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POSSIBILIDADES DE IDENTIFICAÇÃO DE MANCHA DO SÊMEN APÓS A LAVAGEM DE ROUPAS E ROUPAS DE CAMA EM CASOS DE INVESTIGAÇÃO DE ABUSO SEXUAL

POSSIBILITIES OF SEMEN STAIN IDENTIFICATION AFTER CLOTHING AND BEDDING WASHING IN INVESTIGATING CASES OF SEXUAL ASSAULT

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RESUMO

Identificar manchas de sêmen em roupas e roupas de cama é um componente crucial na investigação de casos de agressão sexual. Em alguns casos, roupas e roupas de cama já foram lavadas antes de serem removidas e enviadas para exame forense. Não há dados suficientes sobre as melhores práticas para lidar com vestígios de sêmen nas roupas após a lavagem. O objetivo desse trabalho foi estudar a possibilidade de identificar vestígios de sêmen nas roupas após a lavagem usando técnicas amplamente utilizadas. Para simular evidências físicas típicas, amostras de sêmen de doadores foram aplicadas a peças de roupas feitas de vários tecidos. As roupas foram lavadas sob várias condições (com a ajuda de agentes não biológicos e biológicos (contendo enzimas) e em várias máquinas de lavar). Após a lavagem, as manchas de lavagem foram caracterizadas pela presença de sinal de fluorescência, espermatozóides (método Koren - Stokis), determinação de fosfatase ácida, antígeno prostático específico e semenogelina, além de resultados de pesquisas sorológicas de acordo com o sistema AB0 e Análise de DNA. A análise de roupas usando esses métodos mostrou-se eficaz em condições experimentais. No entanto, a presença de enzimas como componentes detergentes projetados para destruir manchas biogênicas afeta significativamente os resultados da identificação de manchas de sêmen. Foi estabelecido que o perfil genético completo pode ser obtido a partir de manchas de sêmen, mesmo após lavar três vezes. São necessárias estratégias diferentes para detectar, selecionar e identificar manchas de sêmen, dependendo das circunstâncias de um caso. Recomenda-se examinar roupas e roupas de cama, mesmo que as amostras tenham sido previamente lavadas.

Palavras-chave: *medicina forense, abuso sexual, identificação de manchas de sêmen, lavagem, perfil genético*.

ABSTRACT

Identifying semen stains on clothing and bedding is a crucial component in investigating cases of sexual assault. In some cases, clothing and bedding have already been washed before they were removed and sent for forensic examination. There is insufficient data on best practices for handling traces of semen on clothes after washing. This work aimed to study the possibility of identifying traces of semen on clothes after washing using widely used techniques. To simulate typical physical evidence, donor semen samples were applied to pieces of clothing made from various fabrics. The clothes were washed under multiple conditions (with the help of non-biological and biological (enzyme-containing) agents, and across numerous washing machines). After washing, the washing stains were characterized by the presence of fluorescence signal, spermatozoa (Koren – Stokis method), the determination of acid phosphatase, prostate-specific antigen and semenogelin, as well as the results of serological research according to the AB0 system and DNA analysis. Clothing analysis using these methods was shown to be effective in experimental conditions. However, the presence of enzymes as detergent components designed to destroy biogenic stains significantly affect the results of the identification of semen stains. It has been established that the full genetic profile can be obtained from semen stains even after washing three times. Different strategies are needed to detect, select and identify semen stains depending on the circumstances of a case. It is recommended to examine clothing and bedding, even if the specimens were previously washed.

