



RESEARCH PAPER

Detectability of seminal stains on fabrics after various washing steps

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Background and aims: Biological evidence that can help in individualization by DNA profiling is considered crucial evidence in a court of law and helps in the timely delivery of justice. In sexual assault cases, the evidence having the utmost importance is detecting semen in the genital swab or on the victim's clothes. However, semen stains may be washed before seizure by investigating agencies, and some semen can remain as evidence even after washings. This study aims to find the most suitable methods for detecting semen stains after various washing steps. **Material and methods:** White cotton fabric pieces were stained uniformly with semen and air-dried for ten days. The stains were washed in two batches, i.e., with detergent and without detergent, for different time intervals. The screening, UV examination, acid phosphatase test, Florence microcrystal test, and Barberio microcrystal test were used. For the confirmation, a prostate-specific antigen (PSA) test and microscopic examination were used. **Results:** UV Examination gave positive results up to moderate wash, and acid-phosphatase gave a positive result in all washes when done with water only on the contrary with detergent, it gave result only in the soft wash. In the confirmatory test, the PSA Test showed high sensitivity showed positive results in all washings, whereas in the microscopic examination, sperms/sperm heads could be detected in all washed stains, but with detergent the presence of sperm was limited up to the soft wash only. **Conclusion:** Washings of semen-stained fabrics with detergent significantly reduced the possibility of detecting semen and sperms for almost all tests conducted, compared to fabrics washed without detergent. PSA test showed distinguishable results even after several steps of washings. Thus the test of choice for the detection of semen on washed fabrics is PSA.

Keywords: Semen stains; washing; acid phosphatase; microcrystal tests; PSA; microscopic examination.

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INTRODUCTION

Sexual violence is one of the most prevalent crimes across the world for both children and adults. Prevalence is thought to be much higher than the reported and published figures. In sexual assault cases, the detection of semen is the first and crucial step for further investigation. Often the investigators get a trace amount of semen in the exhibits, which may be due to the stains on the clothes are washed out,¹ or due to delayed collection of vaginal swabs. First step

for detecting seminal stains on the fabrics is presumptive test, followed by confirmatory tests.^{1,2} There are very limited studies showing the comparative performance of various tests on seminal stain after washing. This study aimed to compare and identify a suitable method for the detection of seminal stains on fabrics after various washings.

Undiluted semen has very strong photoluminescence with a broad excitation spectrum ranging from 350nm-500nm wavelengths.³ When visualized under alternate light source

(ALS), due to the conjugated proteins Flavin and Choline, dried semen stains will fluoresce.³ It gives Blue fluorescence on the exposure to UV light (350-500nm), which can help in its detection on fabric.⁴ The acid-phosphatase test is one of the most commonly used presumptive tests that use alpha-naphthol phosphate and fast blue B reagents, which test for seminal acid phosphatase in the stains. Acid Phosphatase catalyzes the hydrolysis of various phosphate esters to remove phosphate and the alcoholic group from the substrate and forms an insoluble coloured precipitate with stable diazonium salts (Zinc double salts).^{5,6} In the Florence crystal test, the extracted stain or a piece of stain mixed with Florence reagent (Potassium Iodide+Iodine+ Distilled water),^{7,8} brown coloured needle or rod-shaped choline iodide crystals, confirms the presence of semen on the fabric.⁹ Another useful crystal test is the Barberio crystal test, in which spermine reacts with an aqueous or alcoholic saturated picric acid solution; it forms characteristic yellow needle-shaped crystals of spermine picrate. Both spermine and choline are constituents of seminal fluid which can be detected even when a person is aspermic. Barberio test is considered more reliable than the Florence test as it shows fewer false-negative tests than the Florence test.¹⁰

Microscopic examination for the detection of sperms is considered a confirmatory test for the identification of semen. The most commonly used method for sperm staining is Christmas tree stain; some other stains also in use are aniline blue and eosin,¹¹ hematoxylin, and eosin. Being the most useful confirmatory method, it also has one demerit that sometimes a person can be aspermic or azoospermic to natural or clinical conditions, which is why other chemicals and microcrystal methods are employed. Prostate-Specific Antigen (PSA) test is another popular confirmatory test for the detection of semen. The semen from azoospermic males will still contain this antigen which is present in the seminal plasma. An essential aspect of detecting PSA involves detecting it on contaminated or scarce samples, including laundered fabrics and decomposed cadavers.¹ The one-step ABA card and Cancheck PSA kit are some of the commonly used commercial PSA test kits.

