Detection of Secondary Transfer of Human Spermatozoa between Items of Clothing during a Domestic Washing Machine Cycle using the Quantifiler® Trio DNA Quantification Kit.

By

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In

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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 have 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:

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Table of Contents

Title Page..............................................................................................................................................i
Declaration...............................................................................................................................................ii
Acknowledgements .................................................................................................................................. iii

Part One

Literature Review ..................................................................................................................................... 1

Part Two

Manuscript ............................................................................................................................................... 44
Detection of Secondary Transfer of Human Spermatozoa between Items of Clothing during a Domestic Washing Machine Cycle using the Quantifiler® Trio DNA Quantification Kit: A Literature Review
# Table of Contents

List of Figures ..........................................................................................................................3
List of Tables ..............................................................................................................................4
List of Abbreviations ..................................................................................................................5
Abstract ......................................................................................................................................6
Introduction .................................................................................................................................6
Discussion ....................................................................................................................................10
Presumptive Tests for the Identification of Semen .....................................................................10
  Visual and Alternative Light Source Screening ....................................................................11
  Acid Phosphatase Test .........................................................................................................14
  Prostate Specific Antigen ....................................................................................................19
Confirmatory Testing for the Identification of Semen ...............................................................20
  Rapid Stain Identification Detection-Semen Test .................................................................20
  Picroindigocarmin-Kernechtrot Staining .............................................................................22
Introduction to DNA Analysis ..................................................................................................23
Effects of Laundering on Semen Identification .......................................................................25
Experimental Design ................................................................................................................31
  Stain Preparation and Fabric Types ....................................................................................31
  Laundering ............................................................................................................................32
  Sample Analysis ...................................................................................................................32
Experimental Aims and Hypothesis ............................................................................................33
Conclusion .................................................................................................................................35
References ....................................................................................................................................36
List of Figures

Figure 1: Structure of a Fully Matured Sperm Cell\textsuperscript{8} \hspace{1cm} 8

Figure 2: Photoluminescence spectra of dry untreated semen\textsuperscript{22} \hspace{1cm} 12

Figure 3: Mean time taken for a positive acid phosphatase reaction using the direct method\textsuperscript{33} \hspace{1cm} 17

Figure 4: Mean time taken for a positive acid phosphatase reaction using the indirect method\textsuperscript{33} \hspace{1cm} 18

Figure 5: ABACard\textsuperscript{®} p30 test indicating both positive and negative results\textsuperscript{35} \hspace{1cm} 19

Figure 6: RSID-semen test indicating both positive and negative results\textsuperscript{35} \hspace{1cm} 21

Figure 7: Summary of the general forensic DNA workflow\textsuperscript{41} \hspace{1cm} 23

Figure 8: An example EPG of the DNA samples taken from unstained socks washed at 60°C with DNA profiles of two potential contributors\textsuperscript{50} \hspace{1cm} 26
List of Tables

Table 1: The Washing Procedures used by Spector\textsuperscript{51} \hspace{1cm} 27

Table 2: Comparison AP and Spermatozoa Detection of Six Studies\textsuperscript{52} \hspace{1cm} 29
List of Abbreviations

ABS=Australian Bureau of Statistics
ALS= Alternative Light Source
AP= Acid Phosphatase
DNA= Deoxyribonucleic Acid
EPG=Electropherogram
ICST= Internal Child Sex Trafficking
nm=Nanometres
PCR=Polymerase Chain Reaction
PSA/p30= Prostate Specific Antigen
RSID-Semen= Rapid Stain Identification Detection-Human Semen
SAP= Seminal Acid Phosphatase
Sg= Semenogelin
STR= Short Tandem Repeat
WL= Wood’s Lamp
Abstract

Semen is a biological body fluid and is among the most informative types of evidence recovered at a crime scene. In sexual assault cases, seminal fluid can be a source of deoxyribonucleic acid (DNA). This DNA, recovered from items of interest, links victims and persons of interest, corroborates conflicting stories or exonerates innocent parties. After a sexual assault occurs, items of clothing may undergo laundering to remove any evidence pertaining to the event. Forensic analysts will take this into consideration when testing for seminal fluid and when commenting on the persistence of spermatozoa after laundering. Methods of obtaining DNA profiles from laundered fabric samples have been successfully achieved, however, there remains limited information on this subject. Furthermore, the current knowledge of secondary transfer during the laundering process is minimal. Currently, there is limited scientific literature that establishes the viability of obtaining a DNA profile from the secondary transfer of semen during laundering. This review aims to identify the research gap in the determination of secondary transfer of seminal fluid during laundering and, moreover, aid in the conceptual understanding of the secondary transfer of biological fluids during laundering in both investigatory and court scenarios.

Introduction

Forensic analysis has become a key element in investigatory work relating to crimes, particularly sexual assault cases. Significantly, a 22% increase in reported sexual assaults nationwide was recorded by the Australian Bureau of Statistics (ABS) from 2010 to 2016\textsuperscript{1}. Evidentiary information is presented in criminal proceedings to support the Court. To prove beyond reasonable doubt, the rules of evidence must be adhered to, with the aim to inform
the direction of a case. These rules govern what information can be placed before the court; for example, in sexual assault cases, seminal fluid is the source of deoxyribonucleic acid (DNA). DNA found on items of interest, links victims and persons of interest, corroborating conflicting stories or exonerating innocent parties. To bring these items of interest before a Court, a series of steps must be undertaken; the biological fluids (if present) need to be identified. Once identified, a DNA profile of the biological fluid is commenced and a report is written to the court outlining the results of the DNA profiling. Specifically, in the field of forensic science, the identification of these biological fluids is vital in furthering the direction of the investigation.

Biological body fluids, such as semen, are among the most informative types of evidence recovered but are often difficult to identify due to their similarities or inability to be viewed with the naked eye. Seminal fluid is a complex mixture comprised of a suspension of sperm cells in a liquid matrix and spermatozoa. The four urogenital glands within a male create the seminal fluid. Of those four glands, the seminal vesical gland contributes up to 60%, the prostate contributes up to 30% and the epidermis and bulbourethral glands produce the remaining 10% of the secretion mixture. The sperm component (figure 1) of this complex mixture is a small portion of whole semen, making up just 1% to 5% of the total volume of seminal fluid.
Spermatozoa, the cellular component, is comprised of three regions; the head, midpiece, and tail. The genomic material (DNA) from the paternal donor is housed in the head while the mitochondrion; the energy store, are contained in the mid-piece of the sperm. The last component, the tail, is comprised of many filaments permitting motility\textsuperscript{8,9}. The quality of the seminal fluid being produced differs from male to male with the average ejaculate being 3.5 milliliters containing 10-50 million sperm cells per milliliter and can be negatively impacted by diet, smoking, drug use, and genetic predisposition\textsuperscript{10}. This, in turn, can alter the amount of male ejaculate present in each individual sexual assault. Other than the negative factors listed above, health conditions such as oligospermia (abnormally low sperm count), and aspermia (no production of sperm), affect the spermatozoa count in males, while a medical vasectomy surgically prevents the release of sperm during ejaculation\textsuperscript{11}. Despite this, males who have any one of these conditions can still produce seminal fluid that can be detected in a forensic examination. The secretions from the seminal vesicle and prostate gland still produce results relevant to a forensic investigation even in the absence of spermatozoa\textsuperscript{12-14} due to the other cellular material that is present. The antibodies and leukocytes along with

\textbf{Figure 1: Structure of a Fully Matured Sperm Cell}

Divided into the head containing DNA, a mid-piece containing mitochondria, and the tail providing motility. This diagram was taken from \textit{Anatomy and Physiology} \textsuperscript{8}. 
the many proteins, enzymes, and biochemical molecules are all properties that can be exploited when screening for semen in a forensic context\(^7\). The two most commonly screened biomolecules for the detection of semen are the Seminal Acid Phosphatase (SAP) and the Prostate Specific Antigen (PSA, or p30)\(^{15}\). It should be noted that despite both biomolecules being able to be detected in seminal fluid, they are used only in the presumptive testing due to their presence in other body fluids, in various concentrations, in both males and females\(^{16}\).

The ability to detect seminal stains on items such as bedding and clothing is of great significance to forensic analysts when working on sexual assault cases\(^{3,4}\). Presumptive and confirmatory tests are used for the detection of seminal stains and to highlight an area for subsequent DNA profiling. Popular non-destructive presumptive tests include the Acid Phosphatase Test, Prostate Specific Antigen Test, and Visual/Alternative Light Sources\(^3\) while popular confirmatory tests include Rapid-Stain Identification Detection of Human Semen™ and microscopy for spermatozoa indetification\(^{17}\). Once semen has been confirmed, DNA profiling can be utilised. It should be noted that despite the recovery of spermatozoa and subsequent DNA profiling the challenge for investigators lies in not imposing bias interpretations on the manner of deposition.

Methods of obtaining DNA profiles from laundered fabric samples have been successfully achieved\(^{18}\) but there is still a limited scope of information on this subject and furthermore even greater limitations on the knowledge of secondary transfer during the laundering process. Kafarowski et al.\(^{18}\) first reported that transfer of spermatozoa occurred during
washing in 1996. Further studies reported that complete DNA profiles can be obtained from laundered clothing items after fresh semen deposition and from laundered clothing items that have had up to an eight-month period between deposition of seminal fluid and laundering, including multiple washes\(^{19}\). However, in current scientific literature, it is unknown if a viable DNA profile can be obtained from the secondary transfer of semen after laundering. This literature review intends to determine the experimental design to investigate the potential of secondary transfer of semen and furthermore provide results that will aid in the conceptual understanding of the secondary transfer of biological fluids during laundering in both investigatory and court scenarios.