Keywords: *forensic medicine, sexual abuse, identification of semen stains, washing, genetic profile.*

1. INTRODUCTION:

Identification of traces of a crime with the use of physical evidence, in particular, biological fluids such as blood and semen, is an essential point in forensic examination and is often the key in criminal investigations. Subsequently, the findings are used as evidence in court (Rainn.org., 2014). Sometimes traces of sexual violence are present in small quantities or mixtures or have been previously destroyed, so their identification is often tricky (Martínez et al., 2015). Currently, amid a general increase in the number of sexual assaults, especially against children, the number of targeted removal of crime evidence by criminals has increased. The use of such simple measures as washing in an automatic washing machine with the addition of laundry detergent leads to difficulties in the subsequent identification of traces of the crime. The use of enzyme-containing synthetic detergents further complicates this task and forces experts to improve forensic techniques and expand the range of research methods used (Galitsky, Mussabekova, 2007; Hofmann et al. 2018). Enzymes are designed to destroy stains of biological origin, including group-specific antigens and DNA structure. Detergents containing enzymes remove stains faster than other detergents and are very useful in washing at low temperatures (Gurtovaya, 2007).

Serological, immunological, and molecular genetic methods are traditionally used to identify traces of semen, including micro traces of biological origin (Harbison, Fleming, 2016). Development of molecular biology methods provides an improvement in genetic analysis methods and expands expert capabilities (Harbison, Fleming, 2016; SWGDAM Recommendations for the Efficient DNA Processing Of Sexual Assault Evidence Kits Scientific Working Group on DNA Analysis Methods, 2016) since genetic material (DNA) is a stable biological structure and retains its the ability to be tested for three years (Karni et al., 2013). In some cases, biogenic traces on bedding or clothes which can prove the fact of sexual violence are preliminarily and purposefully destroyed by washing, which leads to a decrease in the amount of DNA available for forensic examination (Brayley-Morris et al., 2015). Clothing after wash is often not investigated since it is assumed that prolonged storage of physical evidence also leads to the impossibility of detecting traces of semen and their subsequent identification (Forensic evidence collection in sexual assault & rape, 2014). However, in recent years, the possibilities of genetic analysis have improved significantly, and studies have shown that a small amount of DNA is required to obtain a full genetic profile from semen stains efficiently. This analysis is feasible despite several preliminary washes of clothing (Rainn.org., 2014; Harbison, Fleming, 2016; SWGDAM Recommendations for the Efficient DNA Processing Of Sexual Assault Evidence Kits Scientific Working Group on DNA Analysis Methods, 2016; Brayley-Morris et al. 2015, Farmen 2008). The empirical studies published have shown that biogenic stains can persist on pieces of clothing after washing in a washing machine using various washing programs, detergents and temperatures (Galitsky, F. Mussabekova, 2007; Hofmann et al. 2018; Gurtovaya, 2007; Brayley-Morris, 2015; Schlagetter, Glynn, 2012), and that DNA profiles can be obtained by examining traces on washed pieces of clothing, and bedding (Farmen, 2008; Schlagetter, Glynn 2012; Jobin, De Gouffe, 2003; Nussbaumer et al., 2003; Edler et al., 2017; Kulstein, Wiegand, 2018) However, not enough information has been published about the possibilities of using traditional methods to study semen stains after washing.

The purpose of this work was to study the influence of modern synthetic detergents on semen stains, as well as to evaluate the effectiveness of using various traditional forensic medical research methods and the possibility of carrying out a molecular genetic examination in the study of physical evidence (clothing, bedding) after washing. The results of such experimental studies can be used as the basis for the development of forensic protocols

in the investigation of specific types of crimes (Rainn.org., 2014; SWGDAM Recommendations for the Efficient DNA Processing Of Sexual Assault Evidence Kits Scientific Working Group on DNA Analysis Methods, 2016).

2. MATERIALS AND METHODS:

2.1. Sample preparation

To simulate a typical physical evidence, semen samples were used (for a period not exceeding 1 hour) from 12 donors who did not take further part in the experiment. All donors were anonymous, and samples were labeled with letters A–M. The material was taken following the rules adopted by the Ethical Commission of KSMU. In all cases, written informed consent was obtained from semen donors. The research bioethics committee approved the study of the National Research Ethics Service (Protocol No. 56, dated December 21, 2017) for the use of human tissues. Donor ejaculate was preliminarily checked for semen. To create the experimental samples listed in Table 1, several ejaculates of each donor were used.