It is observed that sometimes, garments with semen stains are already washed before seizure by investigating agencies. The reasons behind such washings may be intentional by criminals to destroy evidence or by mistake by the victim who is not aware of the significance of such evidence. However, it is expected that some semen remains as evidence even after washings. Therefore this study was initiated to find the most suitable methods for detecting semen stains on fabrics after various washing steps.

MATERIAL AND METHODS

Collection of semen samples and ethics:

Semen samples were collected from the sperm bank of 'Mayo Test Tube Baby and Endoscopy Centre, Bhopal,' ethical

guidelines were followed with informed consent from the volunteers.

Preparation of seminal stain samples:

Collected semen was uniformly spread on cotton fabric cut in square pieces. After spreading semen, cloth pieces were allowed to air-dry for about ten days in a cleanroom. A total of eight stain samples were prepared on cotton fabric. The eight samples included one positive control, one negative control, and the remaining six samples for six different types of washings. After the samples were prepared, each sample was stored separately and was denoted from samples A to F. After sampling; the washing setup was categorized as washing with water only (sample A, B & C) and washing with detergent and water (sample D, E & F). The unwashed sample was taken as a positive control, and the unstained sample was taken as a negative control. Washing was categorized into three types- soft, moderate, and hard based on the wash cycle. Based on the washing time interval, the samples were subcategorized as A1, A2, B1, B2, and so on. Washing was done by simulating the wash cycle of a machine by taking cloth pieces in 50ml tubes and mixing them in a vortex mixer in respective time intervals. After washing, the samples were air-dried and subjected to analysis with different methods of detection.

Tests for the Detection of Seminal Stains:

Acid Phosphatase Test: Buffer was prepared by mixing 5ml glacial acetic acid and 10g of sodium acetate, and volume was made up to 500ml. Then 0.63g of sodium- α -naphthyl phosphate (0.25%) was added in 250ml buffer and 1.25g naphthyl diazo blue B(0.5%) was added to 250ml buffer and mixed thoroughly. To test the method, we took pieces of all stain samples, i.e., negative control, washed samples, and two different control samples washed at different intensities and placed on a piece of filter paper. After that, 1 or 2 drops of reagent-1, i.e., sodium- α -naphthyl phosphate in buffer was added and allowed to soak for few seconds; then few drops of reagent-2, i.e., naphthyl-diazo-blue-B in buffer, were added.¹¹ If the purple colour appeared within 1min, then the presence of semen is confirmed, and if the colour appears after one minute, then it is considered to be due to non-prostate acid phosphatase.⁸

Barberio Test: Extract was prepared from stains with dilute HCL, after which a drop of the extract was placed on the microscopic slide, and the saturated aqueous or alcoholic picric acid solution was added and covered with a coverslip. It was observed under a microscope for the detection of the appearance of spermine picrate crystals.¹²

Florence Test: 1.65g potassium iodide and 2.54g iodine were added in 30ml distilled water. The stains were extracted with normal distilled water or dilute HCL in 1.5ml microtubes for about 1-2 hours. Then the extract of semen stain was placed on a microscopic slide, and a drop of Florence reagent was

added, covered with a coverslip, and analyzed under a compound microscope to detect the crystals.^{12,13}

Microscopic Examination: Aniline blue (1g aniline blue in 10 ml phenol (1% aq) +30 ml distilled water) and eosin yellow (1g in 100ml distilled water) was prepared and mixed to prepare the final stain. We prepared extracts of sample stains in saline (8.5g of NaCl in 1000ml distilled water) by keeping

overnight. Then these extracts were smeared on the microscopic slides, and the smears were allowed to dry. Smears were air-dried, kept in the ether: ethanol (1:1) overnight for fixation. After fixation, slides were air-dried and stained with dye and analyzed for sperm under microscope.¹¹

Table 1 Results of various tests for detection of semen/sperms after different washings with detergent