**Discussion**

The following section aims to address various techniques found within the literature to identify seminal fluid in a forensic environment. This includes how seminal fluid is identified both presumptively and confirmatory, and discussing the way DNA is extracted, quantified and profiled using differential extraction, Quantifiler Trio® quantification, and Powerplex 21® profiling. The final discussion section will focus on the effects of laundering on presumptive and confirmatory testing of seminal fluid, and the ability to generate a viable DNA profile from these laundered items.

**Presumptive Tests for the Identification of Semen**

Presumptive testing uses both non-destructive light sources and chemical agents to detect the presence of biomolecular components within a body fluid\(^{15}\). Non-destructive light sources include visualisation with the naked eye, if possible, and alternative light sources
(ALS) using ultraviolet (UV) light at wavelengths between 360nm and 450nm with orange goggles\textsuperscript{20}. The ALS technique utilises the fluorescent properties of semen to cause excitation between the wavelength of light that is being emitted and the conjugated proteins Flavin and Choline\textsuperscript{21}. The presumptive tests that use chemical agents to cause a reaction with two biomolecules, SAP and PSA, are the Acid Phosphatase Test (AP) and the Prostate Specific Antigen Test (p30)\textsuperscript{15}. If a presumptive test returns a positive result further confirmatory testing is needed to identify if the sample should be sent for analysis to possibly obtain the profile of the offender. If a presumptive test returns a negative result, for example, AP testing for semen on washed undergarments yet there is a witness account of ejaculation on the crotch, further confirmatory testing of that area could be utilised to determine if further DNA analysis is required.

\textit{Visual and Alternative Light Source Screening}

When exhibits are first bought into the laboratory under a sexual offence, the forensic examiners must first determine if any visual signs, such as potential semen stains, can be noted, then further testing will be undertaken to confirm. Seminal fluid is a coagulated off-white mixture when first ejaculated and as drying occurs it becomes an off-white to faint yellow, crusty in appearance stain. When visualised under ALS, due to the conjugated proteins Flavin and Choline, dried semen stains will fluoresce\textsuperscript{21}. Stoilovic\textsuperscript{22} reported that undiluted semen has a very strong photoluminescence (figure 2) with a broad excitation spectrum ranging from 350nm-500nm wavelengths.
Due to this broad excitation range, there are many other molecules that can demonstrate fluorescence at these wavelengths, therefore, ALS is considered a non-destructive presumptive test for seminal fluid. Light sources that assist in this fluorescent identification are the Polilight® and the Wood’s Lamp (WL)\(^{21}\).

The Polilight® is an adaptable light source producing white light, UV light and light with wavelengths between 310nm and 650nm. The light sources between 310nm and 650nm are light sources that can visualise the excitation spectrum of semen\(^3,^{21,23}\). The Polilight® has a range of wavelengths that allows for the fluorescence of semen on multiple different backgrounds that may possible fluoresce themselves. The blue light 450nm setting (with orange goggles) is the most commonly used screening bandwidth for general screening purposes\(^{23}\). Vandenburg\(^{23}\) conducted a study that demonstrated that seminal fluid, even when diluted, could be detected in a variety of fabric types using the Polilight®. With the
advances in technology the Polilight-FLARE® Plus 2 has been produced, this is still a handheld ALS just like the previous Polilight®, but has increased intensity to view excited semen through narrow bands of light. The wavelengths, emitted in narrow 30 to 40nm increments²⁰, for this new technology include 365nm, 415nm, 450nm, 505nm, 530nm, 545nm, 595nm, and 620nm, with the increase in the intensity of the beam the application for examination of exhibits and crime scenes also increases¹⁰.

Previously before the Polilight® was introduced the Wood’s Lamp, a device that emits wavelengths between 320nm and 400nm, was used to detect semen stains. This device was small, safe to use and inexpensive in a forensic setting¹,²⁰,²⁴. The major disadvantage of the Wood’s Lamp was its inability to distinguish between semen and other common products such as background fluorescence or other biological fluids. In 1999 Santucci²⁴ conducted a study that asked 41 physicians to analyse 29 semen samples with the Wood’s Lamp to distinguish between semen and other products, none of the physicians could make that distinction. To build on this knowledge, Nelson²⁰ conducted a similar study to Santucci²⁴ were 48 physicians, all formally trained in forensic evidence collection, and 18 physicians, who all received formal training in the WL, analysed semen samples labelled A–N with the WL. Of the total 66 physicians nil were able to detect semen using the WL²⁰. Recently published reports (as of 2000) claimed that WL should be used in the aid of the recovery of semen²⁵,²⁶, however, Santucci²⁴ reported that the 360nm light emitted from the standard WL does not fluoresce for semen nor can it differentiate from other common products²⁰. Due to these studies²⁰,²⁴ the advancement of technology to the Polilight® and now the Polilight-FLARE® for the identification semen stains in a forensic setting is now in place.
Acid Phosphatase Test

The seminal acid phosphatase (SAP) test is one of the most commonly used presumptive tests in a forensic setting due to being cost-effective, fast in the identification of semen and its ability to pinpoint locations of possible semen staining. SAP is a water-soluble enzyme found in high concentrations in human seminal fluid and acts as a catalyst in the hydrolysis of organic phosphates causing a colour change to occur when SAP reacts with diazonium salts. The presumptive testing for SAP is mainly performed with the alpha-naphthyl acid phosphate reagent; a colour metric indicator, changing the reagent pigment from orange alpha-naphthyl acid phosphate to a purple-blue colour in the presence of acid phosphatase. The most popular colour metric reaction developer is the alpha-naphthyl phosphate with Brentamine Fast Blue, but other substrate/colour combinations include the beta-naphthol with FAST Garnet B and alpha-naphthol with Fast Red AL are also used. The alpha-naphthyl phosphate with Brentamine Fast Blue reaction occurs when naphthol is released from sodium α-naphthyl phosphate by the acid phosphatase enzyme causing the formation of a purple azo dye, a positive reaction, due to naphthol coupling to the buffered Brentamine Fast B salt reagent.

Focusing on the alpha-naphthyl phosphate with Brentamine Fast Blue AP test, there are two main methods for presumptive semen testing; the indirect method and the direct method. The indirect method, completed via the swab technique or via a blot screening test, involves the testing not being directly performed on the exhibits. The swabbing technique involves dampening a cotton swab with deionised water, swabbing the area of interest with light force and applying the AP test directly to the swab. The indirect (blot) screening test
involves a piece of dampened filter/blotting paper to be pressed onto the surface of the exhibit to transfer seminal stains that may be present to the paper. The filter/blotting paper is then tested with the AP reagent producing a colour change from orange to purple if AP is present\textsuperscript{30}. Over time the presence of SAP will induce a colour change indicating the presence of semen, it is worth noting that false positives can occur, so context needs to be considered\textsuperscript{30}. The direct method involves applying large amounts of liquid AP to the sample and observing to see if a colour change occurs\textsuperscript{31}. Like the indirect method of using the alpha-naphthyl phosphate with Brentamine Fast Blue AP test, in the presence of semen a colour metric change will occur from orange to purple\textsuperscript{3,27}. Each method, direct and indirect (blot), for AP testing has its negative effects such as a decreased recovery of spermatozoa for microscopy due to large amounts of liquid for direct testing, while indirect (blot) testing can have sampling errors due to the blotting paper not being replaced precisely onto the area that was being sampled and also increased chances of spermatozoa loss to the blotting paper\textsuperscript{31}.

A study conducted by Lewis\textsuperscript{31} in 2013 looked at the effects of applying AP reagent directly to the surface of exhibits opposed to indirectly applying AP reagent to the damp blotting paper that had been in direct contact with the surface of the exhibit. Lewis\textsuperscript{31} used a series of semen dilutions (1 in 50 up to 1 in 3000) to assess the sensitivity of the direct and indirect AP testing, the effect of AP on histological staining, using haematoxylin and eosin, on spermatozoa and the possibility of obtaining false positives from the vaginal material. This study\textsuperscript{31} also included initial investigations into what effect AP reagent has for subsequent DNA testing. Each dilution series contained three pairs of semen-stained knickers; one pair
tested using the indirect AP method and the other two pairs tested using the direct AP method. The indirect method only used damped screening paper rather than dampening both the screening paper and the knickers. The two direct methods used two different application types; a spray bottle (noted as ‘direct spray’) and an aerosol (noted as ‘direct aerosol’). The direct aerosol method was found to be the most sensitive, being able to detect AP in dilutions up to 1 in 3000\textsuperscript{31}. Lewis\textsuperscript{31} concluded that the direct AP aerosol method decreased the potential for sampling errors on items such as bed linen and undergarments and that there was no significant difference between the direct and indirect methods for the number of spermatozoa found. It should be noted that this study is a standalone study without any replication and therefore further work would need to be undertaken to increase reliability.

