method for detecting choline and was found to have no false positives for any other body fluids, fruit or vegetable products; positive results could still be obtained from samples up to 10 years old and from samples taken from deceased females.

Raman spectroscopy is a forensic technique used to identify the properties and structure of materials based on their different vibrational transitions. There are many applications that involve the identification of different materials such as fibres, drugs, ink, paint and condom lubricant. The theory behind Raman spectroscopy involves the inelastic scattering of a monochromatic, non-destructive, low intensity laser by a sample that is either a solid, liquid or gas; no sample preparation is needed, and no reagents are required to complete the analysis. One of the biggest advantages that Raman spectroscopy has over other tests is that the sample size can be as low as several picograms and the sample will not be destroyed so that further analysis can be undertaken. A Raman spectrum will reveal a specific vibrational signature for a sample based on the energy of the scattered laser light; this feature facilitates the identification of unknown substances.

A study conducted by Virkler (2009) determined the level of spectral diversity from different sperm donors to determine whether Raman spectroscopy was a reliable method in identifying a sample as semen. A spectroscopic signature for human semen was developed and it was
found that a spectrum of dried semen contained three major spectral components, tyrosine, a component containing a protein and possibly choline and a component consistent with spermine phosphate hexhydrate. There were no significant visual differences in the Raman spectra from the multiple donors and the spectrum of dried semen varied considerably when compared to spectra of blood and saliva; this shows the potential for Raman spectroscopy in identifying semen at crime scenes.

In a study conducted by Coyle (2009) swabs were screened in-situ for the presence of condom lubricant using FT-Raman spectroscopy. The main purpose of the study was to determine if the process had any effect on subsequent DNA analysis, particularly the effect of DNA recovery and the quality of DNA profiles obtained. 24 cotton swabs were dosed, in duplicate, with different biological materials (these included saliva, low buccal scrapings, high buccal scrapings, touch and semen) and stored at 4 degrees for a few days until ready for Raman testing; series A samples were not analysed using Raman Spectroscopy whereas series B Samples were subjected to Raman testing. DNA was extracted from all swabs using the Qiagen EZ1 BioRobot and amplified using the AmpFLSTR SGM Plus PCR Amplification Kit. The EZ1 purified samples were quantified using a real-time PCR methodology to determine the optimal amount of DNA template required for a multiplex PCR reaction; a comparison of these results between the Raman and non-Raman samples was performed but were found to have no significant difference. When comparing the quality of DNA profiles obtained Coyle (2009) documented that there was full concordance between the untreated and Raman treated
samples, however, admits that further work does need to be carried out as only duplicates were used within the study\textsuperscript{21}.

\textit{Alternative Light Sources as a Screening Tool}

For larger exhibits, forensic examiners can utilize the fluorescent properties of semen to visually identify potential seminal stains with the assistance of alternative light sources; these include the light sources such as the Wood’s Lamp and the Polilight\textsuperscript{®}\textsuperscript{22}. On first appearance, dried semen stains can appear to be off-white to faint yellow in colour but when visualised under alternative light sources they fluoresce due to the presence of Flavin and Choline conjugated proteins\textsuperscript{22}. Semen that is undiluted and dry has a very strong photoluminescence; Stoilovic (1991) reported that the excitation spectrum of semen was broad and that fluorescence could be generated with wavelengths ranging from 350-500nm\textsuperscript{23}. However, there are many molecules that demonstrate similar excitation fluorescence, and consequently alternative light sources are considered a presumptive test for the presence of semen.

The Wood’s Lamp is a device that emits wavelengths between approximately 320-400nm; it is small, inexpensive, safe and easy to use. The specificity of the Wood’s lamp is low and various studies have cast doubts on the validity of the Wood’s Lamp in the detection of semen stains\textsuperscript{1,24,25}. A study conducted by Santucci (1999) asked 41 physicians to analyse 29 semen samples with the Wood’s Lamp and it was demonstrated that none of the physicians were able to distinguish between semen and other common products using the Wood’s Lamp\textsuperscript{24}. A similar study was conducted again by Nelson (2002) with 66 physicians, 18 of which were provided
training using the Wood’s Lamp. Again, neither the 18 trained physicians or the remaining 48 physicians could detect semen using the Wood’s Lamp$^{25}$.

The Rofin Polilight® is a versatile light source that produces light at wavelengths between 310-650nm, also including the white light and UV options, and is suited to exploit the excitation spectrum of semen$^{1,22,26}$. The Polilight® has bandwidths that range from 100nm for the 450nm setting (blue light), this is the most commonly used for general screening processes, to 27nm for the 555nm setting (green/orange)$^{26}$. The range of wavelengths provided by the Polilight® allows for the fluorescence from semen stains to be observed on a number of different backgrounds, including those backgrounds that fluoresce themselves. The Rofin Polilight-Flare® Plus 2 is brighter than any other handheld alternative light source and has the ability to exploit the excitation of semen through narrow bands of light; the wavelengths include 365, 415, 450, 505, 530, 545, 595 and 620 nm. The intensity of the beam profile can be manipulated to suit the application making it ideal for examinations of crime scenes and exhibits$^5$. In a study conducted by Vandenberg (2006) it was demonstrated that the Polilight® was able to detect seminal fluid on a variety of fabric types, even when the stain was diluted$^{26}$.

**Confirmatory Testing Methods**

The most reliable and widely accepted confirmatory test is through visual identification of spermatozoa using a microscope. Semen is the only body fluid that contains sperm cells and the large amounts of DNA in the heads of the sperm cells can be treated with a stain to make them more visible$^{28}$. The Christmas tree stain has gained popularity in recent years becoming
the stain of choice; it is known for its characteristic colours of red and green which stain the sperm head and tail respectively. The two main reagents that are used to produce this distinctive stain are Picroindigocarmine, this stains the neck and tail portions of the spermatozoa green, and Nuclear Fast Red, this stains the spermatozoa heads red and the tips of the heads a pink colour. Additional techniques include treating the semen sample with proteinase K to denature any epithelial cells to allow the unaffected sperm heads more visible underneath the microscope. Other stains that have been used for microscopic sperm identification include hematoxylin and eosin, Baecchi’s, Papanicolaou’s and Wright’s but as none of these are more effective than the Christmas tree stain it remains the stain of choice.

There are several different working practices that exist for semen extraction and identification that have arisen due to local methods being set up and amended to meet local needs; for example, a laboratory whose customers rarely require DNA profiling will use a quick and simple method that enables the detection and identification of semen rather than a more complex extraction method required for use for PCR. A comparison of methods for the extraction and detection of semen on swabs and cloth samples was conducted by Allard (2007). Nine laboratories were supplied with swabs and pieces of cloth stained with different concentrations of semen ranging from neat to 10,000 and asked to use their standard tests to identify seminal fluid and/or spermatozoa. It was documented that all laboratories obtained a moderate to strong positive AP result up to a 1 in 25 dilution, after this stage the results were variable with only two laboratories recording strong results up to a 1 in 200 dilution, whilst the remaining seven recorded weak or negative results after the 1 in 40. Allard (2007) concluded
that the differences in results could be due to the filter paper used, the length of time the paper is left on the cloth, pre-wetting of the cloth and AP reagent. The effects of different filter papers have since been documented by Lewis (2012) stating that filter paper does have an effect on the reaction time resulting in a positive result when using AP. It was documented that all laboratories that carried out a full set of tests detected sperm at 1 in 200, however, Allard (2007) noted that concentrations varied from few sperm to 3+ (Few = less than 5 on the entire slide, 3+ between 5-10 per field of view). The highest sperm counts were seen on slides from those who spun down the samples and used the pellets when preparing their slides. It was also noted that sperm concentrations were lowest when small volumes of water were used suggesting that the volume of water needs to be sufficient enough to ensure maximum removal of the spermatozoa from the cloth.

