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Evaluating the persistence of laundered semen stains on fabric using a forensic light source system, prostate-specific antigen Semiquant test and DNA recovery-profiling

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Abstract

Semen stains on the clothes of victims of sexual assault can remain as evidence even after garments have been laundered. In this study, we aimed to investigate the effectiveness of commonly preferred methods to detect semen stains in two different fabric types that were laundered with different washing machine programmes and washing powders, and to obtain a DNA profile from the semen stains. For this purpose, a comprehensive study was performed on semen-stained underwear using three different methods for stain detection, confirmation and identification: a forensic light source (FLS) system, the prostate-specific antigen (PSA) test and DNA recovery profiling. With FLS applications, stronger fluorescence was achieved in wash protocols performed at a low temperature (30°C) on semen-stained cotton underwear. DNA recovery between 13.45 and 55.00 ng/µl was obtained by modifications in the DNA extraction step when the effect of temperature and washing powder on DNA recovery was evaluated, and these were enough for short tandem repeat (STR) typing in all samples. This study shows that when semen-stained underwear is washed after a month, some semen stains can be determined by FLS and PSA, and all stains can be identified by STR analyses.

Keywords

Laundered semen stains, forensic light source, PSA test, DNA recovery, DNA profiling, STR

Introduction

Sexual violence continues to be a major problem for both children and adults. As a significant number of victims of sexual assault cases do not complain to the judicial authorities,[1,2] the prevalence is thought to be even higher than published figures.[3] In addition, the number of cases that are not reported because of psychological traumas experienced by the victim is very high.[4–6] It was reported in a study evaluating US National Crime Victimization Survey study data that 65% of sexual violence cases were not reported to the police between 2006 and 2010.[7] The results of the same study revealed that sexual crimes constitute the second highest group of crimes that are not reported after theft crimes.[7] Victims of sexual assault are afraid of seeking help for various reasons such as shame and guilt, fear of the perpetrator, social pressure, anxiety that they will not be believed, fear of family members hearing and anxiety of being traumatised again through the judicial process.[8–10] In particular, the perpetrators of child sexual abuse can be individuals within the family, as well as individuals outside the family, and these can be the hardest case groups to identify.[11] Likewise, the cases of female victims living in underdeveloped countries may often remain hidden for long periods of time because the victims are afraid of

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social or familial pressures.[12–14] As is known, the biological samples considered to belong to the perpetrator are obtained from an examination of the victim's genital area after the assault, and the investigations on the clothes constitute the most important evidence group.[15–17] When there is a delay in the collection of biological evidence after sexual assault, the semen stains on the victim's clothing can remain detectable for a long time, especially through DNA analysis.[18,19] In fact, several studies published in recent years have shown that semen stains on clothes can also be detected after laundering.[15,20–22] However, further studies are needed to determine the conditions under which the laundered semen-stained clothing can constitute evidence.

Possible semen stains on the victim's clothing can be determined by using various screening and verification tests in the elucidation of sexual assault cases.[15,16,23,24] In the next step, DNA is isolated, and subsequently, the DNA profile of the attacker can be determined.[15,16,23] In this study, we aimed to investigate the effectiveness of commonly preferred methods in the detection of semen stains on two different fabric types laundered using different washing machine programmes and different washing powders, and to obtain a DNA profile from the semen stain.