The Lewis\textsuperscript{31} study was an initial study observing the effects of the AP reagent on DNA testing. As mentioned previously, each of the samples were either indirect, direct spray or direct aerosol tested with AP. The DNA from these samples were extracted and quantified using Lightcycler™ and were amplified at 28 cycles using the AMPF/STR® SGM Plus (Applied Biosystems) STR profiling test. One test was run for each sample and if multiple results were present the strongest result was reported\textsuperscript{31}. Each of the three methods produced similar quantities of DNA and qualities of DNA profiles up to and including 1 in 750 dilutions. Furthermore, the direct application results were generally lower than the blot indirect method results, therefore suggesting that AP reagent does have a detrimental impact on DNA profiling.
AP testing has a current two-minute cut-off period meaning that if no reaction has occurred within two minutes the result is considered negative, despite there being little to no scientific basis for this. Furthermore, Lewis\textsuperscript{32} conducted a literature search in 2012 to determine if there was any basis for the two-minute cut-off period of AP testing. Lewis documented that up to the 1 in 40 semen dilution could be detected before the two-minute cut-off period. After that two-minute cut-off period, greater dilutions showed positive reactions between 5 and 10 minutes, though these reactions were considerably faint. Redhead\textsuperscript{33} also utilised the direct and indirect method of AP testing to question the two-minute cut-off period by applying each method and checking every minute up to 20 minutes for a positive result. Figures 3 and 4 show the direct and indirect AP method tests and the mean time for a positive AP reaction.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Mean time taken for a positive acid phosphatase reaction using the direct method.}
\end{figure}

This diagram was taken from Redhead\textsuperscript{33}
From these results, the research clearly demonstrates that the current two-minute cut-off point is inadequate for the detection of weak stains. Furthermore, the diluted semen stains have the potential of yielding DNA profiles from presumptive AP tests with positive results up to 16 minutes\textsuperscript{33}. This coincides with Lewis\textsuperscript{32} providing clear evidence that the current cut-off point for forensic casework may need to be re-examined so that potential results are not missed.
Prostate Specific Antigen

Prostate-specific antigen (PSA) is employed in forensic sexual assault cases when no spermatozoa were detected including cases where offenders may be vasectomised or aspermic males\textsuperscript{12}. PSA is a protein present in high concentrations in semen due to the prostate contributing to the final mixture of seminal fluid that is ejaculated. PSA is a glycoprotein\textsuperscript{12} that breaks down semenogelin(Sg) I and II, and assists in the liquification of the matrix following ejaculation\textsuperscript{34}. Like SAP, PSA is also present in both male and female body fluids\textsuperscript{34}, therefore, PSA like SAP is not considered a confirmatory test for the identification of semen. Two commercial kits the ABACard\textsuperscript{®} and the p30 test rely on mobile monoclonal anti-human PSA antibodies that bind to human PSA. Once bound they migrate along a strip to immobilise polyclonal anti-human PSA antibodies forming a visible line\textsuperscript{12}. Figure 5 demonstrates this migration by showing a positive and negative result for the ABACard p30 test.

![ABACard p30 Test: Positive Result (Human semen at 10000-fold dilution), Negative Result (Pig Semen)](image)

\textbf{Figure 5: ABACard\textsuperscript{®} p30 test indicating both positive and negative results.}

This diagram was taken from Pang et al.\textsuperscript{35}
The ABAcard® test is a p30 immunological assay that in the presence of PSA will migrate via capillary action toward the test sample indicating a colour change (figure 5) forming an antibody-antigen complex\textsuperscript{12}. Peonim \textit{et. al.}\textsuperscript{16} looked to compare the effectiveness of the PSA test and the AP test for semen detection from human vaginal samples. The PSA test beat the AP test for semen detection with a sensitivity of 80.4\% vs 65.5\% respectively but combined (AP+PSA) were better than the individual tests. What is noteworthy is that while PSA (92.3\%) was more convenient the specificity of AP was higher (92.3\%)\textsuperscript{16}.

**Confirmatory Testing for the Identification of semen**

Confirmatory tests confirm the presence of semen and two well-known test approaches include the Rapid Stain Identification Detection-Human semen (RSID-semen) kit and microscopy. Each of these tests is considered confirmatory due to the chemical immunoassay reaction or the visual identification\textsuperscript{35-37}.

**Rapid Stain Identification Detection-Semen Test**

The RSID-semen strip test is similar in sensitivity to the PSA test while being specific for human semen. Due to being human specific, the RSID-semen test does not cross-react with other bodily fluids. The RSID-semen test uses monoclonal anti-human Sg antibodies to produce a positive reaction colour change (figure 6) like that of ABAcard p30 test\textsuperscript{35}.
Pang et al. 35 looked at introducing RSID-semen strip test as an alternative method for the detection of semen. The study compared the sensitivity and specificity of RSID-semen and ABAcard® p30 tests. The sensitivity according to the manufactures for RSID-semen test is that as little as 1ul of Sg human seminal fluid can give a positive result while the ABAcard® test claims that to achieve a positive result PSA as low as 4ng/ml is possible. The study concluded that Sg was detected and is an effective approach to semen identification and offers the same sensitivity as the ABAcard p30 test35. Furthermore, both tests did not consume the DNA present in the samples therefore further aliquots could be taken for DNA extraction35.
Picroindigocarmin-Kernechtrot Staining

One of the most reliable confirmatory tests is the visual identification of spermatozoa using microscopy. The widely accepted technique is enhanced by exploiting biochemical properties of the three sections of a sperm (figure 1)\textsuperscript{8,36,37}. The head of the sperm carries the DNA and can be stained to become more visible. Over recent years the “Christmas Tree”, or Picroindigocarmin-Kernechtrot stain has gained popularity as the stain of choice for forensic laboratories and is known for its characteristic red and green colour\textsuperscript{36,37}. The nuclear fast red or Kernechtrot, stains the head of the sperm red and the trip of the acrosomal cap pink while the Picroindigocarmin stains the mid-piece and tail a green/aquamarine\textsuperscript{17,37}. This cytological observation is considered the gold standard for the confirmation of seminal fluid and often spermatozoa can be identified even if previously discussed presumptive tests are negative. To increase the staining efficiency, proteinase K is used to denature the epithelial cells so that the unaffected sperm heads become more visible under the microscope\textsuperscript{17}. Other stains that are used for microscopy include Baecchi’s, Papanicolaou’s, Wright’s and hematoxylin and eosin but none are as effective as the Christmas Tree stain\textsuperscript{17}.

The study by Allery et al.\textsuperscript{40} compared three staining methods in the cytological detection of spermatozoa. It was noted that Hematoxylin-eosin is the stain that is most commonly described in scientific literature\textsuperscript{37-39} but the Picroindigocarmin-Kernechtrot stain has risen in popularity\textsuperscript{3,17,40}. The study looked at 174 cervicovaginal samples each prepared on three glass slides; air dried, fixed in alcohol and ether and stained with either Hematoxylin-eosin, Picroindigocarmin-Kernechtrot or Alkaline Fushin. The microscope to view each of the slides had a magnification of x40. The results indicated that the Picroindigocarmin-Kernechtrot
detected the highest percentage of spermatozoa followed by Hematoxylin-eosin and Alkaline Fushin at 35.1%, 34.7% and 28.4% respectively. Due to the significant differences between Alkaline Fushin and the other two staining methods, it has been ruled out as a gold standard for the detection of spermatozoa. The Picroindigocarmin-Kernechtrot detected the highest percentage of spermatozoa per microscopic field followed by Hematoxylin-eosin and Alkaline Fushin at 8.3, 4.6 and 4.2 respectively. From these two results, the study noted that Picroindigocarmin-Kernechtrot had the advantage of spermatozoa detection over Hematoxylin-eosin and Alkaline Fushin. These advantages allow for the slides to be read with ease decreasing the risk of negative results while making the working conditions better. The information found in the study will be applied to the decision-making when determining which staining method this study will undertake in its experimental design.

Introduction to DNA Analysis

DNA or STR analysis (as seen in figure 7) involves the extraction and purification of DNA, from there quantification and multiplex Polymerase Chain Reaction (PCR) occurs and finally capillary electrophoresis and electropherogram analysis.

![Figure 7: Summary of the general forensic DNA workflow](image)

This diagram was taken from Nutton

Despite the variabilities in the commercial kits readily available for DNA extraction, their main mechanism involves isolating and purifying the DNA sample without the extraction of
PCR inhibitors\textsuperscript{41,42}. Chelex\textsuperscript{®} Resin and Silica columns are two popular methods used to extract and purify DNA. The Chelex\textsuperscript{®} Resin utilises the chelex ion exchange resin with its affinity to bind to metal ions, such as magnesium (Mg\textsuperscript{2+}) and iron (Fe\textsuperscript{2+}) to extract not only DNA but enzymes, DNases, and other PCR inhibitors\textsuperscript{41}. The Chelex extraction method can be used on a wide range of samples including blood, bone, semen, saliva, hair, and teeth\textsuperscript{43}. The Silica columns extract and purify DNA simultaneously through a lengthier procedure. One kit that is commercially available that utilises this method is the Qiagen\textsuperscript{®} DNeasy\textsuperscript{®} Blood and Tissue Kit. The DNA is essentially absorbed into the silica disc due to the buffering system present within the kit\textsuperscript{44}. The sequential process that each of the methods offer allows for a one directional, easy to follow, work flow\textsuperscript{45} ready to move to the next step, DNA quantification.

