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A Pilot Study: The Effects of Repeat Washing and Fabric Type on the Detection of Seminal Fluid and Spermatozoa.

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HIGHLIGHTS

- Sperm may persist on cotton and terry towel clothing following six wash cycles
- Sperm may be viewed microscopically even when ALS and AP are negative
- If context suggests laundering of garments, microscopy should be considered

ABSTRACT: In sexual assault cases and more specifically those involving childhood sexual abuse (CSA), victims may have had their potentially semen-stained clothing washed multiple times before a criminal investigation commences. Although it has been previously demonstrated that spermatozoa persist on cotton clothing following a single wash cycle, items of clothing washed multiple times are not routinely examined in these cases because of the assumption that the

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laundering process would have removed all seminal fluid and spermatozoa. The aim of this study was to examine the persistence of seminal fluid and spermatozoa on a range of fabric types including cotton, nylon, terry towel (100% cotton), polyester fleece, satin and lace which were laundered up to six times. Three techniques were used for the detection of seminal fluid and spermatozoa: an alternative light source, acid phosphatase test and microscopy. The study demonstrated that spermatozoa persisted on cotton and terry towel following six wash cycles. This data emphasises the need to recover and examine items of clothing and bedding of victims for semen, even if the item has been washed multiple times.

KEYWORDS: child sexual abuse, child sex trafficking, forensic science, forensic biology, semen, spermatozoa, persistence, washing, Polilight-Flare® plus, acid phosphatase.

Introduction

In 2004, childhood sexual abuse (CSA) was declared a “silent health emergency” by the World Health Organization (WHO) [1]. The long term negative effects of CSA have been found to be extensive, including increased risk for violence, depression and suicide [2]. A 2010 Australian study, surveying 1,745 adolescents (812 males and 933 females) found 17% of females and 7% of males were identified as having some type of unwanted sexual contact (touching, fondling, kissing, display of sex organs, etc.) prior to age sixteen [1].

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Studies have found that children involved in sexual abuse rarely acknowledge their own victimisation for several reasons; this could include fear, shame and normalization of sexual behaviours. In such cases, extended periods of time would separate the alleged offence and police investigations, further complicating forensic analyses [3].

Despite the knowledge of potential health risks and the forensic significance of semen, a multi-perpetrator rape study identified that only 20% of offenders wore a condom during a sexual attack [4]. Similarly, a study of child sex trafficking (CST) cases found that offenders did not commonly use condoms but would instead ejaculate directly onto the body, clothing or bedding of the victim [3]. It was identified that in some CST cases, victims hid their clothes for extended periods of time before washing, or washed items of clothing multiple times to remove any visible stains in order to avoid having to discuss the assault with a parent or carer [3].

A small number of studies have demonstrated that seminal fluid is undetectable using the acid phosphatase (AP) test or alternative light sources when items of clothing have been laundered in a washing machine, using various detergents and washing protocols [5-8]. Other studies have demonstrated that spermatozoa continue to persist following washing [9-11]. Interestingly however, none of these studies have investigated the persistence of seminal fluid and spermatozoa beyond one wash cycle or on a fabric type other than cotton, simulating circumstances more commonly encountered in CSA and CST cases. Therefore, the aim of this study was to examine the persistence of seminal fluid and spermatozoa on a range of fabrics types

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including cotton, nylon, terry towel, polar fleece, satin and lace that were laundered up to six times by utilising an alternative light source, AP test and microscopy.

Materials and methods

Preparation of stains

Seminal fluid was collected from one healthy 29-year-old male, over a two-week period, stored at 4°C and used as a homogenous stock. The stock was evaluated for sperm count in accordance with WHO guidelines for the routine counting of spermatozoa [12] in order to establish that it was a representative sample of the normal male population. Fabric samples were chosen to best provide a range of samples encountered in casework. For experiments whereby ALS was utilised, black fabric was selected in order to limit the effect of fabric autofluorescence and false positive results. A mixed 1ml sample of seminal fluid was deposited onto a range of coloured fabric test materials including cotton, lace, polar fleece, satin, nylon and terry towel; this was done in duplicate for all fabric types and labelled A and B. Control samples (unwashed seminal fluid stains) were also made in duplicate and stored in paper bags for the duration of the trial.