Methods

Sampling and transfer to fabric procedure

This study consisted of three replicates of 12 different algorithms of different fabric types and washing programmes. In order to create the semen stain, 36 new undergarments were purchased, half of which were cream-coloured polyester and half white-coloured cotton. The ejaculates were taken from a healthy volunteer donor with a normal sperm count at a specific time interval (at least 48 hours between ejaculations) and stored at +4°C. To determine the location of the semen stains on each undergarment, two circles were drawn with a waterproof pen. In order to ensure the homogeneity of the stock semen sample, the semen was shaken for about 10 minutes at room temperature and then added to the fabric following pipetting for staining. Each undergarment was stained with 750 µl of semen (72 samples in total). Then, the stained underwear was dried at room temperature and stored at room temperature for 1 month. After this, they were washed using different washing machine programmes and different washing powders. In addition, a biological sample (blood) was taken from the analyst who performed the laundry and all laboratory analysis for control purposes, considering the possibility of contamination. This research was approved by the Ethical Committee of the University of Istanbul University-Cerrahpasa, Cerrahpasa Medical Faculty (REC: 604.01.02-A-36) according to the ethical (Declaration of Helsinki) and legal standards in Turkey.

Laundering protocol

The semen-stained underwear was laundered in a domestic washing machine for 60 minutes using a 1000 rpm programme at 30°C or 60°C with three different types of washing powder: biological (Sodasan), non-biological (Alomatik) and non-biological washing powder with stain remover added (Kosla). In the washing process, 60 g of washing powder was used each time, but no fabric softener was used. To avoid the possibility of DNA transfer between the underwear, all undergarments were washed separately in the washing machine and dried at room temperature.

Testing for seminal fluids

Each stain created on each undergarment (36 samples) was investigated with a forensic light source (FLS) system to detect the location of the stain. Then, the semen-stained area was cut out and tested for the presence of prostate-specific antigen (PSA) with a Seratec® PSA Semiquant cassette (cat. no. PSM400F; Seratec®, Göttingen, Germany). The semen-stained area in the second set of underwear (36 samples) was cut out with sterile scissors and used for short tandem repeat (STR) typing after DNA extraction.

Investigation of seminal fluid using a FLS system (Forenscope).

In this study, the detectability of the stain on semen-stained cotton and polyester underwear that was laundered with different washing programmes and washing powders was investigated by a FLS system that can provide light at different wavelengths and using different filters with its own imaging. During the investigations, the Forenscope Mobile Multispectral UV-VIS-IR Imaging System® (Grimed Ltd, Voor, The Netherlands) with Android OS was used. This mobile system records with a 13 MP CMOS sensor which is capable of imaging wavelengths of 330–1080 ± 20 nm. The system's internal camera has a lens with a value of f/2.2 and is capable of continuous autofocus. The system's camera is integrated into a tablet with Android OS. All the images in the tablet are saved in the forensic software as high-resolution images at a resolution of 4160 × 3120. Other light and filter combinations used were as follows: 5.500–6.500 K range white light + visible filter, 360–370 nm range ultraviolet light + custom yellow filter, 405–425 nm range violet light + custom yellow filter, 440–455 nm range blue light + custom yellow filter, 360–370 nm range ultraviolet

light + custom orange filter, 405–425 nm range violet light + custom orange filter, and 440–455 nm range blue light + custom orange filter. All stains were photographed and saved using the device's imaging system. To prevent exterior lighting affecting the images and in order to take the photographs from a standard distance, a specialised dark room headpiece was utilised. The evaluation criteria of the obtained images were defined by the first author as four different categories: negative, weak, moderate and strong.

Immunoassay membrane Semiquant test from semen-stained underwear. The presence of PSA on semen stains was examined with a Seratec® PSA Semiquant test. According to the manufacturer's instructions, the cut underwear pieces were kept in 1 ml of buffer solution for two minutes. About 120 ml of this solution was added to the sample well. At the end of the 10-minute incubation period, positive, weak positive and negative results were determined according to the manufacturer's instructions.

DNA isolation from semen-stained underwear. The semen-stained fabric was cut into 1 cm^2 sections, placed in a sterile tube and incubated with 2 ml of sterile phosphate-buffered saline for 48 hours at room temperature. For DNA isolation, a Quick-DNA™ MiniprepPlus kit (Zymo Research, Irvine, CA) was used, with a minor modification to the protocol. In the first stage of DNA isolation, lysis buffer containing 1 M DL-dithiothreitol (anhydrous m.w.=154.25) was applied instead of proteinase K. Agarose gel electrophoresis and spectrophotometric (Nanodrop, BioSpec) methods were used for DNA integrity and quality evaluation.