Therefore, to ensure consistency, the ejaculates of each donor were initially pooled to obtain a stock solution before applying it on the pieces of clothing. Semen was placed on various items of new children's school clothes from different fabrics: cotton (T-shirts, 100% cotton), semi-synthetic (blouses, gabardine), synthetic (tights, 100% nylon), wool (pants, wool 90%, viscose 10%), wool blend (socks, 70% wool, 20% cotton, 10% Coolmax) and blended (shirts, cotton 65%, polyester 30%, elastane 5%). Before the experiment, all clothes were previously washed twice in a washing machine and dried to remove the surface treatment of the fabric and allow the semen to be absorbed deeper into the fabric. All applied semen stains were dried for at least 24 hours before washing. In some cases, 1 ml of semen from different donors was applied to clothing, and 2 ml of semen were combined as a 1:1 mixture of semen from two donors. The first applied stain of one donor was dried, and then the second semen was additionally applied to it to imitate traces of semen from two different sources. After applying the stains, clothes have been dried for 48 hours at room temperature. Prepared experimental samples of clothing with semen stains were stored individually in paper bags at room temperature in a dark room. Tables 1a, 1b and 1c summarize the main parameters and results of the study.

Before the first wash, all clothes were examined using an alternative light source (Forensic Light Source – Mini-Crimescope Advance) at a wavelength of CSS (532 nm) using an orange filter. To indicate the position of semen stains and provision of the subsequent accuracy of sampling for the study, the location of semen stains on clothes was marked by a water-insoluble pen.

2.2. Washing conditions

For the research, three brands of household automatic washing machines with different types of loading were used (top-loading 1: Whirlpool TDLR 70220; frontal loading 1: Bosch WLG 2416 M; frontal loading 2: Siemens WS12T540OE). The standard wash mode lasting 90 min (~ 30 ° C) and the spin cycle 1200 rpm were used; the quantity of detergent was assigned according to the manufacturer's recommendations. Three brands of regionally popular synthetic detergents were used for washing: biological (enzyme-containing) liquid detergent Persil® Power-Liquid ®, enzyme-containing detergent Tide Original and non-biological washing powder ECOVER ZERO NON-BIO. Fabric conditioners were not used in experimental washes. After washing, clothes were dried for 12 hours at room temperature and stored individually in paper bags until the next wash. Clothes # 19-23 without applied stains (clean) were also washed with clothes having semen stains to study the possibility of transferring DNA from clothes with semen stains to other clothes inside the washing machine. To identify the source of any reconstituted DNA, buccal smears were taken from all semen donors and from a laboratory technician directly working with experimental samples. For further research, the clothing was conditionally divided into five sections, from which the materials for the study were taken.

2.3. Sample processing and analysis

After each test washing, experimental clothing samples were examined using alternative light sources (Forensic Light Source - Mini-Crimescope Advance), were tested for the presence of acid phosphatase (Phosphatesmo K) (Macherey-Nagel, Germany) and semen using immunological tests SERATEC ® PSA Semiquant (Seratech, Germany) and RSID™ - Semen for serological determination of blood group in the AB0 system with the use of polyclonal reagents (anti-A, anti-B, anti-H) (Hematolog LLC, Moscow, Russia) and DNA analysis. Since soluble components of seminal fluid tend to migrate outwards (Kobus et al., 2002), a systematic approach was used for identification accuracy. Plots with semen stains and with an area of 0.8 cm^2 have been cut out on each piece of clothing. Microscopic sections were taken in the middle between the center and the edge of stains to test for the presence of the acid phosphatase (AP), PSA, Sg and establishing the group of AB0 system, and for DNA analysis that has been done at stain centers and the edge according to the present scheme blots (Figure1).