MHS-5, also known as seminal vessel-specific antigen (SVSA), is an antigen that will react only with the epithelium in human seminal vesicles. In human semen the major gel-forming proteins are semenogelin I and semenogelin II; both of these proteins contain SVSA and are recognised by the MHS-5 antibody. The Sema® is an ELISA kit that detects semen by exploiting the reaction between SVSA and the MHS-5; the method is highly sensitive but not nearly as specific as PSA tests and so is no longer used. Alternatively, the identification of human semenogelin in a strip membrane form is now used and is known as the RSID-Semen test. The RSID strip test is just as sensitive as the PSA test as well as being specific to human semen. The antigen is unique to human semen, and therefore, does not cross react with any other substances or body fluids in males, females or with semen from other mammals; some would consider it as
alternative confirmatory method to microscopy when dealing with cases involving aspermic or oligospermic males\textsuperscript{14}.

\textbf{The Effects of Washing on the Presence of Acid Phosphatase and the Retention of Spermatozoa on Fabric}

Occasionally, items of interest have been washed following an alleged sexual assault and analysts may be requested to comment on the effects of laundering on seminal fluid and the persistence of spermatozoa\textsuperscript{30}. In some cases of internal child sex trafficking cases (ICST), it was found that children hid their semen stained clothes from parents or carers to avoid having to discuss the assault\textsuperscript{31}. It was found that victims would often store the clothes for a period of time, ranging from several hours to a number of years, before then washing them to rid them of the visible stains\textsuperscript{31}.

There are several studies that have indicated that spermatozoa from seminal stains on cotton fabric persist even after the fabric has been washed\textsuperscript{2,32,33} Spector (1971) studied the effects of washing on the detection of spermatozoa in seminal stains by machine washing white undershorts made of cotton. The shorts were initially washed in cold water with detergent before being stained with semen. After being left to dry the shorts were subjected to one of 12 different washing procedures and with one of three different types of laundry detergent (X-recommended for hot washing, Y-recommended for cold washing and Z-recommended for either hot or cold washing, containing enzymatic activity):
TABLE 1—Washing procedures as used by Spector (1971)

<table>
<thead>
<tr>
<th>Garment Number</th>
<th>Soak Time</th>
<th>Hot/Cold Wash</th>
<th>Detergent Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 hours in cold water</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2 hours in hot water</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>2 hours in cold water</td>
<td>Hot</td>
<td>Detergent X</td>
</tr>
<tr>
<td>4</td>
<td>2 hours in hot water</td>
<td>Cold</td>
<td>Detergent Y</td>
</tr>
<tr>
<td>5</td>
<td>Overnight</td>
<td>Hot</td>
<td>Detergent X</td>
</tr>
<tr>
<td>6</td>
<td>Overnight-detergent X</td>
<td>Hot</td>
<td>Detergent X</td>
</tr>
<tr>
<td>7</td>
<td>Overnight-detergent X</td>
<td>Hot</td>
<td>Detergent X</td>
</tr>
<tr>
<td>8</td>
<td>Overnight-detergent Y</td>
<td>Cold</td>
<td>Detergent Y</td>
</tr>
<tr>
<td>9</td>
<td>Overnight</td>
<td>Cold</td>
<td>Detergent Z</td>
</tr>
<tr>
<td>10</td>
<td>Overnight-detergent Z</td>
<td>Cold</td>
<td>Detergent Y</td>
</tr>
<tr>
<td>11</td>
<td>Overnight</td>
<td>Hot</td>
<td>Detergent Z</td>
</tr>
<tr>
<td>12</td>
<td>Overnight-detergent Z</td>
<td>Hot</td>
<td>Detergent Z</td>
</tr>
</tbody>
</table>
Spermatozoa were found after all washing procedures with the exception of procedure 12\textsuperscript{32}. Spector (1971) concluded that detectable traces of semen were not readily removed by washing but also admitted to the limitations of the study. “Normal washing procedures” are endless and variables such as fabric type, its colour and the amount of semen are all influencing factors, in addition to detergent type and washing method\textsuperscript{32}.

A similar study was conducted by Kafarowski (1996) in that they examined the retention and transfer of spermatozoa in clothing by machine washing; the study was conducted to test the likelihood of the transfer of spermatozoa during machine washing\textsuperscript{33}. A single semen stain was deposited on a clean pair of cotton panties by natural drainage after vaginal intercourse; this was replicated by two additional couples. Each pair of semen stained panties was then independently washed with three other pristine pairs of cotton panties. Other clean items such as pillowcases, tea towels, t-shirts and socks were added to the wash to simulate a normal load. It was found that in all three independent trials, trace quantities of spermatozoa were found on clothing due to transfer during machine washing. Similarly, to the results found by Spector (1971), Kafarowski (1996) found that following machine washing spermatozoa could still be found in all three of the original semen-stained panties\textsuperscript{33}.

A third study by Jobin (2003) examined nine pairs of nylon panties and nine pairs of cotton panties. The panties were pre washed and stained with semen from three different donors, washed again with detergent and machine dried\textsuperscript{2}. In concordance with the previous studies, microscopic examination of the stains demonstrated that spermatozoa were present on each
set of cotton panties and nylon panties. However, Jobin (2003) went a step further and compared the results from the cotton panties and the nylon panties. The cotton panties were found to have on average more sperm per field than the nylon panties, +2.9 and +1.3 respectively. The ratings refer to the number of spermatozoa identified per microscopic fields of view, these were as follows: 1 sperm in few fields= +1, 1 sperm in many fields= +2, few sperm per field= +3 and many sperm per field=+4.²

It is scientifically accepted that when items have been washed resulting in the dilution of semen, AP activity may be reduced to the point in which it cannot be detected by the SAP test. In a study conducted by Farmen (2008) 30 pairs of cotton briefs were machine washed; 10 pairs at 40 degrees, 10 pairs at 60 degrees and 10 pairs at 60 degrees with a fabric softener (brand not stated)³⁴. None of the 30 pieces of laundered underwear yielded a positive result for AP using the acid phosphatase test, however, DNA was recovered from all 16 of the randomly selected panties tested. The briefs washed at 40 degrees were found to have twice the amount of DNA than those washed at 60 degrees and the use of softener had no significant effect on DNA³⁴. When examining the briefs microscopically, those washed at 40 degrees demonstrated higher spermatozoa counts, however these results may be influenced by degradation issues associated with the storage conditions of the items³⁴.

Despite results obtained by Farmen (2008) other studies have shown that it is possible to obtain a weak AP result from seminal stains associated with fabric exhibits that have been machine washed without detergent in cold water or submerged in water for up to 144
hours\textsuperscript{30,35}. In a study conducted by Crowe (2000) 12 cotton t-shirts of the same colour and style were examined for seminal persistence. Two t-shirts were taken to three different dry cleaning companies and washed with a variety of solvents, detergents and spot cleaners and the remaining six were washed using a domestic top loader washing machine, in either cold (approximately 18 degrees) or warm (approximately 30 degrees) water, either with or without washing powder and either with or without spot cleaning\textsuperscript{30}. It was found that t-shirts that were dry cleaned demonstrated positive reactions to the AP test except when spot cleaned and all machine washed methods gave a negative result to AP except on a cold wash without detergent suggesting that water temperature effected results \textsuperscript{30}.