DNA profiling. Each amplification was conducted with 1 ng of DNA using AmpFISTR Identifiler Plus kits (Applied Biosystems, Foster City, CA). STR genetic loci were amplified the AmpFISTR Identifiler Plus PCR Amplification kit (Applied Biosystems) on a 96-well GeneAmp1 PCR System 9700 (Applied Biosystems) for 28 cycles. Amplification products were separated by capillary electrophoresis on an ABI PRISM1 3130xl Genetic Analyser (Applied Biosystems), and data were analysed using the ABI PRISM1 GeneMapper1 ID software v.3.2 (Applied Biosystems). In this study, we included 15 autosomal STR markers (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA) and Amelogenin (Applied Biosystems AmpFISTR Identifiler Direct User Guide, 2012).

Statistical analyses

Data from three independent studies of all algorithm results generated by washing conditions were analysed with descriptive statistics using IBM SPSS Statistics v21.0 (IBM Corp., Armonk, NY).

Results

The 72 fabric samples stained on cotton and polyester underwear (light coloured) with semen taken from a single donor were kept at room temperature for 1 month. They were then washed using different washing machine programmes and washing powders. After that, the FLS application, PSA test, recovery of DNA and DNA STR typing were used to analyse the fabric samples.

Table 1. Results of PSA test and FLS application of samples.

Fabric	Washing powder	Washing temperature	Analyses (PSA test) 1	2	3	Analyses (FLS)
Cotton	Biological	30°C	++	++	++	Strong
		60°C	++	++	++	Strong
	Non-biological	30°C	+	+	+	Strong
		60°C	–	–	–	Moderate
	Stain remover	30°C	+	+	+	Strong
		60°C	–	–	–	Moderate
Polyester	Biological	30°C	++	++	++	Weak
		60°C	+	+	+	Negative
	Non-biological	30°C	+	+	+	Weak
		60°C	–	–	–	Negative
	Stain remover	30°C	+	+	+	Weak
		60°C	–	–	–	Negative

'++', '+' and '-' indicate positive, weak positive and negative results for prostate-specific antigen, respectively.