In preliminary trials, it was noted that the different fabrics influenced the manner of seminal fluid spread due to various absorptivity and wicking rates. To accommodate this, for stains made on multi-coloured fabrics, the stained area was encircled using a black marker and divided into six equal parts for microscopy sampling to ensure that sampling was uniform. Stains made on black

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fabrics were encircled using a yellow wax pencil. The entire spread of the seminal fluid was marked as best as possible given the difficulty in evaluating it visually against the black background. For this reason, any attempt at dividing the stain into equal portions for microscopy would have been error-prone and therefore the black fabric swatches were used for AP and ALS testing only and not microscopy. The seminal fluid stain was air-dried for 12 hours at room temperature before being stored in paper bags until examination.

Observation of fluorescence using a light source

The light source employed was the Polilight-Flare® II Plus (Rofin, Australia). The Polilight-Flare® plus intensity and beam profile of the light can be changed to suit the application, making it for the examinations of crime scenes and exhibits [13]. Only stains deposited on the black control and test fabric swatches were visualised at a wavelength of 415nm and 450nm and observed with yellow and orange filters, respectively; this was to avoid any false positives due to background fluorescence of the lighter coloured fabrics [6]. Fluorescence was recorded on an arbitrary scale as either strong positive (++), weak positive (+) or negative (-).

Acid phosphatase test

A one litre solution of acid phosphatase was made by dissolving 10 mL of glacial acetic acid, 20g sodium acetate, 2g sodium 1-napthyl phosphate and 4g fast black practical grade in distilled water. The solution was refrigerated overnight, filtered and then adjusted with concentrated sodium hydroxide to pH 5. Each black coloured control and test fabric sample was swabbed with a cotton swab previously moistened using distilled water and the acid phosphatase solution dropped

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directly onto the swab. Multi-coloured fabrics were not used to avoid any chance of further dilution of the sample before microscopy. Results were recorded using a non-linear scale ranging from '+++' to '-' (Table 1).

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Detection of spermatozoa through microscopy.

Each control (unwashed) and test multi-coloured sample was swabbed with a moistened cotton swab and microscopy slides prepared with Christmas tree stain in order to visually identify any cellular materials, including spermatozoa, associated with the stain. The Christmas tree stain is a reliable confirmatory visual test for the the presence of semen and is based on the differential staining ability of the sperm head and tail using nuclear fast red and picroindigocarmine, yielding a crimson colour to the head and green-blue-gray colour to the tail. Nuclear fast red was commercially procured (Sigma Aldrich) and picroindigo carmine was prepared by dissolving 40g picric acid and 10g indigo carmine in distilled water. In addition to slides prepared from the swabs, spermatozoa were isolated from the multi-coloured samples by excising a portion of the stain and vortexing it in 150µL of distilled water for 120 seconds; portion sizes differed depending on the spread of the seminal fluid on the fabric during initial deposition. The fabric was then removed and the sample was centrifuged at 14,000 rpm for three minutes. 2µL of the cell pellet was then transferred to 1-1.2mm thick clear glass/frosted end microscopic slides which were then prepared with a Christmas tree stain [14]. Spermatozoa were identified based on morphological and staining characteristics (green tail and a red head with light pink cap). Sperm density was recorded using a

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non-linear scale ranging from 'few' to '+4' (Table 2). Each rating referred to the number of spermatozoa identified per microscopic field of view (FOV) at 400x magnification on an Olympus BX51 compound microscope fitted with an Olympus DP70 camera. Fields of view that contained spermatozoa with a tail were recorded as 'T'.

Effects of repeat washing.

The semen stained fabrics (all except control samples) were washed independently in a non-biological (OMO-Sensitive) detergent using a domestic LG Inverter Direct Drive 9.5kg top loader washing machine. The washing machine programme included a 15-minute wash cycle, 15-minute rinse cycle and 12-minute spin cycle in cold water (20°C). Additional untreated items such as pants, tops, socks and tea-towels were added in duplicate to simulate a normal washing load. Once washed, samples were air-dried on a clothes airer at room temperature overnight. The laundered fabrics were then placed individually into brown paper bags and stored at room temperature until further examinations. Samples were washed, stored and examined up to six times, if seminal fluid or spermatozoa continued to be detected.

Results and discussion

Observation of fluorescence using a light source.