2.4. Research methods

After each wash, clothes No. 1-18 in the areas designated by the marker were examined using an alternative light source (Forensic Light Source - Mini-Crimescope Advance). The fluorescence intensity of each stain was evaluated using a relative scale as negative, weak, moderate, and strong. Then, ten semen stains (1 cm^2) were tested on each sample of clothing according to the instructions for use. The following tests were conducted: on the presence of acid phosphatase using Phosphatesmo K indicator paper, Koren – Stokis spermatozoa staining (0.5% solution of erythrosine in 25% ammonia), followed by microscopy using a light microscope (detection of whole semen cells was used as evidence which clearly distinguished the red-painted head, neck, tail, or its fragment), and also SERATEC ® PSA Semiquant, RSID ™ -Semen tests. For the serological study of semen stains according to the AB0 system, an absorption/elution reaction was used with anti-A, anti-B polyclonal reagents with a titer of 1: 200, anti-H with a titer of 1: 128. Results were evaluated using the criteria shown in Table 2.

To estimate the amount of DNA extracted, samples were taken after each washing. Two methods of material removal were used: flushing (sample was wiped back and forth with a moistened swab once over the entire surface of the stain) and cutting (chopped pieces removed according to the scheme, Figure 1). The amount and profile of the extracted DNA were determined. All samples were subjected to the genetic analysis: DNA was extracted using the EZ1 DNA Test Kit (Qiagen) with the BioRobot EZ1 (Qiagen) according to the manufacturer's instructions. Before applying BioRobot EZ1 during the cell lysis stage, 1M DTT was added to all samples to improve DNA release. Extracted DNA was eluted in 50 µl of sterile deionized water.

Additionally, DNA was extracted from buccal samples using the EZ1 and BioRobot DNA Test Kit under the manufacturer's instructions. DNA was eluted in 100 µl of sterile deionized water. Negative control was used to monitor possible contamination during DNA extraction. Evaluation of the quality and quantity of human DNA isolated from biological material was performed using the QuantifilerTM Human DNA Quantification Kit reagent kit on the ABI Prism ™ 7500 Sequence Detection System manufactured by Applied Biosystems (USA).

2.5. Statistical analysis

Comparisons of the parameters under study depending on the fabrics, the type of washing machine used, the type of load, the kind of washing powder, and the differences in the amounts of DNA obtained from semen stains under different washing conditions were evaluated using the Kraskel - Wallis H-test.

3. RESULTS AND DISCUSSION:

3.1. Analysis of semen stains after several washes using traditional methods

3.1.1 Semen stain detection with an alternative light source

To establish the effectiveness of using an alternative light source when detecting semen stains after washing, 180 semen stains were

examined with an estimate of their fluorescence. After the first (single) washing, most of the dyes (75%) showed strong fluorescence, 25% - 5%, and moderate - weak (Figure 2).

However, with each subsequent wash, the fluorescence intensity decreased. After the second wash, the fluorescence intensity was 35%, 40%, and 20%, respectively, while 5% of the stains showed a negative result. After triple washing, a negative result was obtained for 22% of the stains. A large part of the semen stains (50%) demonstrated weak fluorescence signal, while the percentage of stains with moderate fluorescence decreased to 25%. The fluorescence intensity varied significantly depending on the size of the semen stain (the stains formed by the mixture of two donors were more extensive in diameter), while the stains of smaller diameter (from one donor) had less intense fluorescence. Different washing conditions, such as washing machine brand or type of loading as well as the model and brand of washing powder, did not have a statistically significant effect on the fluorescence intensity. No acid phosphatase was detected in traces of semen with weak fluorescence. This suggests that fluorescent components are easily removed. Besides, it should be noted that the white color of clothes was optimal for observing fluorescence. It is known that after several washes, low-intensity fluorescence on colored fabrics can be missed out (Kobus et al., 2002). Consequently, the use of an alternative light source to identify semen stains on washing clothes can only be considered as a preliminary examination result, which will provide an expert with additional opportunities to find traces of the crime.

3.1.2 Detection of the acid phosphatase presence in semen stains

A study of semen stains for the acid phosphatase (AP) presence has shown that after a single wash, 84% of the semen stains were AP-negative (Table 3).