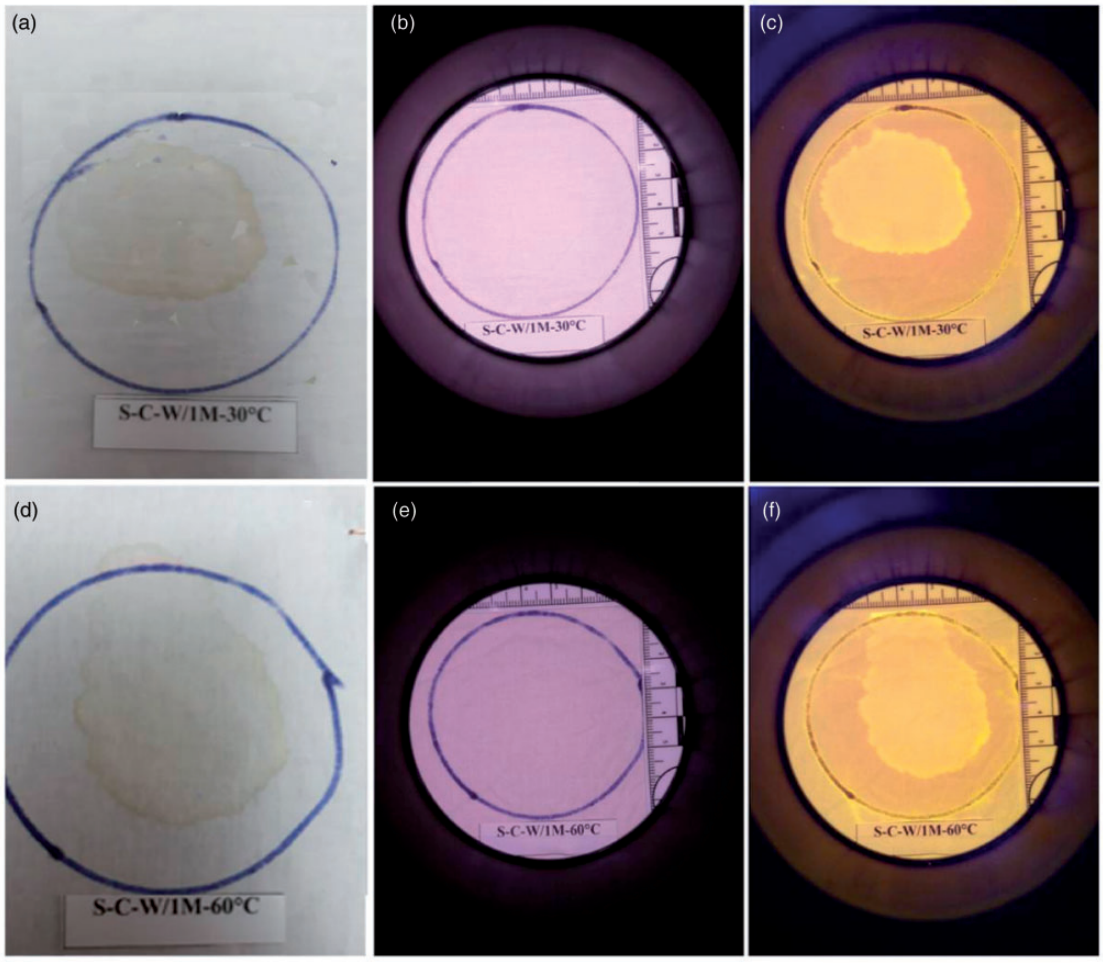


Figure 1. Semen stains on white underwear. (a) Normal view. (b) Laundered at 30°C (viewed with white light+visible filter). (c) Laundered at 30°C (viewed with blue light 445 nm with a custom orange filter). (d) Normal view. (e) Laundered at 60°C (viewed with white light+visible filter). (f) Laundered at 60°C (viewed with blue light 445 nm with a custom orange filter).

FLS testing

The best images were taken with a 13 MP CMOS sensor, under a 440–455 nm range blue light positioned in a circular orientation and filtered with a custom orange filter specific for the camera. Strong fluorescence was obtained in the examination of the FLS after washing the semen-stained cotton underwear at 30°C. Similarly, strong or moderate fluorescence was observed after washing at 60°C, depending on the washing powder used (Table 1 and Figure 1). In contrast, weak fluorescence was observed after washing the polyester underwear at 30°C, and no fluorescence was obtained in any examinations after washing at 60°C (Table 1).

Immunoassay membrane Semiquant test

Strong positive results were obtained with the PSA Semiquant test after the semen-stained cotton underwear was laundered with the biological washing powder at both temperatures (Table 1). The PSA Semiquant test showed weak positive results after washing cotton underwear at 30°C with both non-biological washing powder and stain remover, but it did not give any positive result after washing at 60°C (Table 1).

Washing the semen-stained polyester underwear with biological washing powder at 30°C resulted in strong positive PSA Semiquant tests. However, when the temperature was increased to 60°C, the results were weak positive. On the other hand, after washing at 60°C with both non-biological powder and stain remover, the PSA Semiquant test result for the polyester underwear was negative.

DNA recovery

After laundering all types of semen-stained fabrics, the best DNA recovery results were obtained from the

Table 2. DNA recovery results (ng/μl) for different washing conditions of semen-stained cotton and polyester underwear.