In 1991, Stoilovic demonstrated that the excitation spectrum of semen was broad and that the fluorescence could be generated with wavelengths ranging from 350-500nm. It was demonstrated

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that the detection of seminal stains was based on their photoluminescence and was very much dependent on the nature of the material in which it was deposited [15]. It is well recognized that materials of lighter colours, such as white, can display strong fluorescence due to optical brighteners in the fabrics and detergents; this background fluorescence can mask the stain leading to false negatives [6]. The most informative state for the observation of fluorescence emitted by seminal stains on the different fabric types using the Polilight-Flare® Plus was at 415 nm viewed with a yellow filter. These observations were in line with a previous study that reported seminal stains on dark fabric colours were best visualised using this combination [16]. However, this combination in comparison to the alternative combination comprising of a wavelength of 450 nm with an orange filter highlighted smaller fibres that strongly fluoresced upon exposure to light (Fig 1). This generated background interference with the detection of the seminal stains on the different fabric types.

Detection of seminal fluid via alternative light source.

The variability in absorption abilities of the different fabric types was not apparent when visualising the control (unwashed) seminal stains using the Polilight-Flare® II Plus; this both supported and contradicted results documented in previous studies by Vandenburg (2006) and Kobus (2002) respectively (Table 3).

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It was found that control seminal stains on the highly absorbent polyester fleece and terry towel were just as easily detected by the Polilight-Flare® II Plus when compared to the material that had very little absorbency, such as nylon, where the stain appeared to remain on the surface of the fabric (Table 3). These results can be supported by those reported by Vandenberg (2006) where seminal stains observed with the Polilight® on less absorbent materials did not appear to fluoresce with greater intensity than those observed on more absorbent materials [16]. Kobus (2002), however, disagreed and documented that highly absorbent fleece fabrics poorly fluoresced [6].

Effects of washing on detection of seminal fluid via alternative light source.

In contrast to the strong fluorescence displayed by all control (unwashed) samples, seminal fluid deposited on the more absorbent fabrics such as cotton, polar fleece and terry towel displayed weak fluorescence while fabrics that appeared less absorbent such as satin, nylon and lace did not exhibit fluorescence following one wash (Table 4); Vandenburg (2006) reported that weak fluorescence could still be detected in some stains following washing, however did not state on which fabrics. This suggests, the fluorescence of the seminal fluid was protected from the washing process to a greater extent in the more absorbent fabrics than the less absorbent ones on which the seminal fluids apparently remained on the surface. Following the second wash cycle, no fluorescence was observed on any of the fabrics tested suggesting the remaining fluorescent properties were removed within the second wash.

Effects of washing on the detection of seminal fluid via the acid phosphatase test

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All control (unwashed) seminal stained fabrics produced a '+++' reaction when tested for acid phosphatase using the AP test. It is important to note that the initial cut-off time for detecting AP was two minutes, however, in a study conducted by Lewis (2012), it was documented that despite a literature search there was no scientific basis for the two-minute cut-off period. It was concluded that in cases where more dilute semen samples are expected, increasing the cut off time would increase the likelihood of yielding a positive AP test result [17]. Therefore, the cut off time was extended from two minutes to ten minutes.

It was assumed that the ability of the AP test to detect seminal fluid would be similar, if not better than the alternative light source based on results reported by Vandenberg (2006) [16]. The acid phosphatase test targets the large amounts of the acid phosphatase that the male prostate gland produces and secretes into seminal fluid. However, following the first wash cycle, only cotton returned a weak positive result after five minutes (Table 4). Given the lack of fluorescence on silk, nylon and lace samples, the negative results displayed for AP were not surprising. However, the inability to detect seminal fluid using the AP test on terry towel and polyester-fleece samples, which did display weak fluorescence was unexpected.

Considering the results, the potential cause of the negative results for polyester fleece and terry towel could be perhaps due to the sampling method used. In this study the swabbing method was used rather than the more favourable blot method [5,7-11]. The primary reason for not choosing the blot method was because it involves the addition of water to the sample which would result

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in the dilution of the seminal fluid beyond a single wash cycle. On the other hand, the swabbing method does not involve unnecessary dilution of the seminal fluid which was more suitable for the aims of this study. This method however, in contrast to the blot method in which a large amount of pressure and fluid is applied to draw the seminal fluid out of a stain, only allows for the surface of the fabric to be sampled with reduced water and pressure applied. Therefore, we postulate that the inability to detect seminal fluid using the AP test on absorbent fabrics, despite fluorescence, could be due to the insufficient wetting of the surface area and pressure that the swabbing method utilised when attempting to draw seminal fluid from stains deposited on those materials. It is important to note that this study included a very small sample number, hence why it would be important to confirm if the sampling method was indeed responsible for the above-mentioned results; a study comparing the two sampling methods and with a larger sample size would need to be conducted. Even though there were a limited number of replicates, the results do indicate the effect of the sampling method on the detection of acid phosphatase on different terry towel and polyester fleece.