Most of the semen stains after a single wash in a top-loading machine or a non-biological washing powder were AP-positive with a moderate or strong degree of color, with statistically significant differences between groups ($p \leq 0.05$). In this connection, it can be assumed that these type of wash are less effective in removing biogenic traces. However, precise conclusions are impossible due to limited resources and an insufficient number of comparative studies (other brands of washing machines and non-biological washing powder). After the second wash, AP-negative results were found in 95% of cases, and only 5% of stains showed a slightly positive effect. After the third wash, the results were negative in 100% of cases. In previous studies, it has been reported that AP testing results may be negative or positive (Harbison, Fleming, 2016; Schlagetter, Glynn, 2012; Kobus et al., 2002; Crowe et al., 2003; Stefandou et al., 2010). However, experimental studies showed that only 16% of semen stains were AP-positive as a result of a single wash, 3% of which had strong fluorescence signal, that fully confirms previous studies (Noël et al., 2019) on the ineffectiveness of using tests for detecting acid phosphatase in identifying semen stains after washing.

3.1.3 Detection of spermatozoa in semen stains after washing using microscopy

In the morphological study, spermatozoa were identified microscopically only in 2% of semen stains after washing (Table 4)

The staining intensity of spermatozoa in semen stains after laundry was identical to the color of spermatozoa found in normal conditions. It should be noted that positive results were obtained in semen stains on cotton fabrics, which is most likely due to the heterogeneous structure of the fabric fibers. Studies have shown that the use of synthetic detergent, regardless of its brand or form (powder, liquid) according to the instructions for a single wash in an automatic washing machine, increases the likelihood of destruction of whole spermatozoa up to 98%. This indicates that this method has low efficiency in the study of semen stains after washing. Also, in some cases, spermatozoa may initially be absent in some diseases or be destroyed as a result of putrefactive changes due to improper storage of physical evidence or exposure to environmental factors (Martínez et al. 2015; Harbison, Fleming, 2016; Forensic evidence collection in sexual assault & rape, 2014; Chambers et al., 2010; Dale et al., 2008)

3.1.4 Detection of prostate-specific antigen in semen stains

Tests for the detection of prostate-specific antigen (PSA) and semenogelin (Sg) can be used in the absence of spermatozoa. Identification of semen stains after a single wash using the SERATEC ® PSA Semiquant test showed positive results in 92% with varying degrees of the color of the test line (Table 5).

60% of PSA-positive results were characterized by a moderate degree of the test line color, 30% - intensive and only 2% - weak. As a result of double washing, the number of PSA-negative results increased to 57%, while PSA-positive results of different intensities were 10%, 30%, and 3%, respectively. After triple washing, only 12% of PSA-positive semen stains were observed, of which 9% had mild color intensity.

It should be noted that PSA-positive results were observed while analyzing semen stains on mixed and cotton fabrics. Analysis of the results did not reveal significant differences depending on the type of detergent or brand, or type of washing machine loading. The presence of PSA-positive semen stains after three washes confirms previous studies (Schlagetter, Glynn, 2012; Edler et al., 2017). Although the detection of PSA remains a highly effective method for detecting the semen presence, the interpretation of the results should take into account the presence of false-positive results with other human biological secretions (Dale et al., 2008; Hochmeister et al., 1999).

3.1.5 Identification of semen stains using the RSID ® Semen test

Identification of semen stains after a single wash using RSID ® Semen test showed positive results with varying degrees of the color of the test line in 94% of stains (Table 6) and only 5% of them showed negative results.