SI No	Washing Time	After washing with detergent and water						
		Samples	UV light Examination	AP Test	Barberio Crystal Test	Florence Crystal Test	PSA Test	Microscopic Examination of sperms/ Sperm head
1	5mins	A1	++	+++	++++	++++	++++	+++
2	10mins	A2	++	++	++	+++	+++	++
3	15mins	B1	+	+	++	+++	+++	-
4	20mins	B2	-	-	+	++	++	-
5	25mins	C1	-	-	+	+	++	-
6	30mins	C2	-	-	+	+	+	-
7	Unwashed	Positive control	++++	++++	++++	++++	++++	++++
8	Unwashed	Negative control	-	-	-	-	-	-

Grading: +++++ Very strong positive, +++ Strong positive, ++ Mild positive, + Weak positive, - Negative

Table 2 Results of various tests for detection of semen after different washings without detergent

S.No	Washing Time	After washing with water only (without detergent)						
		Samples	UV light Examination	AP Test	Barberio Crystal Test	Florence Crystal Test	PSA Test	Microscopic Examination of sperms/ Sperm head
1	5 min	D1	+++	+++	+++	+++	++++	+++
2	10 min	D2	++	+++	+++	+++	+++	++
3	15 min	E1	++	+++	++	+++	+++	++
4	20 min	E2	+	++	+	++	++	+
5	25 min	F1	-	++	+	+	++	+
6	30 min	F2	-	++	-	-	+	-
7	Unwashed	Positive control	++++	++++	++++	++++	++++	++++
8	Unwashed	Negative control	-	-	-	-	-	-

Grading: +++++ Very strong positive, +++ Strong positive, ++ Mild positive, + Weak positive, - Negative

RESULTS

The efficiency of UV light to detect washed semen stains was observed after each wash, and the fluorescence intensity of each stain was evaluated using a relative scale and categorized as very strong positive, strong positive, mild positive, weak positive, and negative.⁴ In samples washed with detergent for 5, 10 and 15 mins showed positive results, whereas samples washed for longer than 15 mins gave a negative result (**Table 1**). In washing without detergent, samples gave a positive result up to 20 mins (**Table 2**).

In the acid-phosphatase test, it is observed that semen could be detected even after 30 mins of washing without detergent (**Table 2**). However, semen could not be detected at 20min of washing with detergent (**Table 1**). Results of Barberio and Florence's crystal tests are also displayed in **Table 1** and **Table 2**. All the samples were also tested for the presence of PSA antigen in stain samples, which showed positive results for both types of washings in all conditions tested, as mentioned earlier. Results so microscopic examination for the detection of sperm or sperm head is also tabulated above, which showed that washing with detergent significantly reduces the chances of sperm detection in stains (**Table 1, 2**).

DISCUSSION

In several countries across the world, after the incidence of sexual offenses, it can be kept hidden for a long time without reporting due to several reasons, and semen-stained clothes of victims can often be subjected to washings before the case is notified to the investigating agencies.^{14,15} This study has shown the potential of all the methods which are currently being used or have been used previously for the detection of semen. However, when the methods have been used in conditions where washing of the stains has been taken as an important factor, the tests showed limitations in detecting semen. Washing of semen-stained fabrics with detergent reduced the possibility of detection of semen for almost all tests in general, i.e., AP test, PSA test, microcrystal examination, and microscopic examination. The acid phosphatase test gives more reliable and suitable results for detecting semen stains after washings compared to detection under UV light. PSA test proved to be the most robust method as it gave positive results in fabrics after all the washings with & without detergent. The findings of this work are somewhat similar to the observations in a study by Ragne Kristin Farnen et al.¹⁶

CONCLUSION

This study highlighted the sensitivity of the detection methods of semen stains. UV Examination proved to show limitations in detection of fluorescence in both washings (with detergent and without detergent), whereas in Acid phosphatase test proved ineffective in the case of washing with detergent as it only gave results up to moderate wash. PSA showed

promising results in both types of washes. However, in microscopic examination the sperm were visible under a microscope only in washing with water, and in washing with detergent, the sperms were visible only up to soft wash. Hence this study concludes that the most effective method for detecting the seminal stains on fabrics after different washes is the PSA test. This study can contribute in choosing the appropriate method for detection of semen stains when there are chances that a very low amount of semen is present on the fabric and the stains have been washed, or the evidence seized from the crime scene is in less quantity.

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Conflict of interest: None declared.

Ethics considerations: Semen sample was collected from Mayo Test Tube Baby and Endoscopy Centre, Bhopal, with ethical considerations and consent of the semen donors. All data were treated confidentially, and the study was conducted in accordance with the Declaration of Helsinki.

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