Joshi (1981) studied the effects of water immersion on seminal stains in order to simulate a case in which a victim was raped, killed and thrown into a well\textsuperscript{35}. At 72 hours’ strong positive results were seen for both the AP test and for spermatozoa detection, after the 72-hour mark AP results were still strong but the spermatozoa were found to be losing their tails. At 120 hours of immersion the AP activity decreased, taking more time for a colour change to emerge and the density of tail-less sperm increased. At 144 hours, AP activity was still detectable but only well-defined sperm heads could be seen\textsuperscript{35}.

The studies conducted by both Crowe (2000) and Joshi (1981) demonstrated that it is possible to obtain positive AP results, however, these results were obtained with certain restrictions in the washing protocol\textsuperscript{30,35}. As previously stated by Spector (1971) “Normal washing procedures” are endless and variables such as fabric type, its colour and the amount of semen are all
influencing factors, in addition to detergent type and washing method\textsuperscript{32}. In the study conducted by Crowe (2000) it is mentioned that t-shirts that were dry cleaned still showed positive results in a AP test except when spot cleaned. Crowe (2000) admitted to the limitations within the study stating that cotton t-shirts would normally be washed rather than dry cleaned and unless stains were visible to the cleaner they would not be spot cleaned; as seminal stains are generally not visible on fabric they would not be spot cleaned. It was also stated that all machine washed methods gave a negative result to AP except on a cold wash without detergent, again, the limitations of the washing technique need to be considered when determining the effects of washing on AP results\textsuperscript{30}. The study by Joshi (1981) demonstrated that AP was still detectable after 144 hours of water immersion. When putting these results within the context of the case and others that are similar the results are viable. However, when determining whether washing affects AP activity the results from this study should be carefully considered depending on the context of the forensic exhibit; this is because water immersion is not the same a full wash cycle in a washing machine; this would involve, at a minimum, agitation of the sample\textsuperscript{35}.

The study conducted by Spector (1971) is a great starting point for research into the effects of washing on AP and the retention of spermatozoa on fabrics, but, disappointingly it appears that the research hasn’t been taken further; in respect to the number of washes it takes to fully rid a sample of detectable AP and spermatozoa. It was also noted that in many of the studies water temperature is defined as either warm, hot or cold, with the exception of Farmen (2008) who defined warm and hot as 40 °c and 60 °c respectively. Cold, warm and hot
can be defined as anything and could vary between different studies. This is why it could be a possibility that the variance in results between studies could be due to different water temperatures and not the variables in which each study is manipulating as originally thought; this cannot be confirmed as the actual temperatures are unknown. Temperatures need to be defined so that the studies can be compared accurately and subsequent studies have parameters to work within.

It was also found that studies used detergents but didn’t state brand name and type, again, this needs to be considered as a factor affecting results. No studies, to date, have compared different brands of washing detergents and their effects on AP and spermatozoa retention on fabrics. The studies were also limited to the same fabric type, with only Jobin (2003) including nylon within the study; it would be interesting to know how different fabrics types hold sperm and whether or not it will have an effect on retaining spermatozoa and AP².

The following table is a summary of results from the above six studies. All studies used cotton with the exception of Jobin (2003) who used both cotton and nylon. Spermatozoa was found after being washed regardless of whether samples were washed in cold, hot or warm water, with or without detergent, machine washed or soaked; this is with the exception of a hot wash with enzymatic detergent used by Spector (1971). The results from the AP tests show conflicting results from one study to the next, however, as Spector (1971) admitted these variations in results could be due to variables such as fabric type, its colour, amount of semen deposited, detergent, washing method and drying method³².
<table>
<thead>
<tr>
<th>Author</th>
<th>Washing Technique/Detergent Used</th>
<th>Fabric Type</th>
<th>Spermatozoa</th>
<th>AP Result</th>
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Peonim (2013) investigated the sensitivity and the specificity of the Prostate specific antigen (PSA) test in comparison to the AP test by examining vaginal swabs from 2450 rape cases between 2008 and 2010. Each swab was tested for semen using one of three methods: sperm detection by microscopy, the AP enzymatic test and the presence of PSA using immunochromatographic rapid kit test; the microscopy results for the presence of sperm was used as a gold standard for comparing the efficiencies of the AP and PSA tests. It demonstrated that the AP test was more specific than PSA for detecting semen (96.4% and 92.3% respectively) but was less sensitive (65.5% and 80.4% respectively). The receiver operating characteristic (ROC) area of the AP and PSA tests were 0.8091 and 0.8639, respectively; an ROC curve is a graphical plot that illustrates sensitivity as a function of fall-out or probability of false alarm. Peonim (2013) states that the fact that the ROC for the PSA was significantly higher than that of the AP test suggests that the PSA is better than the AP test for semen detection.

Vanderburg (2006) investigated the accuracy of Polilight® in comparison to the AP test by examining forty casework items (these included underwear, clothing, bed sheets and quilts); this was achieved by examining the items using the AP test subsequent to Polilight®. False negatives were defined as results that were identified as being negative using the Polilight® but were AP positive and the presence of spermatozoa was confirmed through cytological identification; it was found that the occurrence of false negatives for the Polilight® was one in 40 or 2.5%. False positives were results that were identified as being positive using the Polilight® but AP negative; the occurrence of false positives was found to be quite high (20 in 40 or 50%) Out of the 40 exhibit items Vanderburg (2006) found that both the Polilight® and
the AP were consistent with their results when examining 19 (47.5%) of the items; this suggests that the Polilight® has a tendency to detect stains in general, whether they happen to be seminal stains or not\textsuperscript{26}. In conclusion, it was found that the Polilight® was more sensitive than specific, but given the small number of false negatives it remains a very useful presumptive test for detecting areas for further analysis. In addition, all stains identified either by AP or Polilight® would require further examination, e.g. through cytological examination to confirm the presence of spermatozoa; this highlights the need to consider multiple approaches used in conjunction with each other\textsuperscript{26}.

The Effects of Fabric Type and Fabric Absorbency on Fluorescence

Studies have demonstrated that some materials can show strong background fluorescence under alternative light sources due to optical brighteners in detergents and the physical and chemical structure of the fabrics fibres; these include white fabrics, fleecy material or dark coloured fabrics\textsuperscript{22,26} Kobus (2002) investigated the effect of fabric fluorescence by illuminating semen stains under different filter settings on a Polilight®: these included UV, 415nm, 450nm, 505nm and 530nm. It was found that white cotton, pink satin and pink fleecy material all showed strong background fluorescence under certain conditions; this reduced the contrast between the semen stain and the fabric making the stain difficult to see or in some cases masked the fluorescence of the stain entirely\textsuperscript{22}. Vanderburg (2006) also investigated the effects of fabric colour on semen fluorescence and agreed that material colour affected the strength of the stains appearance\textsuperscript{26}. Semen stains on pink nylon, red cotton and pink polka dot cotton fabrics were reported as inhibiting the detection of the seminal stains, however,
semenal satins on the same fabric types but in different colours were easily detected\textsuperscript{26}. This suggests that the fabric colour affects the contrast between the semen stain and the fabric. As background fluorescence can mask semen stains the ability to produce fluorescence at larger wavelengths can be of an advantage\textsuperscript{22}. When observing the fluorescence of semen stains on a variety of materials using the Polilight\textsuperscript{®} Vanderburg (2006) found that the general wavelength was set to 450nm, whilst wearing orange goggles. Other wavelength/ goggle combinations included 415nm/yellow goggles, for stains on dark materials, and 505nm/red, to maximise contrast on materials that produced high background fluorescence\textsuperscript{26}.