. D*etection of spermatozoa through microscopy*

All control samples contained an abundance of spermatozoa with tails and were recorded as +4T, however, following the first wash cycle, spermatozoa scores were found to vary based on fabric type (Table 4); for example, few for lace, +2" for nylon and +4T for cotton. As time progresses the loss of tails is expected due to degradation of the sperms cells in the sample.

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Despite the differences in the ability to detect seminal fluid on lace and polar fleece, both recorded spermatozoa counts of few (four and one, respectively, on the entire slide) following the first wash (Table 4). On further investigation, it was noted that despite the differences in the ability of lace and polar fleece to absorb seminal fluid, they have a similar structure leading to the similarities in spermatozoa detected. We hypothesise that because lace has a very loose construction, it allows the spermatozoa to move freely through the fabric without becoming entrapped within the fibres. Similarly, polar fleece has a knitted construction; knitted fabrics have a looser fibre construction which enables the fabric to stretch [18]. Hence, we suggest that due to the looser construction of the fibres and the stretch that this provides, spermatozoa were more easily washed away following the first wash cycle.

Nylon was found to have a slightly higher sperm count of +2 following the first wash cycle (Table 4). A similar finding of average sperm count of +1.3 was reported in a study conducted by Jobin (2003) [11]. Nylon is a woven fabric and more specifically, made using a plain weave (Figure 2); the difference in the fibre construction of nylon in comparison to lace and polar fleece may account for the ability of nylon to retain more spermatozoa following washing.

Satin, unlike nylon, lace and polar fleece continued to yield results up to the third wash cycle; +4, +4 and few for the first, second and third washes, respectively (Table 4). Like nylon, satin is a woven fabric, however, satin is made using a slightly looser satin weave giving rise to its silky appearance (Figure 2) [19]. It is difficult to explain the reason for detection of spermatozoa in satin longer than

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nylon despite having a looser weave construction. There are several variables that could be considered such as fabric absorption and synthetic versus non-synthetic materials, however, as this was only being a pilot study these were not explored. Further work on fabric construction will need to be explored before a solid explanation can be made.

Cotton and terry towel showed the greatest spermatozoa retention of the six fabrics used within this study; retaining spermatozoa up to the sixth wash. Sperm counts of +4 were documented for the first and second wash on cotton before decreasing to +2 and few (4 on entire slide) for the third and fourth wash, respectively. However, for the fifth and sixth washes sperm count numbers increased back up to +4 (Table 4). This gradual decline and then subsequent incline in spermatozoa numbers could be due to the distribution of spermatozoa throughout the stain. The portions excised and used for extraction in the analysis of spermatozoa following the third and fourth wash cycles, could have had low sperm counts originally despite stirring of the sample before application and therefore the results obtained were not due to the wash cycle itself. Similarly to nylon, cotton is a woven fabric made using plain weave (Figure 2) [19]. The construction of plain weave in comparison to satin weave is what we suggest gives rise to the difference in results; tighter the weave, more likely the spermatozoa entangle within the fibres and thus making it difficult to remove them via washing.

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Terry towel yielded similar results to that of cotton, with sperm counts continuing to be recorded as +4 up to the sixth wash (Table 4). Based on these results it was not surprising to learn that terry towels are a woven material made up of cotton or a cotton-polyester blend [20]; in the case of this study the terry towel used was 100% cotton explaining the similarity between the two.

We therefore report what we believe to be the first controlled demonstration of the recovery of spermatozoa cells after six domestic washing machine cycles. This presents significant implications for the management and collection of exhibits related to sexual offences whereby witness reports may indicate laundering of exhibits have taken place post-assault. In many forensic biology laboratories, negative ALS and AP results are likely to preclude microscopic analysis for spermatozoa cells. We warn against this and suggest that if case context indicates that a garment has been laundered following a sexual assault, further microscopy may be pertinent in identifying offender cellular material for DNA analysis. With the knowledge that spermatozoa cells could be retained for up to six wash cycles, microscopy should still be considered in order to rule out the presence of any forensic evidence that may be used in a conviction.