DNA quantification is used as a preliminary check to determine if further investigation using STR profiling should occur. The process of quantification selects single loci to be amplified by PCR with an intercalating fluorescing dye. As each subsequent round of amplification occurs the increasing PCR product is detected from this increasing fluorescence\textsuperscript{45}. The Quantifiler\textsuperscript{®} Trio Quantification kit from ThermoFisher Scientific is an improved quantification technique that utilises a four-dye multiplex system to give details on gender composition and human specificity\textsuperscript{46}. A study undertaken by Vierra-Silva \textit{et al.}\textsuperscript{47} showed that the Quantifiler\textsuperscript{®} Trio could successfully quantify samples with as little DNA as 10pg when contaminated with other products. Furthermore, this multiplex system assesses the DNA samples prior to STR analysis which can be useful for degraded samples that may not have a producible result\textsuperscript{46}. 
The DNA profiling system routinely used in most Australian laboratories is the Profiler Plus (Life Technologies) but new systems such as the Y-Filer (Life Technologies) and Powerplex® 21 are being introduced⁴⁸. These new systems have an increased sensitivity which allows the acquisition of DNA profiles from samples that previously couldn’t be analysed. Ballantyne et al.⁴⁸ analysed 134 total samples and using the Powerplex 21 analysis 94 (70%) of those samples displayed a positive amplification result, compared to 27% and 23% for the Y-Filer and Profiler Plus respectively. Even though this study focused on the environmental background DNA and the possible causes of contamination in the laboratory, this increased sensitivity was seen in the Powerplex® 21 system can be applied to casework samples were previously undetectable amounts of DNA could now be detected⁴⁸.

**Effects of Laundering on Semen Identification**

After an alleged sexual assault, items of interest that may have details pertaining to the event may be washed, analysts must be aware of this situation and may be required to comment on the persistence of spermatozoa and what effects, if any, washing has on seminal fluid⁴⁹. There are a small number of empirical studies that demonstrate spermatozoa can persist on clothing items after laundering in a washing machine and that DNA profiles can be obtained from these stains¹⁸,⁴⁹-⁵³. Victims of sexual assault, specifically victims of internal child sex trafficking (ICST) rarely acknowledge their own victimisation⁵⁰ which may cause a long lag time between the offence and the subsequent investigation. Brayley et. al.⁵⁰ noted that victims, especially children, hid their semen-stained clothes from parents or carers for a period, ranging from several hours to several years. Due to this lag in exhibit examination, the study set out to test if viable DNA profiles could be recovered from laundered semen stains. This study deposited either 1ml of semen, 2ml of semen or a 1:1
ratio of semen from two donors onto items of clothing that were then air dried and stored for 8 months to simulate delays in evidence recovery. The semen-stained clothing items were all laundered in a Hotpoint BHWM129 domestic washing machine using a 90 minute, 1200rpm spin cycle program either using biological or non-biological detergent at 30°C or 60°C. The results showed that a range of DNA from 6 to 18μg was recovered from a single source semen stain after one wash on cotton t-shirts and polyester trousers. Each stain resulted in a DNA profile that could be matched back to the semen donor. This was the first study to demonstrate a profileable DNA can be recovered from laundered semen stains.

Brayley et al. also undertook an investigation into the potential for secondary transfer between clothing items in a washing machine at 60°C. The results of this preliminary investigation yielded a complete major single source profile matching the donor from the previously unstained sock item. An example electropherogram (EPG) of this result is shown in figure 8.

Figure 8: An example EPG of the DNA samples taken from unstained socks washed at 60°C with DNA profiles of two potential contributors. The semen donor D1 and the user of the washing machine W. This diagram was taken from Brayley et al.
Spector\textsuperscript{51} studied the effect laundering had on the detection of spermatozoa by washing white cotton undershorts in a washing machine using both cold and warm washes with 3 different detergent types, the washing procedures Spector used are listed in table 1.

**Table 1: The Washing Procedures used by Spector\textsuperscript{51}**

This diagram was taken from Nolan\textsuperscript{52}

<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>
Except for procedure number 12, spermatozoa were found on all items after laundering. The conclusion of this study was that detectable traces of seminal fluid could not be readily removed during the wash cycle. As discussed above Spector admitted there were limitations for this study including endless variables such as fabric type, semen amount, detergent type and wash cycles that could all play a role in reproducing these results. Kafarowski et. al. did a similar study that examined the retention and secondary transfer of spermatozoa in clothing items in a washing machine, this study was one of the first that looked at the likelihood of secondary transfer. In the three independent trials, trace amounts of spermatozoa were found on all clothing items, this was due to transfer in the washing machine. Kafarowski et. al. and Spector both found that spermatozoa could still be detected on the original semen-stained items following a machine wash. The study conducted by Jobin like Kafarowski et. al. Spector and Brayley et. al. also had microscopic identification of spermatozoa present on each of the sets of panties. From these four studies, it can be widely accepted that spermatozoa have persisted on items of clothing or bed linen that have been laundered.

A further three studies, Crowe et. al., Farmen et. al., and Joshi et. al. all looked at the ability to obtain AP results and spermatozoa results from washed items. Both Crowe et. al. and Joshi et. al. detected strong positive results of AP even after spot cleaning and water immersion for up to 72 hours respectively. However, Farmen et al. washed 30 pieces of underwear, all with the seminal fluid present before washing, and none of those 30 garments produced a positive AP result. A further interesting result is that 16 randomly selected panties of the 30 had a recoverable amount of DNA. This discrepancy in results
between the three studies could come down to Spector's observations that “normal washing procedures” have endless variables all which will influence how semen is recovered.

A summary of all these studies results can be seen in table 2 and compares the AP and spermatozoa detection of all six studies.

Table 2: Comparison AP and Spermatozoa Detection of Six Studies.

This diagram was taken from Nolan.

<table>
<thead>
<tr>
<th>Author</th>
<th>Washing Technique/Detergent Used</th>
<th>Fabric Type</th>
<th>Spermatozoa</th>
<th>AP Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spector (1971)</td>
<td>Soaked</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Cold</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hot</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Machine Washed</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cold/No Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cold/Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cold/Enzymatic Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hot/No Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hot/Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hot/Enzymatic Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kafarowski (1996)</td>
<td>Machine Washed</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Warm/Phosphate free Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Warm/Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Nylon</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Farmen (2008)</td>
<td>Machine Washed</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Warm (40 °C) /No Detergent</td>
<td>Cotton</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hot (60 °C) /No Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Crowe (2000)</td>
<td>Machine Washed</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cold/No Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cold/Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cold/Detergent/Spot Cleaned</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Warm/No Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Warm/Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Warm/Detergent/Spot Cleaned</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dry Cleaned</td>
<td>Cotton</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Solvent A/Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Solvent A/Detergent/Spot Cleaned</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Solvent B/Detergent</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Solvent B/Detergent/Spot Cleaned</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Solvent C/Detergent I</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Solvent C/Detergent II</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Joshi (1981)</td>
<td>Soaked</td>
<td>Cotton</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
A study conducted by Nolan\textsuperscript{52} looked at the effects of washing and wash duration on the detection of seminal fluid and spermatozoa identification on different fabric types (cotton, nylon, towel, polar fleece, satin, and lace). For the identification of seminal fluid and spermatozoa, this study utilised the presumptive ALS and AP testing and confirmatory microscopy. Of the 6 fabric types, florescence from the ALS test was observed on cotton, towel and polar fleece after a single wash while the only fabric to produce a positive AP test after a single wash was cotton. The identification of spermatozoa via microscopy produced higher rates of detection with all fabric types detecting spermatozoa after a single wash. After multiple washes, varied results were recorded with satin recording spermatozoa detection results after the third wash, while fabric types such as towel and cotton recorded spermatozoa detection after six washes\textsuperscript{52}. This study concluded that the absorbency of the fabric influenced the relationship between spermatozoa retention and its detection using both presumptive and confirmatory testing. Therefore, from these results, it is plausible that due to the persistence of spermatozoa on a range of fabric types after multiple wash cycles that the spermatozoa could transfer from one item to another during just one wash cycle.

Overall, the research on the effects of laundering on seminal stains indicate that spermatozoa can be found after a wash cycle on items of interest and that previously unstained garments before washing can have secondary transfer from items of stained garments during a wash cycle. Though the subject of laundering on spermatozoa recovery has been researched, the idea that spermatozoa can transfer between items of clothing during laundering has not. The minimal amount of research in this area indicated a research
gap and this study endeavoured to determine if secondary transfer of spermatozoa can occur during washing between items of clothing.

**Experimental Design**

To address the research gap, the experimental design included several aspects from the current literature with slight modifications to account for the lack of knowledge in this area. The way in which the stains are prepared, and the fabric types were chosen is to contend with what may happen in a real case situation. The laundering process was based on what could be considered your average wash cycle based on the literature and the sample analysis aspects have all been chosen due to information gathered from the literature.

**Stain Preparation and Fabric Types**

Lewis\textsuperscript{31} prepared the seminal staining from fresh unfrozen semen and once applied onto knickers, were left to air dry in a fume hood for up to one week before testing. Kafarowski et. al.\textsuperscript{18} prepared the semen-stained samples allowing for air drying for a minimum of 48 hours. Nolan\textsuperscript{52} collected semen over a two-week period to homogenise the semen samples and applied 1ml of semen to several fabric types that were left to air dry for 12 hours before washing. All three studies allowed the semen to be air dried onto the samples at varying time lengths, due to the time constraints on this project the samples with semen stains will only be left to air dry for a minimum of 24 hours before testing. Stained cotton bedsheets and unstained cotton undergarments were decided upon as these fabric types have an increased popularity of use in everyday lifestyle while also allowing for comparison with the previous studies\textsuperscript{18,31,52}. 
Brayley et al. used a domestic washing machine with a program duration of 90 minutes and a 1200rpm spin cycle at temperatures of 30°C and 60°C. Spector used multiple detergents and wash temperatures to launder the samples. To minimise the number of variables and to introduce some control into this project the wash cycle will be a normal wash cycle in a domestic washing machine on a cold wash with a spin cycle of 1000rpm. The washing machine will hold one stained bedsheet and one pair of unstained cotton undergarments to simulate a “normal” wash cycle for an everyday household.