The variability in the fluorescence of semen depending on the absorbency of the material in which it was deposited was studied by both Vanderburg (2006) and Kobus (2002), however, both with conflicting results\textsuperscript{22,26}. In the study conducted by Kobus (2002), a solution of rhodamine 6G in ethanol was deposited on white cotton, pink and dark grey fleecy fabric and pink satin fabric. Undiluted semen was deposited on fleecy fabrics, polyester and cotton and the rate of absorption and fluorescence observed; the semen stains were then diluted by pipetting water onto the stain, left to dry and their fluorescence observed\textsuperscript{22}. The rhodamine solution was found to be rapidly absorbed by fleecy fabrics and had weak to no detectable fluorescence when observed with a Polilight\textsuperscript{®}; the conclusion was that the detection of any fluorescent material would be markedly inhibited by its absorption into the fabric\textsuperscript{22}. It was documented that neat semen remained on the surface of the fabric when first applied due to its high viscosity and when still wet, it showed strong fluorescence. Additionally, the absorption rate of semen varied depending on fabric type and previously washed fabrics absorbed semen
faster than unwashed. It was demonstrated that the fluorescence of the semen initially decreased after being absorbed into the fabric, however, after the stain dried the fluorescence increased. When applied to the thick fleecy fabrics no absorption of the neat semen occurred until after it was diluted; this lead to rapid absorption on the semen and permanent loss of the fluorescence.

However, Vanderburg (2006) found that seminal stains deposited on highly absorbent materials such as blue velour and dark green polar fleece were easily detected with the Polilight®, although less visible with the naked eye. On materials that appeared to have very little absorbency, such as nylon where the stain appeared to sit on the surface of the material, the fluorescence did not appear to be significantly greater than on more absorbent materials such as cotton and polyester.

The Effects of Washing and Laundry Detergents on the Fluorescence of Seminal Stains

To investigate the effects of washing on the fluorescence of seminal stains Kobus (2002) prepared semen stains on white cotton, pink polyester and pink fleecy materials, washed the samples with detergent (the brand is not stated) and examined using a Polilight®; a semen stain that was stored for six months at room temperature was also treated in the same way. New unwashed white cotton was also washed, allowed to dry and semen deposited onto the cloth; this was used to compare the fluorescence of seminal stains deposited on both new washed fabrics and new unwashed fabrics. It was found that weak fluorescence could still be seen in some stains after the washing process and that the fluorescence of older stains was stronger.
after washing than that of the fresh stains. Kobus (2002) suggested that the ageing process has enabled the fluorescent components of semen to be more resistant to removal when washed and this is again said when weakly fluorescent washed stains gave a negative result when tested with acid phosphatase\textsuperscript{22}. The fluorescence of the semen stain on the pre-washed white cotton was found to have significantly reduced in comparison to that of the unwashed white cotton. It was suggested that this difference could be due to the removal of the surface treatments applied to the fabric through the washing process, this would allow for the semen to be more easily absorbed into the fabric leading to the decrease in fluorescence\textsuperscript{22}.

Due to the fact that seminal stains can be removed by simply washing the fabric the effects of using laundry detergents is important, more so in determining whether or not detergents can lead to false positives through routine washing with detergents\textsuperscript{26}. Several studies have looked at the effects of washing semen stained fabrics both with and without detergents. To examine the effects of laundry detergents under Polilight\textsuperscript{®}, Vanderburg (2006) placed samples of eight commonly used laundry detergents onto white polyester samples and observed their fluorescence both before and after washing\textsuperscript{26}. Two out of the seven products (Preen\textsuperscript{®} and Cold Power\textsuperscript{®}) left behind residual staining after being washed that appeared white in colour and bright under the Polilight\textsuperscript{®} which could be confused with a seminal stain\textsuperscript{26}. Based on the results obtained in the study conducted by Vanderburg (2006) either Sard\textsuperscript{®}, White King\textsuperscript{®}, Earth Choice\textsuperscript{®}, Omo\textsuperscript{®} or Cuddly\textsuperscript{®} (fabric softener) will be used in this proposed study as none of these detergents left staining that could be confused with a seminal stain.

*Microscopic Detection of Spermatozoa: Comparison of Three Staining Methods*
The cytological detection of spermatozoa on vaginal swabs or on material items such as clothing or bedding is one of the few methods currently used to confirm the presence of spermatozoa. The hematoxylin-eosin stain is the most commonly described stain seen in scientific literature\textsuperscript{37,38,39} however the Christmas tree stain has risen in popularity in recent years\textsuperscript{1,29,40}. A study conducted by Allery (2001) aimed at determining the best cytological staining method for detecting spermatozoa by comparing the three most commonly used stains: Christmas tree, alkaline fuchsin and hematoxylin-eosin\textsuperscript{40}. Triplicate slides from 174 vaginal swabs were prepared; they were air dried, fixed with alcohol and stained with one of the three stains. All slides were then visualised microscopically and the number of spermatozoa per field was counted. It was found that Christmas tree detected spermatozoa in 35.1% of samples, hematoxylin-eosin in 34.7%, and alkaline fuchsin in 28.4%\textsuperscript{40}. Statistical analysis of results showed that the difference between alkaline fuchsin and the other two stains was statistically significant, however, the difference between Christmas tree and hematoxylin-eosin was not. When comparing the number of spermatozoa found in each microscopic field according to time, Allery (2001) found that Christmas tree stain gave significantly higher results (8.3) than hematoxylin-eosin (4.6) and alkaline fuchsin (4.1); this was done over a period of three days to simulate time after intercourse\textsuperscript{40}.

The Christmas tree stain appeared to be on par with the hematoxylin-eosin stain whereas the alkaline fuchsin stain was statistically ruled out as being effective in detecting spermatozoa. After closer comparison of both the Christmas tree stain and the hematoxylin-eosin stain, Allery (2001) concluded that both stains were of similar value in detecting spermatozoa, no
matter what the time interval was between sexual intercourse and examination for the presence of spermatozoa. However, Christmas tree stain still had the advantage of detecting more spermatozoa on every slide studied; it was also the easiest to read; making working conditions for the technician better\textsuperscript{40}.

**CONCLUSION**

It has been reported by Kafarowski (1996), Spector (1971), Jobin (2003) and in many other studies that despite washing, spermatozoa can still be detected by cytological means. These studies also investigated the effects of washing on the ability to detect Acid Phosphatase but with conflicting results\textsuperscript{7,32,33}. However, as previously stated there are a number of flaws within these studies that could explain these differences, ie. Failure to define variables such as washing temperature, brand of detergents and length of wash cycles. These variables need to be kept constant and made available so that the change in one variable can be accurately assessed and compared to others already documented within literature.

Farmen (2008) documented specific washing temperatures of 40 and 60 degrees defining the parameters of the study by assessing the effects of wash temperature on AP and spermatozoa. In conclusion, it was found that wash temperature yielded no specific results as no AP was found on any of the items after washing but it did have an effect on the retention of spermatozoa, the colder temperature yielding higher spermatozoa counts\textsuperscript{34}. Based on the results from this study, only one temperature will be used in the proposed study to eliminate the possibility of results being affected by washing temperature.
Vandenberg (2006) highlighted the need for multiple approaches by comparing the ability of AP and the (Polilight®) in detecting seminal stains. It was found that the Polilight® was more sensitive than specific, but given the small number of false negatives (2.5%) it remains a very useful presumptive test for detecting areas for further analysis. Given the results documented by Vandenberg (2006) both AP and an alternative light source will be used in conjunction to identify seminal stains in this proposed study.