Conclusions

In cases of child sexual assault, it is common for offenders to ejaculate directly onto the clothing or bedding of their victims, for victims to hide those clothes for extended periods of time before

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washing and for victims to wash those items multiple times to rid themselves of any reminder that the assault took place [3].

This study demonstrates that alternative light sources, the Acid Phosphatase test and microscopy are all viable methods for the detection of seminal fluid and spermatozoa on items of clothing before washing takes place. However, after items have been washed the ability of ALS and the AP test in identifying seminal fluid stains dramatically decreases leaving microscopy as the ideal method for detection of spermatozoa. Spermatozoa can be obtained from laundered semen stained clothing following a wash cycle, highlighting the importance of testing these items following sexual assaults despite knowledge that they could have been washed. On both cotton and terry towel, spermatozoa can be found in abundance after six wash cycles using microscopy techniques. This emphasises the need to recover and examine items of clothing and bedding of victims for semen, even if the item has been washed multiple times.

Due to this study primarily looking at observational findings, further research to evaluate differences in sperm counts found based on fabric construction (knitted or woven) needs to be conducted. One of the explanations for the cause of differences in sperm counts amongst the fabric types used could be number of replicates used, however, to be certain, the application of statistics using a larger data set to explain if these differences are statistically significant would have to be undertaken. Similarly, it would be of interest to compare spermatozoa counts on fabrics that differ in absorption abilities also. Once these systematic studies have been completed, studies

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to examine if viable DNA profiles can be generated from such items would be of importance and even more useful in sexual assault investigations. It is also important to note that there are limitless variations to the washing protocol used within this study; variables such as washing temperature, detergent type, washing time and drying methods could all be influential factors in the ability to detect seminal fluid and spermatozoa, hence, should be studied and documented in the future also.

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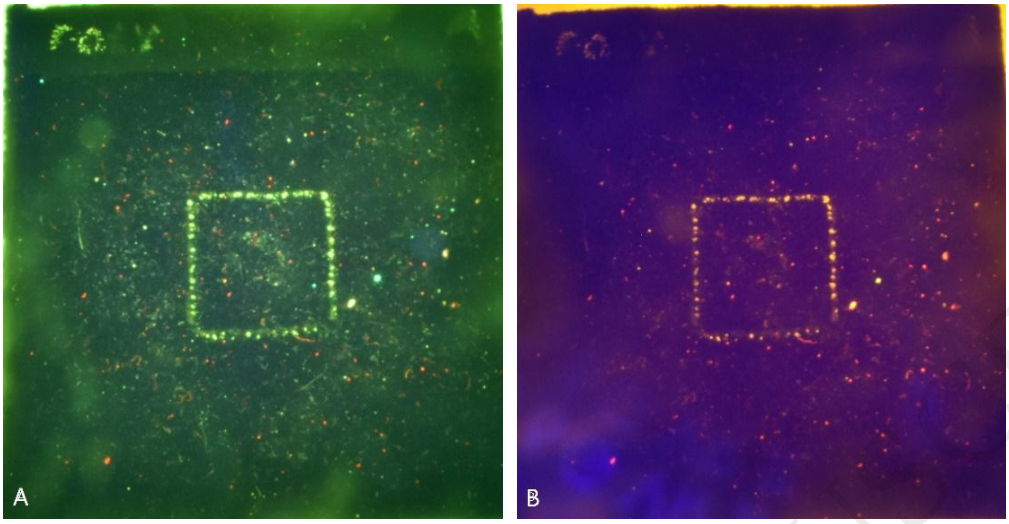

Fig. 1. Seminal stains on black polar fleece following one wash (A) 415 nm excitation viewed through yellow goggles and (B) 450 nm excitation, viewed through orange goggles.