		Polyester			Cotton		
Washing powder	Washing temperature	*n*	*M*	*SD*	*n*	*M*	*SD*
Biological	30°C	3	13.45	2.5	3	19.86	2.5
	60°C	3	26.99	4.4	3	13.74	1.1
Non-biological	30°C	3	16.32	2.7	3	21.47	2.9
	60°C	3	25.86	3.6	3	15.05	2.5
Stain remover	30°C	3	32.26	5.8	3	24.53	2.2
	60°C	3	55.00	6	3	22.38	2.0

	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
D	12,13	31.2, 32.2	7,10	11,12	14,15.2	7,8	11,12	8,11	17,20	10.2, 13.2	15.2,16	7,9	13.2, 18.2	X,Y	11,12	19,19

Figure 2. Short tandem repeat typing of the donor semen sample.

polyester underwear (55.00 ng/μl; Table 2). The DNA recovery studies on semen-stained polyester underwear showed that better results were obtained with the 60°C washing programme compared with the 30°C washing programme with all types of washing powder. In contrast to this, the DNA recovery for the cotton underwear was higher when washing at 30°C than when washing at 60°C with all types of washing powders studied (Table 2).

According to the evaluation of DNA recovery according to washing powder type, the best results were obtained with the stain remover, non-biological washing powder and biological washing powder, respectively (Table 2). When washing the underwear with biological and non-biological washing powder at a low temperature (30°C), the DNA recovery for cotton underwear was found to be higher than that for the polyester fabric. In the DNA recovery studies of underwear laundered at a high temperature (60°C), the result for the polyester underwear was better than for the cotton underwear. When the fabrics were laundered with stain removers at both temperatures, DNA recovery for the polyester fabric was better than for the cotton fabric.

STR typing

The DNA recovery amounts obtained after laundering under different conditions were between 13.45 and 55.00 ng/μl, and these amounts and DNA quality were enough for STR typing. In the DNA isolates obtained from all laundered semen-stained underwear, DNA profiles were obtained that matched the donor and originated from a single source (Figure 2).

Discussion

In many countries throughout the world, after sexual crimes occur, they can be kept hidden for long periods of time for various reasons, and biological evidence on clothing may not be available. This evidence can be extremely important in the decision-making process of judicial authorities. However, the semen-stained clothes of victims can often be subjected to washing in the period before the case is notified to the judicial authorities. This study aimed to reveal the difficulties that can be encountered in all laboratory stages, from the determination of the stain in the laundered semen-stained underwear to the acquisition of the DNA profile. This study was designed for this purpose with three replicates of 12 algorithms that were designed as the experimental model on different samples formed under the same conditions. In this study, the use of a single donor for experiments and analyses introduced some limitations.

FLS is an important method used in the detection of biological stains, which is an important group of evidence after sexual assault.[25] Doubtlessly, the effective use of FLS in determining the location of semen stains, especially in laundered underwear, may vary, depending on several factors such as the type of fabric, storage time and sample quantity.[17] Although there were numerous studies on different types of fabrics and different FLS systems used on different surfaces,[26–28] the studies of FLS on laundered semen-stained fabrics are very limited.[16,22] This study showed that FLS can still be used effectively for the detection of stains on washed semen-stained cotton underwear. Similarly, Noel and colleagues demonstrated that even after

laundering white cotton fabric several times, semen stains could be detected using FLS.[22] In contrast to this, Schlagetter and colleagues reported that they obtained weak fluorescence on cotton fabrics after wet and air-dried semen stains upon both hot and cold laundry protocols and that semen stains on polyester fabrics were not fluorescent.[17] The difference between the results of this study and other studies is thought to be related to the deposition time of the semen stains before washing. In the present study, the stains were kept for 1 month before washing, while Schlagetter did not perform any waiting protocol before the wash protocol.[17] In order to obtain detailed conclusions related to this subject, more extensive studies are needed on different types and colours of fabrics, as well as stains with different storage times.