Both Kobus (2002) and Vandenberg (2006) extensively studied the effects of material type and absorbency on the ability to detect seminal stains with the Polilight®. Kobus (2002) found that white cotton, pink satin and pink fleecy material all showed strong background fluorescence under certain conditions, reducing the contrast between the semen stain and the fabric making the stain difficult to see or in some cases masking the fluorescence of the stain entirely. Vanderburg (2006) agreed that material colour affected the strength of the stains appearance documenting that semen stains on pink nylon, red cotton and pink polka dot cotton fabrics inhibited the detection of the seminal stains, however, seminal satins on the same fabric types but in different colours were easily detected. This suggests that the fabric colour affects the contrast between the semen stain and the fabric, hence, in the proposed study black fabric will be used to avoid background fluorescence.

Conflicting results were documented in regards to the effects fabric absorbency stain fluorescence. Vanderburg (2006) found that seminal stains deposited on highly absorbent
materials such as blue velour and dark green polar fleece were easily detected with the Polilight®, although less visible with the naked eye. On materials that appeared to have very little absorbency, such as nylon where the stain appeared to sit on the surface of the material, the fluorescence did not appear to be significantly greater than on more absorbent materials such as cotton and polyester. Kobus (2002), however, disagreed concluding that the detection of any fluorescent material would be markedly inhibited by its absorption into the fabric. In the proposed study, different fabric types with different absorption abilities will be used and the ability of the Polilight-Flare® Flare 2 to detect the seminal stains after washing will be assessed.

To assess the effects of washing detergents Vanderburg (2006) placed samples of eight commonly used laundry detergents onto white polyester samples and observed their fluorescence both before and after washing. Two out of the seven products (Preen® and Cold Power®) left behind residual staining after being washed that appeared white in colour and bright under the Polilight® which could be confused with a seminal stain. Based on the results obtained in the study conducted by Vanderburg (2006) either Sard®, White King®, Earth Choice®, Omo® or Cuddly® (fabric softener) will be used in the proposed study to avoid misinterpretation of results.

A comparison of methods for the extraction and detection of semen on swabs and cloth samples was conducted by Allard (2007). It was documented that the extractions that yielded the highest sperm counts were seen on slides from those who spun down the extracts and
used the pellets when preparing their slides. It was also noted that sperm concentrations were lowest when small volumes of water were used suggesting that the volume of water needs to be sufficient enough to ensure maximum removal of the spermatozoa from the cloth. Based on the results in the study conducted by Allard (2007) an extraction procedure that uses a large amount of water and centrifuging will be used.

The best cytological staining method for detecting spermatozoa was found by comparing the three most commonly used stains: Christmas tree, alkaline fuchsin and hematoxylin-eosin in a study conducted by Allery (2001). It was documented that the Christmas tree stain appeared to be on par with the hematoxylin-eosin stain whereas the alkaline fuchsin stain was statistically ruled out as being effective in detecting spermatozoa. In a closer comparison of both the Christmas tree stain and the hematoxylin-eosin stain, Allery (2001) concluded that both stains were of similar value in detecting spermatozoa, however, Christmas tree stain still had the advantage of detecting more spermatozoa on every slide studied as it was also the easiest to read. Based on these results the Christmas tree stain will be used in the cytological staining method for detecting spermatozoa.

All of the above studies have demonstrated different alternatives in detecting seminal fluid and spermatozoa on washed forensic exhibits; these consist of the use of alternative light sources (Polilight®), the Acid Phosphatase test and cytological detection using the Christmas tree stain. Each method has its positives and negatives which highlights the need to consider multiple approaches in conjunction with each other. However, all of these studies have failed
to continue on with the washing process to examine the effects of washing and wash duration on the ability to detect seminal fluid and spermatozoa on different fabric types. The future study proposal is to examine the effects of washing and wash duration on the ability to detect seminal fluid and spermatozoa on six different fabric types (cotton, nylon, satin, lace, polar fleece and towel) using an alternative light source (the Polilight-Flare®), the acid phosphatase test and microscopically. It is hypothesized that fabrics with fibres that are more tightly bound, specifically the cotton and towel, will have a greater affinity to spermatozoa than those that don’t and that these fabrics will retain spermatozoa and seminal fluid past the first wash cycle.
REFERENCES


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Part Two

Manuscript

The Detection of Spermatozoa on Washed Forensic Exhibits
**ABSTRACT:** Examining items of clothing and bedding for seminal fluid and spermatozoa is an important aspect in sexual assault cases. The detection of semen or spermatozoa on these items and subsequent DNA profiling provides investigators and the courts with information of high evidentiary value. The effects of washing and wash duration on the ability to detect seminal stains and spermatozoa using an alternative light source, the Acid Phosphatase test and microscopy on a range of different fabric types (cotton, nylon, towel, polar fleece, satin and lace) were assessed. Fluorescence was observed on cotton, towel, and polar fleece material after a single wash. However, after the initial wash, seminal fluid was only detected on the cotton material using the Acid Phosphatase test. All other materials failed to demonstrate any residual acid phosphatase activity after the initial wash. The detection and identification of spermatozoa via microscopy resulted in higher detection rates, with spermatozoa recorded for all fabric types after the first wash. Results varied past this point with satin recording results after the third wash and +4 (more than 10 spermatozoa per field) being recorded for both towel and cotton after six washes. It was concluded that the fabric absorbance influenced results recorded for AP and fluorescence and the variation in sperm count numbers recorded for the different fabric types suggests there may have been a relationship between the knit of the fabric and spermatozoa retention.

**KEYWORDS:** forensic science, forensic biology, semen, Polilight-Flare® plus, acid phosphatase, spermatozoa, persistence, washing.
Introduction

Detecting and identifying body fluids at crime scenes plays a vital role in the provision of intelligence and evidentiary information in forensic science. In 2007, 19,781 cases were reported by forensic biologists as sexual assault cases in Australia (9.2% of the total violent crimes reported). Sexual assault is a crime of violence and victims are more commonly women and children. Examining items of clothing and bedding for seminal fluid is an important aspect in sexual assault cases. The detection of semen and subsequent DNA profiling provides investigators and the courts with information of high evidentiary value, which, when coupled with additional evidence, is vital in assisting the triers of fact to reach a decision.

There are currently a number of presumptive and confirmatory tests used to identify the presence of semen and spermatozoa on forensic exhibits in sexual assault cases. Acid Phosphatase (AP) is a water soluble enzyme that is generally found in high concentrations in seminal fluid and has the ability to act as a catalyst in the hydrolysis of organic phosphates. This forms a product that reacts with diazonium salts resulting in a colour change. There are a number of substrate/colour developer combinations, these include beta-naphthol with Fast Garnet B, alpha-naphthol with Fast Red AL, and the most popular, alpha-naphthyl phosphate with Brentamine Fast Blue. The colour reaction develops over time and the time taken to induce a colour change, in tandem with contextual information, can be used to inform whether or not the colour change is due to the presence of semen. The identification of AP
provides a fast, cost-effective and straightforward way to pinpoint the locations of possible semen stains.

In 1991, Stoilovic demonstrated that the excitation spectrum of semen was broad and that the fluorescence could be generated with wavelengths ranging from 350-500 nm⁴. The Rofin Polilight-Flare® Plus 2 is brighter than any other handheld alternative light source and has the ability to exploit the excitation of semen through narrow bands of light; the wavelengths include 365, 415, 450, 505, 530, 545, 595 and 620 nm. The intensity of the beam profile can be manipulated to suit the application making it ideal for examinations of crime scenes and exhibits⁵.