Sample Analysis

For the initial identification of seminal fluid on the laundered stained bedsheets and pristine underwear, the ALS that will be utilised is the Polilight-FLARE® over the WL due to both Nelson and Santucci finding the differentiation between semen and other common products using the WL could not be done. The presumptive testing for semen stains for this experiment will be the indirect blotting AP test due to there being a large area to examine for the bedsheets and due to unknown whereabouts of the semen stains on the undergarments. The indirect blotting AP method was chosen over the direct AP method because some laundered items may only have small amounts of spermatozoa present and using the direct AP method could increase the risk of losing what spermatozoa are present therefore hindering or inhibiting further DNA profiling. Microscopy will be the confirmatory test used to detect the presence of semen and specifically spermatozoa for this experiment. Picroindigocarmin-Kernechtrot, “Christmas tree”, staining was chosen over Hematoxylin-eosin and Alkaline Fushin due to the result found in Allery et al.
For the DNA analysis a differential extraction, separating the semen and epithelial cells, will be undertaken to minimise the change of mixed profiles arising in the STR analysis. The quantification kit utilised for this experimental design will be the Quantifiler® Trio Quantification kit from ThermoFisher Scientific due to its four-dye multiplex system that allows for gender composition\textsuperscript{46} which will act as a safeguard in case any epithelial cells are not separated from the semen cells correctly. Finally, the Powerplex® 21 kit will be the commercial DNA profiling kit that will produce the STR analysis due to its increased sensitivity over other kits as stated in Ballantyne \textit{et al}\textsuperscript{48}.

\textbf{Experimental Aims and Hypothesis}

The research presented in this literature review indicates that spermatozoa can be found on garments after laundered in a washing machine and moreover a full DNA profile can be recovered. The research also presents the concept of secondary transfer of DNA from one item to another and that DNA profiles have been obtained as a result of the secondary transfer. Due to both concepts being accepted as an occurrence in a forensic setting, the idea that secondary transfer of semen can occur after a wash cycle is plausible. Therefore, the literature review dictates the aim of the experiment to determine if the secondary transfer of seminal fluid can occur during a wash cycle. Subsequently, three hypotheses are to be tested to answer the main aim of this experiment.
Experimental Hypothesis 1

H₀: After a normal cold wash cycle a viable DNA profile using the Powerplex 21® kit is not able to be obtained from a semen covered bedsheet.

H₁: After a normal cold wash cycle a viable DNA profile using the Powerplex 21® kit is able to be obtained from a semen covered bedsheet.

Experimental Hypothesis 2

H₀: During a normal cold wash cycle the secondary transfer of seminal fluid from a bedsheet to an undergarment is not going to occur.

H₁: During a normal cold wash cycle the secondary transfer of seminal fluid from a bedsheet to an undergarment is going to occur.

Experimental Hypothesis 3

H₀: After a normal cold wash cycle a viable DNA profile using the Powerplex 21® kit is not able to be obtained from an undergarment that has a secondary transfer of seminal fluid from a bedsheet.

H₁: After a normal cold wash cycle a viable DNA profile using the Powerplex 21® kit is able to be obtained from an undergarment that has a secondary transfer of seminal fluid from a bedsheet.
This project aims address the research gap by gathering more information on the effects of laundering with a domestic washing machine on bedsheets containing seminal fluid with clean undergarments to determine if secondary transfer of seminal fluid occurs during a wash cycle.

**Conclusion**

Crimes such as sexual assault are on the rise worldwide and there may or may not be attempts by both the victim and the offender to hide any evidence of this crime. The main way people try to hide evidence of sexual assault is through the washing of clothing or bed linen. Due to several studies being able to get a complete DNA profile from laundered items\textsuperscript{12,49-54} the next step is addressing secondary transfer. The research gap is firstly in the reproducibility of these studies but also that there are little to no studies documenting the effects of the secondary transfer of semen onto garments during a washing cycle. To address the research gap, stained bedsheets and unstained cotton underwear will undergo washing together and be analysed using the presumptive AP testing, confirmatory microscopy Christmas Tree staining and Powerplex\textsuperscript{®}21 DNA profiling. This project endeavours to close this research gap by aiming to determine if the secondary transfer of seminal fluid can occur during a wash cycle and, if so, can a viable DNA profile be obtained.
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Detection of Secondary Transfer of Human Spermatozoa between Items of Clothing during a Domestic Washing Machine Cycle using the Quantifiler® Trio DNA Quantification Kit.
Detection of Secondary Transfer from Human Spermatozoa between Items of Clothing during a Domestic Washing Machine Cycle using the Quantifiler® Trio DNA Quantification Kit.

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Abstract

Forensic analysis has become a key component in investigatory work relating to crimes, particularly sexual assault cases. In sexual assault cases, a primary source of evidence is biological fluids, such as semen, as it is a known source of deoxyribonucleic acid (DNA) evidence. After a sexual assault occurs, items of clothing may undergo laundering to remove any evidence pertaining to the event. Hence, it is important for forensic analysts to take this into consideration when testing for seminal fluid and when commenting on the persistence of spermatozoa after laundering. The detection of secondary transfer of spermatozoa between stained cotton bedsheets and pristine cotton underwear during a domestic washing machine cycle were assessed using presumptive Alternative Light Source (ALS) and Acid Phosphatase (AP) Testing and confirmed using Microscopy and the Quantifiler® Trio DNA quantification kit. After one washing machine cycle, all 8 of the stained bedsheets returned positive quantification results for male DNA, while 5 of the 8 pristine underwear returned positive results for transferred male DNA, providing further evidence of the likelihood of secondary transfer.

Key Words: Forensic Science, Laundering, Secondary Transfer, Spermatozoa, DNA
Introduction

Forensic analysis has become a key element in investigatory work relating to alleged crimes, particularly sexual assault cases. The Australian Bureau of Statistics (ABS), between 2010 to 2016, recorded a 22% increase in reported sexual assaults nationwide\(^1\). Evidentiary information in sexual assault cases during criminal proceedings provides support to the Court to determine a verdict. The judicial system governs what evidence can be received in a trial. In sexual assault cases, a primary source of evidence are biological fluids; in particular seminal fluid, which is known to be a source of deoxyribonucleic acid (DNA) evidence. DNA found on items of interest has the ability to link alleged victims and persons of interest, corroborate conflicting stories, or exonerate innocent parties\(^2\). To bring these items of interest before a court, a series of steps must be undertaken, including the identification and analysis of biological fluids if present. Initially, the biological fluids (if present) need to be identified to effectively recover the DNA from the cellular source and later analyse a recovered DNA profile. A report is then written outlining the results, interpretation and conclusions of the DNA profiling.

Biological body fluids, such as semen, are among the most informative types of evidence recovered, but are often difficult to identify due to their visual similarities to other fluids or inability to be viewed with the naked eye\(^2,3\). The four male urogenital glands create a mixture of secretions called seminal fluid. Of those four glands, the seminal vesical gland contributes up to 60% of the fluid, the prostate contributes up to 30%, and the epidermis and bulbourethral glands produce the remaining 10% of the secretion mixture\(^4\). The quality of the seminal fluid being produced differs from male to male with the average ejaculate having
a volume of 3.5 milliliters, containing 10 – 50 million sperm cells per milliliter. This can be negatively impacted by diet, smoking, drug use, and genetic predisposition reducing the spermatozoa count. These individual specific variables can alter the amount of male ejaculate present in each individual sexual assault. Other than the negative factors listed above, health conditions such as oligospermia (abnormally low sperm count) and aspermia (no production of sperm), affect the spermatozoa count in males, while a medical vasectomy prevents the release of spermatozoa during ejaculation. Despite this, males who have any one of these conditions can still produce seminal fluid that can be detected in a forensic examination. The secretions from the seminal vesicle and prostate gland still produce results relevant to a forensic investigation even in the absence of spermatozoa due to the other cellular material present within the secretions.

The ability to detect seminal stains on items such as bedding, and clothing is of great significance to forensic analysts when working on sexual assault cases. Presumptive and confirmatory tests are used for the detection of seminal stains and used to highlight an area for subsequent DNA analysis. Frequently used non-destory presumptive tests include: the Acid Phosphatase Test (AP test), Prostate Specific Antigen Test (p30/PSA test), and Visual/ Alternative Light Sources (ALS Analysis). Confirmatory tests which are preferentially used by forensic scientists include Rapid-Stain Identification Detection of Human Semen™(RSID-Semen) and microscopy for spermatozoa identification. Once the presence of spermatozoa has been confirmed, downstream processing such as DNA extraction, quantification, amplification and, finally, profiling can be utilised to confirm the origin of the sample.
Methods of obtaining DNA profiles from laundered fabric samples have been successfully developed in previous studies\textsuperscript{11}. However, limited information exists on the subject of secondary transfer during the laundering process, thus identifying an area in need of additional research. Kafarowski \textit{et al.}\textsuperscript{11} first reported that transfer of spermatozoa occurred during washing in 1996. Further studies reported that complete DNA profiles can be obtained from laundered clothing items after fresh semen deposition, and from laundered clothing items that have had up to an eight-month period between deposition of seminal fluid and laundering, including multiple washes\textsuperscript{12}. However, in current scientific literature, there is limited information available regarding a viable DNA profile being obtained from the secondary transfer of semen during laundering. This is directly relevant to forensic analysts due to the advances in technology including increased sensitivity and discriminatory power thus allowing the recovery of a partial or full DNA profile which may not have previously been obtainable.