Although the acid phosphatase (AP) test is a frequently used method in detecting semen on stained underwear, in this study, the PSA test was used instead of the AP test because it was reported to give negative results on washed semen stains in the literature.[24] The PSA Semiquant test that is based on the immunological principle is known to work at 10^5 dilutions on unwashed semen stains[29] and at 10^3 dilutions on washed semen stains.[30] The results of this study demonstrated that the laundering temperature and the type of washing powder used have a significant effect on PSA activity. All the tests conducted after laundering at a low temperature (30°C) were positive for PSA. Similarly, the best PSA test results were obtained after laundering the semen-stained underwear with biological washing powder compared with other types of washing powders. In addition, the PSA tests gave more positive results on laundered cotton underwear compared with laundered polyester underwear (Table 1). Similarly, the study by Jobin and colleagues found that semen stains on cotton fabric were positive and those on nylon fabric were negative.[16] Both semen-stained cotton and polyester underwear gave positive results with the PSA test only after laundering with biological washing powder at 60°C. A laundering temperature of 60°C is thought to be more effective when non-biological washing powder and stain remover are used. Likewise, Laffan and colleagues reported that the PSA test results were weakly positive when garments were laundered at 90°C without a waiting time[30] (Quick-DNA™ MiniprepPlus; Zymo Research).

Microscopic examination results are known to be inadequate when the semen-stained underwear is kept for more than 2 weeks due to the degradation of the sperm cells.[24] Thus, direct DNA isolation and STR typing on semen-stained laundered clothes were used in this study. The analyses conducted for this purpose show that the DNA recovery was between 13.45 and 55.00 ng/μl with the DNA Extraction Kit (Quick-DNA™ MiniprepPlus Kit; Zymo Research). With small modifications in the kit protocol, the DNA amount obtained was increased, and the impurities in the DNA samples were removed. So, DNA recovery was enhanced. Similarly, in the study by Farmen and colleagues which compared different extraction methods on semen-stained fabrics laundered after being kept for 24 hours, an average of 0.038 ng/ml DNA recovery could be obtained with the Chelex method and an average of 0.036 ng/ml of DNA recovery with the EZ1 method.[24] On the other hand, in a study conducted by Morris (6–18 μg), where semen-stained fabrics were kept for 8 months, the DNA recovery efficiency was much higher than that of Farmen (0.036 ng/m) and our study (13.45–55 ng/ml).[15,24] The reason for this is thought to be the storage period of the semen-stained fabrics (Table 3). The reason for the lower recovery of DNA compared with Morris's study is thought to be related to the storage time of the semen-stained fabrics in this study (Table 3).[15,24] As stated in Morris and colleagues' study, high amounts of DNA recovery were obtained, regardless of washing temperature, washing powder and fabric type.[15] This result is also confirmed in the present study. However, when the studies on this subject are compared, it can be seen that there are differences between the results (Table 3).[15] Considering the washing temperature, DNA recovery from stained cotton underwear was higher at low temperatures than other temperatures, as indicated in Farmen's study.[24] In contrast to this, a higher yield of DNA recovery from polyester fabrics was achieved at a high temperature (60°C; Table 2). Similarly, Nussbaumer reported that laundering protocols at high temperatures led to a higher DNA recovery yield and stated that the DNA recovery at 60°C is nearly twice as high as the yield at 40°C.[31] However, in the study conducted by Nussbaumer, the type of fabric containing the stain was not specified. It should not be ignored that the difference in DNA recovery between cotton and polyester as related to temperature increase may be caused by the type of fabric as well as the weaving frequency of the fabrics. Therefore, there is a need for studies where the weaving frequency of fabrics is also taken into consideration. In the laundering protocols at the same temperature for different samples in this study, DNA recovery on the cotton samples was achieved at a lower yield by the laundering protocols conducted with biological washing powder compared with non-biological washing powder, as reported in the study by Morris and colleagues.[15] However, unlike the work by Morris and colleagues, DNA recovery from polyester underwear was obtained at lower yields with biological washing powder compared with non-biological washing

Table 3. Forensic studies performed with laundered semen stains.