As a result of the second washing, the number of negative results is significantly increased and reached 52%, while the test line has a weak coloring in 8% of cases, moderate - 35% and intensive in 5 % of cases. As a result of a triple washing when samples have been tested using RSID ® Semen, the number of negative results increased to 85%, which is most likely due to the excess of the endpoint of the semen dilution. Despite this, the obtained results confirmed the results of previous studies (Cooper et al., 2014; Suttipasit, Wongwittayapanich, 2018; Honggang et al., 2012) and showed that the RSID ® Semen test is highly useful for identifying semen on clothes or bedding after washing. Besides, the lack of cross-reactivity concerning other substrates and biological fluids is also a key factor, which is of great importance in cases of sexual violence when mixing of various human secretions is possible (Forensic evidence collection in sexual assault & rape, 2014; Suttipasit, Wongwittayapanich, 2018)

Experimental data have shown that PSA and Sg detection tests are quick and sensitive methods for identifying semen stains after washing. However, the sensitivity of the RSID ® Semen test in this study was slightly higher than that of the SERATEC ® PSA Semiquant test, regardless of whether the stain test result was negative or positive. These results show that SERATEC ® PSA Semiquant and RSID ®. Semen immunochromatographic tests are very effective in identifying traces of semen after washing due to high sensitivity, while cross-reaction with other biological fluids is not typical for RSID ® Semen (Hochmeister et al., 1999; Cooper et al., 2014; Suttipasit, Wongwittayapanich, 2018).

3.1.6 Detection of group antigens A, B, H in the semen stains

Further, we performed a serological analysis to determine the blood group in the AB0 system in tested samples. The corresponding group, antigens A, B, and H, were detected in all semen stains with a positive PSA and Sg test results. Notably, each subsequent wash led to a decrease in the group antigens titer value and a subsequent reduction in t agglutination intensity from intensive (+++) after a single wash to a weakly expressed (+) after a triple wash. Additionally, good fixation of group antigens (medium and robust agglutination intensity) was shown by semen stains on cotton and mixed fabrics (T-shirts, shirts, pants) in contrast to synthetic ones. That is also most likely related to the structure and surface characteristics of the fibers. The natural heterogeneous structure of natural fibers contributes to a stronger fixation

of antigens. It was established that the serological detection of the blood group is quite effective in the study of semen stains after washing, but since this method is routine, its use can be justified only in certain circumstances, including for the preliminary selection of traces before subsequent DNA analysis.

3.2. DNA analysis of semen stains after washing

3.2.1 Efficiency of DNA extraction from the samples

For DNA analysis, differential DNA extraction was performed. DNA yield was quantified only for the spermatozoa fraction. The results of sample analysis obtained by flushing show that this method is not suitable for the study of clothes after washing since the results obtained were significantly inferior to the results obtained in the study of the cut stains (Tables 7 and 8).

Negative results were obtained regardless of the washing conditions, apparently due to an insufficient amount of DNA. After a single wash of cotton and blended fabrics, the DNA concentration obtained from the spermatozoa fraction ranged on average from 0.018 to 0.021 ng / μl. Despite this, genetic profiles were obtained in the traces with the maximum DNA concentration after a single wash. The DNA concentration obtained from the semen fraction after the second wash decreased significantly (0.002-0.005 ng / μl). As a result, in 92% of the samples tested, the amount of DNA was insufficient for subsequent analysis.

On the contrary, the cut-off stains taken after a single wash contained enough DNA to establish the genetic profile (Table 8).

Even though with each subsequent washing, the amount of extracted DNA was continually decreasing, the minimum concentration of DNA required for amplification and obtaining a full profile has been maintained. Analyzing the sums of ranks presented in the resulting report, we can talk about the impact of the method for material removal on the research results. The necessary study performance was ensured when cutting the material with the stain for research, and the worst one was in the case of the removal of the material by flushing the stain (Tables 7 and 8).

Although detergents for washing, in general, have a similar composition, however, the number and ratio of components in them can be very different depending on the brand. The results of the study showed that different detergents could have a different effect on DNA detection and recovery in semen stains. Thus, after the use of non-biological washing powder, it was possible to recover higher amounts of DNA than in the study of semen stains after washing with enzyme-containing agents (Tables 9a and 9b).