This pilot study aimed at addressing the effects of laundering with a domestic washing machine on bedsheets containing seminal fluid with clean undergarments to determine if secondary transfer of seminal fluid is a plausible occurrence. Furthermore, the quantification of male human DNA using the Quantifiler\textsuperscript{®} Trio DNA Quantification Kit is utilised to provide additional evidence to support the likelihood of the secondary transfer event during washing.
Methods and Materials

Six ejaculate samples were collected from a healthy 23-year-old male individual into sterile yellow top containers every second day. These samples were stored frozen over a 12-day period and homogenised into mixtures A and B once all six samples were collected. Each of the homogenous mixtures contained 3 ejaculate samples and swabs of both homogeneous mixtures (A and B) were taken as positive controls, stored in the same conditions as the mixtures, ready for DNA analysis.

Initial Laundering and Stain Preparation

Prior to any experimental work being performed, a Bosch Maxx Classic domestic washing machine was bleached with 1 cup of bleach (household grade) and run on the highest temperature, 60°C 1 hour and 16 minutes normal wash cycle (1000rpm spin cycle) setting as a cleaning cycle. Eight cotton single fitted bedsheets (Target Taupe 250 thread count) numbered 1 to 8 and eight 95% cotton, 5% elastane/lycra underwear (Kmart Ladies size 12) numbered 1 to 8 were washed individually to remove any stain repellents within the fabric and air dried for 24 hours at room temperature. Each of the bedsheets and undergarments were swabbed prior to semen application as negative control samples and stored in the freezer prior to DNA analysis. Each of the bedsheets were folded into a large square and had a 15cm x 15 cm square marked using a permanent marker. Approximately 3ml of semen from homogenous semen mixture A (vortexed to mix before application) was applied via 1ml transfer pipette to the marked squares on bedsheets 1 – 4 and, respectively, 3 ml of homogeneous semen mixture B (vortexed to mix before application) was applied via 1ml transfer pipette to the marked squares on bedsheets 5 – 8. Two small swatches of bedsheet
material also had semen deposition of homogenous mixture A and B to provide positive controls for ALS and AP testing which were recorded as positive control A and positive control B respectively. Once the homogeneous mixtures were applied the bedsheets were left to air dry for 24 hours at room temperature. Each of the bedsheets and underwear were stored separately in brown paper packaging for up to 72 hours to simulate evidence storage procedures when not in direct use throughout the study.

**Subsequent Laundering after Stain Deposition**

The Bosch Maxx Classic domestic washing machine was set to a 45-minute cold normal wash cycle (1000rpm spin cycle) setting for all washing involving each of the replicates 1 – 8. Each replicate wash cycle contained one bedsheet and one set of underwear. In between each replicate wash cycle, the washing machine was cleaned to remove any residual DNA using 1 cup of bleach (household grade) and run on the highest temperature, 60°C 1 hour and 16 minutes normal wash cycle (1000rpm spin cycle) setting. At the completion of each laundering cycle, the bedsheet and underwear were set out to air dry for 24 hours at room temperature and then packaged separately in brown paper packaging for up to 72 hours to simulate evidence storage procedures when not in direct use throughout the study.

**Presumptive Testing: Alternative Light Source and Acid Phosphatase**

Each of the bedsheet and underwear replicates underwent presumptive testing for semen to determine areas of interest. For visual confirmation of staining, a Pollilight-FLARE® Plus (Rofin Australia) ALS was used and stains visualised using orange goggles at 450nm
wavelength. The fluorescence each sample emitted was recorded as follows: +++ = strong fluorescence, ++ = fluorescence, + = weak fluorescence and - = no fluorescence. If the ALS returned a positive result, then the sample was subject to a presumptive AP test. For this study the indirect blotting method was utilised which involved covering the item (either bedsheets or underwear) with a piece of chromatography paper moistened with distilled water and patting firmly over the whole area. The 500ml solution of alpha-naphthyl phosphate with Brentamine Fast Blue AP reagent was freshly made by dissolving 5ml of glacial acetic acid, 10g sodium acetate, 1g sodium-1-naphthyl phosphate and 2g fast black k in distilled water which was refrigerated overnight. The following day the solution was filtered and adjusted to ph5 using sodium hydroxide. The AP was sprayed directly on to the chromatography paper with a pressurised spray bottle. The AP result each sample produced was recorded as follows: +++ = reaction within 5 seconds, ++ = reaction within 30 seconds, + = reaction within 2 minutes, and - = no reaction.

**Confirmatory Testing: Light Microscopy**

To prepare the microscopy slides, portions of bedsheets 1 – 8 were excised in small 2.5cm x 2.5cm squares and cut into smaller pieces to be placed into a 1.5ml Eppendorf microcentrifuge tube with spin basket. The bedsheet excision samples were then soaked with 2ml of sterile purified water, vortexed for 2 minutes and centrifuged for 5 minutes. The spin basket was removed; the pellet was swabbed and smeared onto a microscopy slide that was then heat fixed. Portions from the underwear 1 – 8 were excised in two parts, as shown in Figure 1; 4 randomly selected areas (A, B, D and E) on the outside of the underwear were excised in a total of 2.5cm x 2.5cm squares while portions from the crotch area (C) were
excised in small 2.5cm x 2.5cm squares, and cut into smaller pieces to be placed into 1.5ml Eppendorf microcentrifuge tubes with spin basket.

For the underwear, microscopy slides, the samples were soaked in 150ul pre-lysis buffer without dithiothreitol (DTT), vortexed for 1 minute and centrifuged for 1 minute. To create the underwear microscopy slides, 2ul of supernatant were smeared onto the slides and heat fixed. The positive and negative control swabs were smeared onto separate microscopy slides and heat fixed for staining. Each of the individual slides were stained using the Picroindigocarmin-Kernechtrot (“Christmas Tree”) staining method\textsuperscript{13,14}. The slides were stained with nuclear fast red for 20 minutes and then rinsed using sterile purified water. Picroindigocarmin was then applied to the slides for 30 seconds and rinsed with 95% ethanol before heat fixing. After microscopy analysis using a light microscope at x40 objective lens the number of spermatozoa present on each slide were recorded as follows: $\text{+4} = 8$ or more

Figure 1: The locations of the excisions collected from each set of underwear (1 – 8) to assess DNA transfer, cuttings A, B, D and E collected together in one microcentrifuge tube represent one area of examination and cutting C represents the second area of examination.
spermatozoa in some fields, \( +3 = 5 - 7 \) spermatozoa in some fields, \( +2 = 2 - 4 \) spermatozoa in some fields, \( +1 = 1 \) spermatozoa in some fields and \( 0 = \) no spermatozoa present.

**DNA Analysis**

**DNA Extraction and Purification**

In preparation for the extractions, the positive and negative control swabs were cut from the plastic applicators, and the excisions of fabric were all extracted following the DNA IQ™ System-Small Sample Case Work Protocol section 4.B DNA Isolation from Stains on Solid Material\(^{15}\). The protocol was followed with slight modifications including: i) microscopy slides were made with pre-lysis and no DTT before adding the pre-lysis with DTT to continue the extraction; ii) after step 6, all samples were transferred in to a 96-well plate; iii) from step 6 onwards, every time it stated vortex, manual pipetting was used; and, iv) 30ul elution volume. Once completed all 42 samples were transferred back into Eppendorf microcentrifuge tubes for freezer storage.

**DNA Quantification of the Samples using the Quantifiler® Trio DNA Quantification Kit on the QuantStudio™6 Flex qPCR Instrument**

The 42 extracted DNA samples were quantified using the Quantifiler® Trio DNA Quantification Kit. For all samples the protocol was followed as prescribed by ThermoFisher Scientific\(^{16}\) and each of the reactions were plated into a 384 well plate with two sets of serially diluted standards ready for Real-time Polymerase Chain Reaction (qPCR). The qPCR was run on a QuantStudio™6 Flex which was calibrated to accommodate the expanded dye
set (including JUN®, ABY®, FAM® and Mustang Purple® dyes) for the Quantifiler® Trio DNA quantification kit. The software for this machine was setup following the details in the user guide\textsuperscript{16} with the thermal cycling conditions noted in Figure 2. Minor adjustments to this protocol included: i) adjusting the nominated cycles number to 30 cycles; and, ii) updating the capture point to be at the final 60°C point succeeding the annealing and extension phase.

<table>
<thead>
<tr>
<th>Holding Stage</th>
<th>Cycling Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Incubation</td>
<td>Denaturation</td>
</tr>
<tr>
<td>25.0°C 05:00</td>
<td>95.0°C 02:00</td>
</tr>
<tr>
<td>60.0°C 00:30</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: The thermal profile run method for the QuantStudio™6 Flex qPCR instrument using the Quantifiler® Trio Kit\textsuperscript{16}.

The qPCR report was produced using the QuantStudio™ Real-Time PCR Software v1.3 automated analysis that was used to analyse the results.
Results

Presumptive Testing: Alternative Light Source and Acid Phosphatase

The presumptive testing involved using an ALS Polilight-FLARE® Plus (Rofin Australia) to visualise any possible staining present on either the bedsheets or underwear after a wash cycle. If there was a positive visual identification of the stain, then a continued AP test was undertaken. Figure 3 displays examples of how each fluorescent category appears under 450nm wavelength viewed with orange goggles.

![Image of fluorescent results]

Figure 3: Alternative Light Source analysis using a Polilight-FLARE® Plus (Rofin Australia) at 450nm wavelength visualised under orange goggles producing results for the positive controls A(+A) and B(+B), and replicate 4 (R4) and replicate 6 (R6) of the bedsheets all returning ++++, ++ and + positive results respectively.