The most reliable and widely accepted confirmatory test is through visual identification of spermatozoa using a microscope. Semen is the only body fluid that contains sperm cells and the large amounts of DNA in the heads of the sperm cells can be treated with a stain to make them more visible⁶. The Christmas tree stain has gained popularity in recent years becoming the stain of choice; it is known for its characteristic colours of red and green which stain the sperm head and tail respectively. The two main reagents that are used to produce this distinctive stain are Picroindigocarmine, this stains the neck and tail portions of the sperm green, and Nuclear Fast Red, this stains the sperm heads red and the tips of the heads a pink colour⁷.
Occasionally items of interest have been washed following an alleged sexual assault and analysts may be requested to comment on the effects of laundering on seminal fluid and the persistence of spermatozoa. There are studies that have indicated that when items have been washed resulting in the dilution of semen, Acid Phosphatase activity may be reduced to the point in which it cannot be detected by the Acid Phosphatase test or by alternative light sources such as the Polilight. Other studies, however, have indicated that spermatozoa from seminal stains on cotton fabric persist even after the fabric has been washed.

Spector (1971) studied the effects of washing on the detection of spermatozoa in seminal stains by machine washing white undershorts made of cotton; the shorts were subjected to one of 12 different washing procedures and with one of three different types of laundry detergent. It was documented that spermatozoa were found after all washing procedures with the exception of one procedure that involved washing the undershorts in hot water with an enzymatic detergent. Similarly, a study by Kafarowski (1996) examined the retention and transfer of spermatozoa in clothing by machine washing. It was documented that trace quantities of spermatozoa were found on clothing due to transfer during machine washing and spermatozoa could still be found on the original semen stained items. A third study by Jobin (2003) examined nine pairs of semen stained nylon panties and nine pairs of semen stained cotton panties; the semen stained panties were washed with detergent and machine-dried. In concordance with the previous studies, microscopic examination of the stains demonstrated that spermatozoa were present on each set of cotton panties and nylon panties.
In a study conducted by Farmen (2008), 30 pairs of cotton briefs were machine washed; 10 pairs at 40 degrees, 10 pairs at 60 degrees and 10 pairs at 60 degrees with a fabric softener (brand not stated) and it was documented that none of the 30 pieces of laundered underwear yielded a positive result for AP using the acid phosphatase test. However, despite results obtained by Farmen (2008), other studies have shown that it is possible to obtain a weak AP result from seminal stains associated with fabric exhibits that have been machine washed without detergent in cold water or submerged in water for up to 144 hours. In a study conducted by Crowe (2000), 12 cotton t-shirts were examined for seminal persistence when either machine washed or dry cleaned. It was found that t-shirts that were dry cleaned demonstrated positive reactions to the AP test except when spot cleaned and all machine-washed methods gave a negative result to AP except on a cold wash without detergent. Joshi (1981) studied the effects of water immersion on seminal stains in order to simulate a case in which a victim was raped, killed and thrown into a well. At 72 hours strong positive results were documented for AP test, however, after 120 hours of immersion, the AP activity decreased, taking more time for a colour change to emerge. At the conclusion of the study (144 hours), it was documented that AP activity was still detectable.

The studies conducted by both Joshi (1981) and Crowe (2000) demonstrated that it is possible to obtain positive AP results, however, these results were obtained with certain restrictions in the washing protocol. As previously stated by Spector (1971) “Normal washing procedures” are endless and variables such as fabric type, its colour and the amount of semen are all influencing factors, in addition to detergent type and washing method. In the study
conducted by Crowe (2000), it is mentioned that t-shirts that were dry cleaned still showed positive results in an AP test except when spot cleaned. Crowe (2000) admitted to the limitations within the study stating that cotton t-shirts would normally be washed rather than dry cleaned and unless stains were visible to the cleaner they would not be spot cleaned; as seminal stains are generally not visible on fabric they would not be spot cleaned. These results, however, are irrelevant as the effects of washing and not dry cleaning is the focus within this study. It was also stated that all machine-washed methods gave a negative result to AP except on a cold wash without detergent, again, the limitations of the washing technique need to be considered when determining the effects of washing on AP results. The study by Joshi (1981) demonstrated that AP was still detectable after 144 hours of water immersion. When putting these results within the context of the case and others that are similar the results are viable. However, when determining whether washing affects AP activity the results from this study should be carefully considered depending on the context of the forensic exhibit; this is because water immersion is not the same a full wash cycle in a washing machine; this would involve, at a minimum, agitation of the sample.

The study conducted by Spector (1971) is a great starting point for research into the effects of washing on AP and the retention of spermatozoa on fabrics, but, disappointingly it appears that the research hasn’t been taken further; in respect to the number of washes it takes to fully rid a sample of detectable AP and spermatozoa. It was also noted that in many of the studies water temperature is defined as either warm, hot or cold, with the exception of Farmen (2008) who defined warm and hot as 40 °c and 60 °c respectively. Cold, warm and hot can be
defined as anything and could vary between different studies. This is why it could be a possibility that the variance in results between studies could be due to different water temperatures and not the variables in which each study is manipulating as originally thought; this cannot be confirmed as the actual temperatures are unknown. Temperatures need to be defined so that the studies can be compared accurately and subsequent studies have parameters to work within.

It was also noted that studies used detergents but didn’t state brand name and type, again, this needs to be considered as a factor affecting results. No studies, to date, have compared different brands of washing detergents and their effects on AP and spermatozoa retention on fabrics. The studies were also limited to the same fabric type, with only Jobin (2003) including nylon within the study.

No reports were found in the literature that documented the effects of washing and wash duration on the ability to detect seminal fluid and spermatozoa on a range of fabric types. In this study, seminal stains deposited on a range of different fabric types (cotton, nylon, towel, polar fleece, satin and lace) will be examined to assess the effects of washing and wash duration on the ability to detect seminal stains and spermatozoa using an alternative light source, the Acid Phosphatase test and microscopy.
Materials and Methods

Preparation of Stains

Seminal fluid was collected over a two-week period (in order to make a homogenous stock) and stored at 4°C before use. 1ml of seminal fluid was deposited onto a range of fabric types, including black cotton, lace, polar fleece, satin, nylon and towel as well as multi-coloured cotton, lace, polar fleece, satin, nylon and towel. It was noted that the seminal fluid spread differently amongst the different fabric types, hence, those deposited on the multi-coloured fabric were encircled using a black marker and divided into six even portions to ensure even sampling for the spermatozoa detection portion of the study. The seminal fluid was left for 12 hours to air dry before being placed in paper bags to await examination.

Observation of Fluorescence using a Light Source

The light source used in this study was a the Pollilight-Flare® plus (Rofin Australia. Samples deposited on the black fabric swatches were visualised using the Pollilight-Flare® plus at a wavelength of 415nm in combination with the yellow goggles and at 450nm in combination with orange goggles. Fluorescence was recorded as either strong positive (++), weak positive (+) or negative (-).

Acid Phosphatase

A 1L solution of acid phosphatase was made by dissolving 10ml of glacial acetic acid, 20g sodium acetate, 2g sodium 1-napthyl phosphate and 4g fast black k in distilled water. The
solution was refrigerated overnight, filtered and then adjusted with NaOH to pH5. Each black fabric sample was swabbed with a moistened cotton swab and the acid phosphatase solution dropped directly onto the swab. Results were recorded using a non-linear scale ranging from “+++” to -. Each rating refers to the amount of time taken to achieve a positive result; these are as follows: “+++” = strong positive (results within 30 seconds), “++” = positive (results within 2 minutes), “+” = weak positive (results within 10 minutes), “-” = no positive results/positive results past the 10 minute cut off.