Forensic studies	Morris et al.[15]	Farmen et al.[24]	Jobin et al.[16]	Crowe et al.[23]	This study
Amount of semen	1 ml (single donor); 2 ml (two donors)	0.5 ml	0.190 ml	0.5 ml	0.75 ml
Washing machine programme	30°C and 60°C	40°C and 60°C	Warm water wash, cold water rinse	18°C and 30°C	30°C and 60°C
Washing powder type	Biological and non-biological	Non-biological and non-biological+fabric softener	Concentrate	Washing powder	Biological, non-biological and non-biological+ stain remover
Amount of washing powder	Tablet and capsule	Indefinite	60 ml	About 65 g	About 60 g
Washing machine	Domestic washing machine	Domestic washing machine	Commercial clothes washers	Domestic washing machine	Domestic washing machine
Semen stain storage time	8 months	1 day	Overnight	Only drying	1 month
Fabric types	Cotton, polyester and nylon	Cotton	Cotton and nylon	Cotton	Cotton and polyester
Clothing types	Tights, T-shirt and trousers	Underwear	Underpants and gusset	T-shirt	Underwear
Number of washes	1 and 2	1	1	1	1
Washing time	90 minutes	80 minutes for 40°C and 90 minutes for 60°C	–	–	60 minutes
Washing machine spin cycle (rpm)	1200	700	–	–	1000
Number of semen donors	2	5	3	1	1
DNA extraction method	EZ1	EZ1 and Chelex	Phenol chloroform with purification	Standard organic extraction	Quick-DNA Miniprep Plus
STR genetic loci	PowerPlex1 ESI 16	None	AmpFiSTR	The CTT triplex (CSF1PO, TPOX and THO1)	AmpFiSTR
DNA quantity	6–18 μg	0.038 ng/μl with Chelex; 0.036 ng/μl with EZ1	–	0.1–13 ng/μl	13.45–55.00 ng/μl
Semen tests	DNA recovery and STR typing	DNA recovery AP and PSA test	AP: fast blue; PSA test; microscopy DNA recovery and STR typing	AP: fast blue; microscopy DNA recovery and STR typing	FLS, PSA test, DNA recovery and STR typing

–: not stated in the literature; STR: short tandem repeat; AP: acid phosphatase.

powder. It is thought that this difference may be related to the storage time of the semen stains before washing and to the type of fabric (Table 3).

In terms of stain remover, DNA recovery has been found to be more effective for both cotton and polyester underwear than biological and non-biological washing powders. This may mean that the use of stain remover on semen stains is no more effective than washing powder alone. There are many stain removers on the market known to have an effect on different stains. The stain remover used in this study was not very effective at removing semen stains. In addition, this study is the first in the literature where the semen stains were washed with stain removers.

Conclusions

The present study is comprehensive, and it evaluates the process from the determination of stain localisation to the individualisation stage with the use of different laundered temperatures, different washing powders and different semen-stained fabrics. This study confirmed the knowledge that the amount of DNA recovery obtained from laundered semen-stained clothes varies depending on the type of washing powder, laundering temperature and fabric type. It has been shown that after applying different washing protocols on the formed stains, some semen stains can still be detected by the FLS system and confirmed by the PSA Semiquant test. In addition, a sufficient amount and quality of DNA were achieved for STR analysis in all analyses performed on the stains. Based on the washing temperature, fabric type and detergent type, it is possible to detect semen stains with FLS systems, confirm them with PSA testing and influence the amount of DNA recovery. It is very difficult to locate semen stains with FLS systems on fabrics washed at especially high temperatures. Likewise, it is problematic to obtain results on polyester fabrics with the PSA test. However, an STR profile was obtained from all samples, even those that could not be detected by the screening or confirmation methods.

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