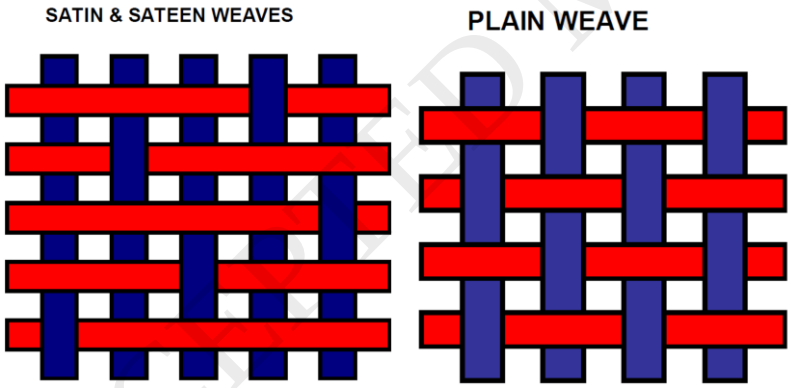

Fig. 2. Weave structures: (a) satin weave and (b) plain weave [19].

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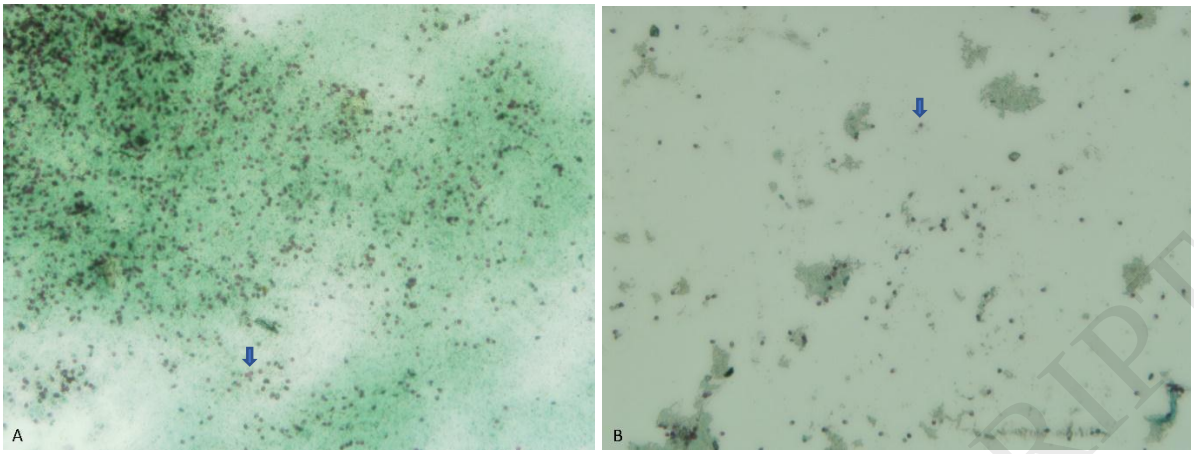

Fig. 3. Microscopic view of spermatozoa (blue arrow) on cotton: (a) control sample (unwashed) (b) sample after 6th wash.

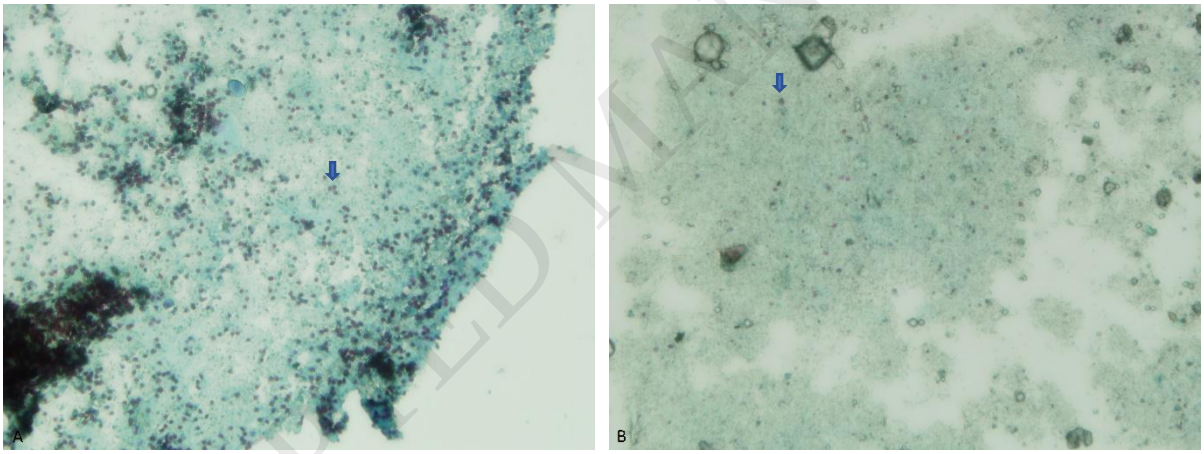

Fig. 4. Microscopic view of spermatozoa (blue arrow) on terry towel: (a) control sample (unwashed) (b) sample after 6th wash.