Detection of Spermatozoa through Microscopy

Each multi-coloured sample was swabbed with a moistened cotton swab and microscopic slides prepared from that swab with Christmas tree stain. Additionally, spermatozoa was extracted from the multi-coloured samples by excising a portion of the stain and vortexing it in 150 µL of distilled water for 60 seconds. The fabric was then removed and the sample was spun down at 14,000 rpm for 120 seconds. 2µL of the pellet was then used for the slides which were prepared with a Christmas tree stain. Spermatozoa were identified based on morphological and staining characteristics (green tail and a red head with light pink cap). Sperm density was recorded using a non-linear scale ranging from “few” to “+4”. Each rating refers to the number of spermatozoa identified per microscopic field of view (FOV); these are as follows: “few” = less than 5 spermatozoa per slide, “+1” = 1 spermatozoa in some fields, “+2” = 1-5 spermatozoa in most fields, “+3” = 5-10 spermatozoa in most fields, “+4” = more than 10 spermatozoa per field. Fields of view that contained spermatozoa with a tail were recorded as T. Spermatozoa were viewed at a 400x magnification.
Effects of Repeat Washing

The semen stained fabrics were washed independently in a non-biological (OMO-Sensitive) detergent using a domestic top loader washing machine. The washing machine programme included a 45-minute wash cycle in cold water (20°C). Other clean items such as pants, tops, socks and tea-towels were added to simulate a normal washing load. Once washed, samples were left to air dry on a clothes horse at room temperature overnight. The laundered fabrics were then placed individually into brown paper bags and stored in a cool, dry place to await subsequent examinations. Samples were washed, stored and examined up to six times if applicable.

Results and Discussion

Observation of Fluorescence using a Light Source

The most informative state for the observation of fluorescence of seminal stains on the different fabric types using the Polilight-Flare® plus was employing the wavelength set to 415nm using the yellow goggles; this was in accordance with a previous study documenting that seminal stains on dark fabric colours were best visualised using this combination\(^{15}\). However, this combination in comparison to the alternative combination comprising of a wavelength of 450nm with orange goggles highlighted smaller fibres that strongly fluoresced under the light. This generated background interference with the detection of the seminal stains on the different fabric types, however, the stains still demonstrated greater fluorescence using the 415nm and yellow goggle combination as shown in Fig 1.
The variability of the absorbency of the different fabric types was not apparent when visualising the control seminal stains using the Polilight-Flare® plus; this both supported and contradicted results documented in previous studies\(^9,15\). It was found that control seminal stains on the highly absorbent polar fleece and towel were just as easily detected by the Polilight-Flare® plus when compared to the material that had very little absorbency, such as nylon (where the stain appeared to remain on the surface of the fabric) (see table 1). These results support what was documented by Vandenberg (2006) who found that seminal stains observed with the Polilight\(^*\) on less absorbent materials did not appear to fluoresce with greater intensity than those observed on more absorbent materials\(^{15}\). Kobus (2002), however, disagreed and documented that seminal stains deposited on highly absorbent fleece fabrics poorly fluoresced\(^9\).
**Effect of Washing on Fluorescence**

Weak fluorescence could still be detected in stains deposited on cotton, polar fleece and towel after one wash but not after two. Although fabric absorbency was not found to have affected fluorescence in neat samples, those fabrics that are considered more absorbent such as cotton, polar fleece and towel continued to fluoresce after the first wash. This suggests that for seminal stains that are deposited on more absorbent materials, the fluorescent properties of the stain are somewhat more protected from the washing process than if the stain was to remain on the surface of the fabric.

**Acid Phosphatase**

All control semen stains produced a +++ reaction when tested for acid phosphatase using the Acid Phosphatase test. However, after the first wash cycle, only cotton produced a weak result turning positive after 5 minutes (Table 1). Initially, the cut off time for detecting AP has been set to two minutes; this meant that if no reaction was seen within two minutes then the test was recorded as being negative. However, in a study conducted by Lewis (2012), it was documented that despite a literature search there was no scientific basis for the two-minute cutoff period; the study also determined whether the make and type of filter paper affected the likelihood of obtaining a positive result\(^\text{16}\). It was concluded that in cases where more dilute semen samples are expected, using a thicker grade filter paper and/or increasing the cut off time would be expected to increase the likelihood of yielding a positive AP test result; this is expected when garments have been washed and hence the reasoning behind why a 10-minute cut off period was used for this study\(^\text{16}\).
TABLE 1-Effect of washing on detection of semen and spermatozoa using three different detection methods.

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<tr>
<th>Detection Method</th>
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<td>Cotton</td>
<td>Towel</td>
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Fluorescence: strong positive (++), weak positive (+) or negative (-).

Acid Phosphatase: “+++” = strong positive (results within 30 seconds), “++” = positive (results within 2 minutes), “+” = weak positive (results within 10 minutes), “-” = no positive results/positive results past the 10 minute cut off.

Sperm Density: “few” = less than 5 spermatozoa per slide, “+1” = 1 spermatozoa in some fields, “+2” = 1-5 spermatozoa in most fields, “+3” = 5-10 spermatozoa in most fields, “+4” = more than 10 spermatozoa per field. Fields of view that contained spermatozoa with a tail were recorded as T.
Fabric absorbency could again be considered as an influential factor affecting the detection using the Acid Phosphatase test. For fabrics that have little absorbency, such as nylon, satin and lace, the seminal stain was left exposed to the cleaning agent and water diluting it enough so that acid phosphatase could not be detected after the first wash cycle. For highly absorbent materials, such as polar fleece and towel, it is a possibility that acid phosphatase wasn’t detected because it was absorbed so well into the fabric that the swabbing method was not sufficient enough to draw the seminal fluid from the stain. Cotton, even though considered absorbent, is not as absorbent as both polar fleece and towel. This meant that the acid phosphatase in the seminal stain had decreased exposure to the cleaning agent and water from the wash cycle but was not absorbed into the fabric so much that it could not be detected by the Acid Phosphatase test after the first wash cycle.

The sampling method must also be considered as a factor affecting results. The indirect (blot) screening method involves pressing damp blotting/filter paper onto the surface of the item of interest (that has also been dampened with distilled water) to transfer any seminal fluid present onto the filter paper. The paper is then subjected to the acid phosphatase reagent that changes colour from orange to purple in the presence of AP. Alternatively, acid phosphatase solution can be deposited onto a swab using a dropper; this method, however, has only been documented as being used for vaginal or anal swabs.

The indirect (blot) screening method appears to be the method of choice as it is well documented throughout literature. However, there are negative factors associated with using the direct/blot method for detection of AP. Large amounts of liquid are applied to
the sample which leads to diffusion of the spermatozoa resulting in decreased recovery for microscopic evaluation and it also increases the chances of losing spermatozoa from the stain onto the blotting paper²⁰. Although the favoured method, applying extra liquid to the fabric would dilute the seminal fluid even more after each wash cycle and hence the reason behind why this method was not used in this study. As the effects of washing and wash duration on the ability to detect seminal stains and spermatozoa on each fabric type was the aim of the study an alternative method needed to be considered.