Each of the laundered bedsheet samples produced a fluorescent result ranging from fluorescence to weak fluorescence with each of the positive controls returning a strong
fluorescence result. The difference in fluorescent levels (Figure 3) were noted in Tables 1 and 2. The tables were separated to view the results by which homogenous mixture was used with each replicate. Table 1 presents the results for replicates using homogenous mixture A.

Table 1: Alternative Light Source and subsequent Acid Phosphatase analysis of the positive control A, negative control, bedsheets 1 – 4 and underwear 1 – 4. The results for the Alternative Light Source were scored as follows: +++ = strong fluorescence, ++ = fluorescence, + = weak fluorescence - = no fluorescence and the Acid Phosphatase results were scored as follows: +++ = reaction within 5 seconds, ++ = reaction within 30 seconds, + = reaction within 2 minutes, - = no reaction.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Alternative Light Source</th>
<th>Acid Phosphatase Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control A</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Negative Control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bedsheet 2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bedsheet 3</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Bedsheet 4</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Underwear 1</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Underwear 2</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Underwear 3</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Underwear 4</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 1 compared homogenous mixture A (positive control A) against bedsheet replicates 1 – 4 and underwear replicates 1 – 4. Of the bedsheets tested with ALS 75% (bedsheets 1,3 and 4) returned a fluorescent result while only 25% (bedsheet 2) returned a weak fluorescent result. The underwear replicates 1 – 4 did not produce fluorescence when visualised and therefore were given the result indicating no fluorescence. The positive control A produced a strong fluorescent result and the negative control produced a no fluorescence result. If the ALS had a positive result then the item underwent AP testing, all the bedsheet replicates were AP tested and if seminal fluid was present a purple reaction
would occur. Zero of the bedsheet replicates 1 – 4 returned a positive result for AP and coinciding with the statement above, the underwear replicates 1 – 4 did not undergo any AP testing due to the negative ALS results. Table 2 presents the results of the ALS and AP testing for replicates using homogeneous mixture B.

Table 2: Alternative Light Source and subsequent Acid Phosphatase analysis of the positive control B, negative control, bedsheets 5 – 8 and underwear 5 – 8. The results for the Alternative Light Source were scored as follows: +++ = strong fluorescence, ++ = fluorescence, + = weak fluorescence - = no fluorescence and the Acid Phosphatase results were scored as follows: +++ = reaction within 5 seconds, ++ = reaction within 30 seconds, + = reaction within 2 minutes, - = no reaction.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Alternative Light Source</th>
<th>Acid Phosphatase Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control B</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Negative Control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bedsheet 5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bedsheet 6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bedsheet 7</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Bedsheet 8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Underwear 5</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Underwear 6</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Underwear 7</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Underwear 8</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2 compared homogenous mixture B (positive control B) against bedsheet replicates 5 – 8 and underwear replicates 5 – 8. The bedsheets tested using ALS came back with 25% (bedsheet 7) returning a fluorescence while 75% (bedsheets 5, 6 and 8) returned a weak fluorescence. The positive control B produced a strong fluorescent result matching that of positive control A and the negative control produced a no fluorescence result. The underwear replicates 5 – 8 did not produce fluorescence when visualised corresponding with the results from underwear replicates 1 – 4. Again, if there was a positive ALS result the item underwent AP testing. Zero of the bedsheet replicates 5 – 8 returned a positive result for AP and, with the underwear replicates 5 – 8 producing no ALS results, there was
no further AP testing. Overall for ALS presumptive testing, the bedsheets replicates 1 – 8 produced positive results covering equally the fluorescence or weak fluorescence categories, while the underwear replicates 1 – 8 kept a uniform negative result. For AP testing the bedsheets 1 – 8 were consistently negative across all replicates and the underwear replicates 1 – 8 did not undergo AP testing.

Confirmatory Testing: Microscopy

The confirmatory method of spermatozoa identification was microscopy. The staining method was effective in identifying the pink spermatozoa heads and the green spermatozoa tails when present, as seen in Figure 4, while also confirming morphological integrity.
Figure 4 gives a general overview of the categorisation of each of the microscopy slides. Both positive control slides showed the pink acrosomal caps and spermatozoa heads with attached green spermatozoa tails, while each of the replicate bedsheet samples only
showed the pink acrosomal caps and spermatozoa heads. Table 3 represents the results of
the microscopy for the bedsheet and underwear samples.

Table 3: The microscopy results of the positive controls, negative control, bedsheet samples and
underwear samples counting the number of spermatozoa present on each slide. The results were
recorded as follows: $\text{+4} = 8$ or more spermatozoa in some fields, $\text{+3} = 5 – 7$ spermatozoa in some
fields, $\text{+2} = 2 – 4$ spermatozoa in some fields, $\text{+1} = 1$ spermatozoa in some fields, $0 = \text{no}
spermatozoa present.\

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Microscopy</th>
<th>Replicate</th>
<th>Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control A</td>
<td>+4</td>
<td>Positive Control B</td>
<td>+4</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0</td>
<td>Negative Control</td>
<td>0</td>
</tr>
<tr>
<td>Bedsheet 1</td>
<td>+4</td>
<td>Bedsheet 5</td>
<td>+2</td>
</tr>
<tr>
<td>Bedsheet 2</td>
<td>+2</td>
<td>Bedsheet 6</td>
<td>+3</td>
</tr>
<tr>
<td>Bedsheet 3</td>
<td>+3</td>
<td>Bedsheet 7</td>
<td>+4</td>
</tr>
<tr>
<td>Bedsheet 4</td>
<td>+3</td>
<td>Bedsheet 8</td>
<td>+4</td>
</tr>
<tr>
<td>Underwear 1</td>
<td>0</td>
<td>Underwear 5</td>
<td>0</td>
</tr>
<tr>
<td>Underwear 2</td>
<td>0</td>
<td>Underwear 6</td>
<td>0</td>
</tr>
<tr>
<td>Underwear 3</td>
<td>0</td>
<td>Underwear 7</td>
<td>0</td>
</tr>
<tr>
<td>Underwear 4</td>
<td>0</td>
<td>Underwear 8</td>
<td>0</td>
</tr>
</tbody>
</table>

The positive controls gave a microscopy result of 8 or more spermatozoa in some fields
which was expected as the spermatozoa were swabbed directly onto the slides without
dilution or agitation and the negative control had no spermatozoa present. All the bedsheet
slides ranged from +2 to +4 results with 37.5% of the bedsheets (1, 7 and 8) returning with
8 or more spermatozoa in some fields, 37.5% of the bedsheets (3, 4 and 6) returning a 5 – 7
spermatozoa in some fields and 25% of the bedsheets (2 and 5) returning a 2 – 4
spermatozoa in some fields result while all the underwear slides produced a 0 result for no spermatozoa present.

**DNA Analysis**

Once the presumptive and confirmatory testing was completed, a total of 42 samples underwent DNA analysis. This included positive controls for both homogenous mixture A and B, negative controls for all bedsheet replicates 1 – 8 and underwear replicates 1 – 8, and bedsheet sample replicates 1 – 8, underwear sample replicates 1 – 8 and underwear crotch sample replicates 1 – 8. All 42 samples were first extracted to a 30ul elution volume and then quantified to determine the quantity (ng/ul.) of male DNA present.

*DNA Quantification of the Samples using the Quantifiler® Trio DNA Quantification Kit on the QuantStudio™6 Flex qPCR Instrument*

After extraction the 42 samples underwent quantification using the Quantifiler® Trio DNA Quantification Kit to determine if the samples contained any male DNA. The amount of male DNA was recorded as a quantity in ng/ul displayed in Figures 5 and 6. The amount of DNA found from the bedsheet replicates 1 – 8 were compared in Figure 5.
Figure 5 illustrates the amount of male DNA found on each of bedsheets that have been laundered. All the bedsheets were found to contain over 100ng/ul of male DNA with replicate 6 containing the most male DNA at 241.11ng/ul, while replicate 8 contained the least amount of DNA at 117.22ng/ul. The remaining replicates 1 – 5, and 7 contained 139ng/ul, 137.79ng/ul, 134.19ng/ul, 195.65 ng/ul, 147.85ng/ul and 139ng/ul respectively. The theory that male DNA, specifically spermatozoa, during laundering will be transferred was tested experimentally with the results presented in Figure 6.

Figure 5: The quantity (ng/ul) of male DNA found on each of the 8 stained bedsheets after laundering.
Figure 6 demonstrates the results of male DNA found on the underwear that prior to washing contained no male DNA, according to the negative controls of the underwear, which returned no quantified male DNA. The excised underwear samples were broken into two areas (Figure 1): the combined four randomly sampled regions (green) which, from here on out, will be referred to as underwear; and the underwear crotch region (purple) which, from here on out, will be referred to as underwear crotch. Of the underwear replicates 1 – 8 replicate 1 contained the most male DNA at 0.004ng/ul while replicate 6 returned a nil value for male DNA. The remaining replicates 2 – 5, 7 and 8 contained 0.03ng/ul, 0.001 ng/ul, 0.001ng/ul, 0.002ng/ul, 0.002ng/ul and 0.001ng/ul respectively. Of the underwear crotch replicates 1 – 8 replicate 1 contained the most male DNA at 0.057ng/ul and replicate 5 contained the least male DNA at 0.022ng/ul. The remaining replicates 2 – 3 and 5 – 8 all...
contained male DNA at 0.051ng/ul, 0.049ng/ul, 0.029ng/ul, 0.044ng/ul, 0.045ng/ul and 0.032ng/ul respectively. Overall, 100% of the stained bedsheets returned positive quantification results for male DNA, while 25% of the pristine underwear and 100% of the pristine underwear crotch returned positive results for male DNA, providing further evidence of the likelihood of secondary transfer.