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Table 1. Scoring method for Acid Phosphatase test

Cut-Off	Result	Score
30 seconds	Strong positive	+++
2 minutes	Positive	++
10 minutes	Weak positive	+
10+ minutes	Negative/Positive result after 10-minute cut-off	-

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Table 2. Scoring method for microscopy.

Spermatozoa Number	Score
Less than 5 spermatozoa per slide	Few
1 spermatozoa in some fields	+1
1-5 spermatozoa in most fields	+2
5-10 spermatozoa in most fields	+3
10+ spermatozoa per field	+4

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Table 3. Fluorescence of seminal fluid on fabrics with various absorption abilities as reported by Kobus (2002), Vandenburg (2006) and within this study.

Study	Light Source	Fabric Type	Fabric Colour	Result-Fluorescence
Kobus (2002)	Polilight PL10	Cotton	White	Strong
		Satin	Pink	Strong
		Polar Fleece	Pink	Weak
Vandenburg (2006)	Polilight PL500	Nylon	Pink	Strong
		Cotton	Red	Strong
		Cotton	Pink Polka Dot	Strong
		Velour	Blue	Strong
		Polar Fleece	Dark Green	Strong
		Nylon	Not Stated	Strong
This Study (2017)	Polilight-Flare II Plus	Cotton	Black	Strong
		Terry Towel	Black	Strong
		Satin	Black	Strong
		Nylon	Black	Strong
		Polar Fleece	Black	Strong
		Lace	Black	Strong

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Detection Method	Fluorescence						Acid Phosphatase						Microscopy					
Fabric Type	Cotton	Terry Towel	Satin	Nylon	Polar Fleece	Lace	Cotton	Terry Towel	Satin	Nylon	Polar Fleece	Lace	Cotton	Terry Towel	Satin	Nylon	Polar Fleece	Lace
Control A	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+4T	+4T	+4T	+4T	+4T	+4T
Control B	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+4T	+4T	+4T	+4T	+4T	+4T
Wash 1A	+	+	-	-	+	-	+	-	-	-	-	-	+4T	+4T	+4	+2	Few	Few
Wash 1B	+	+	-	-	+	-	+	-	-	-	-	-	+4T	+4T	+4	+2	Few	Few
Wash 2A	-	-	-	-	-	-	-	-	-	-	-	-	+4	+4	+4	-	-	-
Wash 2B	-	-	-	-	-	-	-	-	-	-	-	-	+4	+4	+4	-	-	-
Wash 3A	-	-	-	-	-	-	-	-	-	-	-	-	+2	+4	+1	-	-	-
Wash 3B	-	-	-	-	-	-	-	-	-	-	-	-	+3	+4	Few	-	-	-
Wash 4A	-	-	-	-	-	-	-	-	-	-	-	-	+2	+4	-	-	-	-
Wash 4B	-	-	-	-	-	-	-	-	-	-	-	-	+2	+4	-	-	-	-
Wash 5A	-	-	-	-	-	-	-	-	-	-	-	-	+4	+4	-	-	-	-
Wash 5B	-	-	-	-	-	-	-	-	-	-	-	-	+4	+4	-	-	-	-
Wash 6A	-	-	-	-	-	-	-	-	-	-	-	-	+4	+4	-	-	-	-
Wash 6B	-	-	-	-	-	-	-	-	-	-	-	-	+4	+4	-	-	-	-

Table 4. Effects of washing on detection of semen and spermatozoa using three different detection methods.

Fluorescence: strong positive (++), weak positive (+) or negative (-).

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

Sperm Density: "few" = less than 5 spermatozoa per slide, "+1" = 1 spermatozoa in some fields, "+2" = 1-5 spermatozoa in most fields, "+3" = 5-10 spermatozoa in most fields, "+4" = more than 10 spermatozoa per field. Fields of view that contained spermatozoa with a tail were recorded as T