The swabbing method, although not the favoured or conventional method was used in this study to ensure that further, unnecessary dilution of the stain would not take place. However, due to the differences in results across the various types of materials, this may have had a negative effect on the results. As previously mentioned, the blot method involves pressing damp blotting/filter paper onto the surface of the item of interest (that has also been dampened with distilled water) to transfer any seminal fluid present onto the filter paper³. The swabbing method, although to some extent involves pressing down onto the fabric using the swab, does not actively draw seminal fluid from the stain through pressure. The combination of damp filter paper and pressure is what could possibly be needed to sufficiently draw seminal fluid out of stains deposited on highly absorbent materials, such as polar fleece and towel, to obtain the expected positive results.
Detection of Spermatozoa through Microscopy

Initially, the fabric swatches were swabbed with a moistened swab and microscopic slides made up from that swab with Christmas Tree stain. This method, however, produced extremely poor results. When comparing results with those documented within the literature sperm counts were extremely low, few (1 per slide) and 0 for cotton and nylon respectively in comparison to +2.9 and +1.3 for cotton and nylon respectively as documented by Jobin (2003)\textsuperscript{14}. Consequently, to ensure reliable and documentable results spermatozoa was then extracted from the fabric by excising a portion and spinning the spermatozoa down into a pellet which was then suspended onto a slide and stained with Christmas tree stain.

For all six fabric types, spermatozoa was recovered after being washed but with varying results (Table 1). All control samples contained an abundance of spermatozoa with tails and were recorded as 4T. However, after one wash cycle spermatozoa on three out of the six fabrics types decreased dramatically.
Lace and polar fleece recorded spermatozoa counts of few (4 on entire slide) and few (1 on entire slide) respectively following the first wash cycle. No reports have been found in the literature regarding the analysis of seminal stains or spermatozoa on lace. However, due to the nature of the fabric, when the seminal fluid was deposited onto the fabric a large proportion of the seminal fluid leaked through onto the surface underneath. Although very small sections of the lace fluoresced prior to washing, demonstrating some absorbance of the control semen, the low sperm count after one wash could be due to the limited absorbance into the fabric at the initial stage of deposition.

As with lace, no reports have been found in the literature regarding the analysis of spermatozoa on polar fleece. Surprisingly, polar fleece had the lowest sperm count out of the six fabrics despite the fact that fluorescence could still be detected after one wash. Polar fleece can be made up of combinations of cotton, polyester and cotton/polyester blends; in the case of this study the polar fleece used was a 20% cotton-80% polyester blend. However, despite in part being made up of cotton, polar fleece is knitted rather than woven (Figure 2), which means...
that the fabric has more stretch due to the fibres being less tightly bound compared to those that are woven\textsuperscript{21}. This suggests that although the fluorescent properties remained in the fabric due to its absorbent properties the spermatozoa were more readily removed by the wash cycle due to the way the fabric is manufactured, i.e. knitted, rather than woven.

Nylon has been previously documented in literature to retain spermatozoa after the first wash cycle\textsuperscript{14}. A sperm count of +2 was recorded for nylon following the first wash cycle, however, no sperm was detected past this point. The sperm count documented after the first wash cycle was in concordance with the average sperm count documented in a study conducted by Jobin (2003) who documented an average sperm count of +1.3\textsuperscript{14}. Similarly, to cotton, nylon is made using a plain weave, however, the spermatozoa numbers for both fabrics vary drastically suggesting that absorbance of the seminal fluid into the fabric at the initial stage of deposition can affect the retention of the spermatozoa following a wash cycle. Although woven more tightly than satin, nylon failed to record numbers beyond the second wash cycle as the absorbance properties of satin appeared to be greater than those of nylon, again suggesting, absorbance properties of the materials had an overall effect.
Satin, unlike nylon, lace and polar fleece continued to yield results up to the third wash cycle. Interestingly, both the AP test and observation of fluorescence yielded negative results after the first wash cycle but a sperm count of +4 was recorded for both the first and second wash until a sudden drop was recorded at the third wash (few-5 on entire slide). Satin is a woven fabric, done in a similar fashion to cotton, but the satin weave is less tightly bound (Figure 3) which is why satin is left with a silky finish. This suggests that the absorbance of the seminal stains was affected by the silky finish of the fabric (resulting in negative results for AP and fluorescence) but the weave of the fabric was tight enough to retain spermatozoa up to the third wash cycle.
Cotton and towel yielded the best results out of the six fabrics used within this study; retaining spermatozoa up to the sixth wash. Sperm counts of +4 were documented for the first and second wash for cotton before decreasing to +2 and few (4 on entire slide) for the third and fourth washes respectively. However, for the fifth and sixth washes sperm count numbers increased back up to +4 (Table 1). This gradual decline and then subsequent incline in spermatozoa numbers could be due to the distribution of spermatozoa throughout the stain. The portions excised and used for extraction in the analysis of spermatozoa following the third and fourth wash cycles, must have had low sperm counts originally and therefore the results obtained were not due to the wash cycle itself. As previously mentioned, cotton is a woven fabric made using the plain weave (Figure 3). When compared to satin (also a woven fabric) cotton yielded higher sperm counts due to the nature of the weave; as depicted in figure 3, cotton is made using the plain weave which is more tightly bound than the satin weave suggesting that weave type affects spermatozoa retention in fabrics.
Towel yielded similar results to that of cotton, with sperm counts continuing to be recorded as +4 up to the sixth wash (Table 1). Unlike cotton, the towel did not decrease in spermatozoa numbers and remained at +4 throughout all six wash cycles suggesting distribution of the spermatozoa was more even than on the cotton sample. Towels are a woven material made up of cotton or a cotton-polyester blend; in the case of this study the towel used was 100% cotton\textsuperscript{23}. This explains the similarity in spermatozoa counts found on both cotton and towel as they are both made from the same fibres and woven in the same way.

Further research needs to be considered in regards to the application of statistics for the differences in sperm counts found based on fabric manufacturing (knitted or woven). Although this study suggests this could be the cause for the differences in sperm counts amongst the fabric types used, application of statistics using a larger data set would document if those differences are statically significant. Also, as stated in a study by Spector, “Normal washing procedures” are endless and other variables such as washing temperature, detergent type,
washing time, drying methods could all be influencing factors that affect the detection of semen and spermatozoa on washed forensic exhibits and need to be studied and documented in the future\textsuperscript{13}.

**Conclusions**

The type of material and the way in which is manufactured is a factor that was found to affect the detection of AP, fluorescence and spermatozoa. For those fabrics that are more absorbent, such as cotton, towel and polar fleece fluorescence can still be detected following the first wash cycle but was found to be undetectable for subsequent washes. Acid phosphatase could only be detected within the 10 minute cut off period for cotton suggesting that absorbency of the material also affected results. For materials with little absorbency such as lace, satin and nylon, AP could not be detected after the first wash cycle suggesting that absorbance of the seminal fluid into the fabric is needed to somewhat protect the AP from being diluted during the wash cycle. The same result was found for materials which were highly absorbent such as towel and polar fleece, the negative result may be a consequence of the swabbing method used in replacement of the indirect (blot) method for AP detection.

When examining the different fabric types for spermatozoa varying results were found for each fabric. It was found that for fabrics that were knitted or had a loose construction such as the polar fleece and lace respectively retention of spermatozoa within the fabric was low resulting in low spermatozoa counts. For fabrics that were woven such as satin, nylon, cotton
and towel spermatozoa counts were high and lasted past the first wash cycle. However, different variations of weave technique further affected the retention of spermatozoa as displayed by the difference in counts between satin (satin weave) and the cotton and towel (plain weave). It should be noted that six washes should not be considered an end point for the detection of spermatozoa on cotton and towel and further investigation is required to establish the true number of washes in which spermatozoa can no longer be detected.
References


Available from:

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63. Profile on the Production of Towels [Cited 2016 November 9].

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