Discussion

After an alleged sexual assault has occurred it is not uncommon to find the victim, or the perpetrator, will try to erase any evidence of the event. In this instance this includes the laundering of any items such as linen or clothing. The specific effect of laundering on human spermatozoa and the theory of secondary transfer during laundering is relatively unknown\(^\text{11,12}\). Furthermore, the manner of which the semen was deposited; direct deposition or post-coital drainage as opposed to transfer during laundering\(^\text{17}\) will impact on the “one sperm defence” in sexual assault cases. The findings in this study have helped to contribute to the literature available on the secondary transfer of human spermatozoa during a domestic washing machine cycle.

Presumptive Testing: Alternative Light Source and Acid Phosphatase

Presumptive testing includes both non-destructive light sources such as the visual identification or the Polilgiht-Flare\(^\text{®}\) Plus ALS and chemical agents such as AP to detect the biomolecular components present within body fluids\(^\text{18}\). To visualise any stain present on the laundered items ALS was used specifically the Polilgiht-Flare\(^\text{®}\) Plus at 450nm with orange
goggles. This wavelength was chosen, as it is the most commonly used screening bandwidth\(^{19}\). It was noted in this study that all the stained bedsheets still produced a fluorescence using the Polilight-Flare\(^\text{®}\) Plus ALS after laundering while zero of the sets of underwear produced a fluorescence. It is suggested that due to the composition of cotton the spermatozoa have been absorbed into the material rather than just staying on the surface and therefore have been protected against the effects of washing\(^{20}\). Furthermore, the underwear replicates may not have produced a positive fluorescence because the conjugated proteins Flavin and Choline that produce the fluorescence\(^{21}\) may have been lost during the wash. Once the ALS results were determined any items that produced a positive ALS underwent AP testing. This method was used due to the potential loss of spermatozoa that may have been transferred from the item to the blotting paper possibly causing a decrease in downstream results.

There are two main methods for presumptive semen testing using the alpha-naphthyl phosphate with Brentamine Fast Blue AP test; the indirect blot method and the direct method\(^{22}\). Each method has negative effects on spermatozoa recovery, but this study chose the indirect blot method over the direct method because of the large volume of AP that would have been applied to the item of interest possibly washing away any present spermatozoa\(^{23}\). Three studies: Crowe et. al\(^{24}\), Farmen et. al., \(^{25}\) and Joshi et. al\(^{26}\) looked at obtaining AP results and spermatozoa results from laundered items similar to this study. Crowe et. a\(^{24}\) and Joshi et. a\(^{26}\) detected strong AP positive results after spot cleaning and water immersion for up to 72 hours respectively while Farmen et. a\(^{25}\) found that all garments produced a negative AP result despite producing a positive AP result before
This study concurs with the findings of Farmen et al.\textsuperscript{25} that laundered stained items give negative results for AP as all bedsheet replicates produced negative results for AP.

AP may not have been detected on the bedsheets due to the absorbency of cotton. While not considered an absorbent material in comparison to materials such as towel or polar fleece, cotton still has the ability to absorb the semen decreasing exposure to water and cleaning agents\textsuperscript{20}. The decreased absorbency of cotton may not have allowed enough of the stain to be absorbed thus not being able to be detected via AP testing after laundering. Furthermore, the discrepancies in results between the various studies including this one could be accounted for by Spector\textsuperscript{27} observations that “normal washing procedures” have endless variables which influence how semen is recovered.

**Confirmatory Testing: Microscopy**

Microscopy is widely accepted as a confirmatory test for semen due to the visual identification of spermatozoa\textsuperscript{28-30}. The Picroindigocarmin-Kernechtrot (“Christmas Tree”) stain was used for this study due to its rising popularity in scientific literature\textsuperscript{2,10,31} and its popularity as the stain of choice for forensic laboratories\textsuperscript{28,29}. The characteristic red and green colour of the Picroindigocarmin-Kernechtrot stain comes from the nuclear fast red staining the sperm head red and the tip of the acrosomal cap pink while the Picoindigocarmin stains the mid-piece and tail a green/aquamarine\textsuperscript{10,29}. The confirmation of seminal fluid though this cytological observation is considered the gold standard due to
the ability to identify spermatozoa on items of interest that have previously had negative presumptive testing results\textsuperscript{10}.

In this study the presumptive AP testing gave a negative result for all bedsheet replicates 1 – 8 and all underwear replicates 1 – 8. Due to the ability to microscopically confirm the presence of spermatozoa even if negative results were returned this outcome prompted the use of microscopy as one of the main confirmatory tests. All bedsheet replicates returned positive microscopy results ranging from +2 to +4 but only the presence of sperm heads and the acrosomal caps were able to be viewed with the tails having been lost. The loss of the spermatozoa tails is a common occurrence even in refrigerated samples\textsuperscript{32} and due to the extra agitation to dislodge the spermatozoa from the fabric the lack of tails is not unexpected. Despite the lack of tails present the overall ability to view the sperm heads and acrosomal caps, which contain the source of nuclear DNA was achieved confirming the presence of spermatozoa on all bedsheet replicates.

Despite the negative results for AP for the underwear replicates 1 – 8 visual confirmation of spermatozoa using microscopy was undertaken. All sets of underwear contained no visual identification of spermatozoa. The absence of spermatozoa present on the underwear slides may have been due to the method of microscopy slide preparation specifically the use of the pre-lysis buffer to dislodge the spermatozoa into solution. The use of the pre-lysis buffer may have broken down the spermatozoa that may have been present causing a lack of intact spermatozoa to be present but further testing would need to be undertaken to determine if this was a factor. In future to increase stain efficiency and possible yield proteinase K could
be used first to dislodge the spermatozoa and denture the epithelial cells leaving the unaffected sperm heads to become more visible under the microscope\textsuperscript{10}. Furthermore, future studies could use the Rapid Stain Identification Detection Semen strip test (RSID), which has a high sensitivity specificity for human semen while also not consuming the DNA present in the samples allowing for further DNA extraction\textsuperscript{33}. The lack of positive results for the underwear replicates did not mean that human spermatozoa were not present. After a negative microscopy result forensic laboratories stop processing the samples as this is considered a null result. To determine if this result and subsequent stop of analysis was premature further DNA analysis was undertaken to confirm the quantity, (ng/ul), if any, of male DNA present on both the bedsheets and underwear replicates.

**DNA Analysis**

The extraction method undertaken for this study did not involve differential extraction. Differential extraction separates the epithelial fraction and the sperm fraction so that quantification can occur on the sperm fraction only in sexual assault cases. Differential extraction is the preferred method of extraction for items relating to sexual assaults where there will be more than one contributor of DNA on the item of interest\textsuperscript{34}. Due to time constraints and financial limitations it was decided to forgo the differential extraction method and instead focus on targeting the male DNA further downstream. The further downstream analysis used to target the male DNA from each of the samples was the use of the Quantifiler® Trio DNA Quantification Kit.
DNA Quantification of the Sample using the Quantifiler® Trio DNA Quantification Kit on the QuantStudio™6 Flex qPCR Instrument

The purpose of this study was to determine if there was potential for secondary transfer of human spermatozoa during a domestic washing machine cycle between items of clothing. To get a definitive answer to this question the Quantifiler® Trio DNA Quantification Kit was used. This kit was chosen due to its more robust nature and sensitivity in providing additional information on targeting male DNA\textsuperscript{35}. This study determined that it was possible to obtain quantifiable DNA results from the laundered stained bedsheets and furthermore from the laundered pristine underwear. The Quantifiler® Trio DNA Quantification Kit detects DNA at a minimum threshold of 0.0003pg/ul\textsuperscript{36} and in accordance with this threshold 100% of the stained bedsheets produced positive quantification results for male DNA, while 25% of the pristine underwear and 100% of the pristine underwear crotch returned positive results for male DNA. The difference in the amount of male DNA found between the two regions of underwear could be due to the crotch region having a double layer of fabric which would increase its absorbency thus increasing the likelihood of spermatozoa in that region. This result provides further evidence of the likelihood of secondary transfer.

Additional Considerations

This pilot study had to set very specific parameters due to time and financial constraints; because of this, certain parameters went untested and need to be considered when thinking about how they may affect secondary transfer during laundering. Brayley-Morris et. al\textsuperscript{39} did note in their preliminary study that although different parameters may affect the transfer of spermatozoa during laundering the study found similar DNA quantities in clothing.
regardless of detergent type, washing machine type or DNA extraction method used. Furthermore, secondary transfer of spermatozoa during laundering under a wide range of conditions is possible\textsuperscript{17}.

**Conclusion**

The parameters employed to investigate if human spermatozoa could be transferred during laundering in a domestic washing machine were sufficient to obtain male DNA results from previously pristine underwear. This adds to the literature by adding additional evidence confirming the likelihood of the secondary transfer event. Moreover, this adds emphasis that the presence of even just one or a few spermatozoa cannot confirm acts of sexual assault and that all additional evidence needs to be taken into consideration before drawing final conclusions. The current study has provided an insight into the forensic theory that male DNA can transfer from a source on a bedsheets to underwear during laundering. Further research will need to be undertaken to determine if a partial or full DNA profile could be obtained from previously pristine items that have had spermatozoa transfer from stained items during laundering.

**Disclaimer**

The authors of this study have no declared conflict of interest, nor do they endorse any products for the purpose of DNA extraction or quantification within the study conducted.
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