As a result, 2.8 - 3.9 ng/μl of DNA corresponding to the DNA profiles of semen donors was isolated from the stains removed by cutting after one wash, regardless of the washing conditions. Depending on the number of washes, the overall decrease in the average amount of DNA extracted from semen stains has steadily decreased. Thus, the whole amounts of DNA in dyes on cotton fabrics recorded at each subsequent washing was lowering on the average from 3.9 ng / μl after the first washing to 2.57 after the second washing and to 2.07 ng / μl after the third washing. After triple wash, the amount of extracted DNA in some samples remained high and amounted to 1.5-2 ng / μl, regardless of the brand and type of loading characteristic to a washing machine. It should be noted that similar trends were observed in the study of semen stains on mixed fabric types. However, the amount of isolated DNA in them was significantly lower. In stains on synthetic fabrics, a sufficient amount of material for DNA identification was not obtained, which is most likely associated not only with the DNA degradation under the influence of enzymes contained in the detergent but also with the smooth structure of the fibers in this type of fabric. Previous studies have already proved the ability of biological body fluids, such as blood, saliva, and semen to be preserved on the fabrics after a single wash, and showed that in most cases enough DNA could be recovered to establish the genetic profile (Brayley-Morris et al., 2015; Nussbaumer et al., 2003; Edler et al., 2017; Kulstein, Wiegand, 2018; Andrews, Coquoz, 1994). In the present study, cut-off semen stains having washed once yielded sufficient amounts of DNA, which in some cases reached 3.9 ng / μl. As have been previously reported (Brayley-Morris et al., 2015; Nussbaumer et al., 2003), this amount of DNA

is sufficient to obtain a genetic profile. The results of the study show that under standard washing conditions, the DNA amount required for identification and establishing the full genetic profile can be obtained from semen stains after triple washing. It can be assumed that with such high amounts of DNA collected, complete genetic patterns can be established after a higher number of washes, especially if the area of the surface under investigation is large (Brayley-Morris et al., 2015).

3.2.2 Efficiency of DNA profile recovery from several donors

To study the possibilities of distinguishing DNA profiles in cases of sexual violence committed by several persons at the same time (Chambers et al., 2010), semen stains from two donors were applied to cotton and various types of mixed tissues (T-shirts, pants and shirts). Clothing samples tested are described in Table 1.

Since the amount of DNA obtained from semen stains on mixed tissues (trousers, shirts) was less (2.1–2.5 ng / μl) than on T-shirts (3.5-3.9 ng / μl), it was in the study of semen stains obtained from two donors that sufficient quantities of DNA were obtained from T-shirts, allowing to establish complete DNA profiles of both donors, regardless of the number of washes. Although the amount of DNA extracted from pants and shirts was lower than the amount removed from semen stains on cotton fabrics (T-shirts), for some of the samples, full DNA profiles were obtained, which have been corresponded, however, to the profile of only one of the donors. At the same time, the most significant differences in the entire range of the DNA amount for the studied stains were obtained on cotton fabrics ($p \leq 0.05$). Although the study of traces on shirts and trousers showed a wide range of the amount of isolated DNA, it was not possible to establish full profiles of both donors (Table 10b).

This most likely depends on the individual qualities of the donor semen and the type of fabric. At the same time, there are practically no significant differences between the mixed, semi-synthetic, and half-wool material in the entire range of the studied amounts of DNA, throughout all washes. However, in some cases, DNA mixtures of two people were isolated, which could not be reliably divided into major and minor components, and therefore, no precise results were obtained.

3.2.3 Determination of secondary DNA transfer when washing in a washing machine

Previous studies suggest that spermatozoa from semen stains on women's underwear can be found on other underwear when washed in the same washing machine (Kafarowski et al., 1996; Kamphausen et al., 2015; Voskoboinik et al., 2017). To study the possibility of transferring DNA from one clothes to another when washing in a washing machine, clean clothes were washed together with experimental clothes with semen stains (Table 10a). The analysis results for the studied clothes (№№ 19-23) after washing at 30 ° C are presented in table 10b. When conducting the research, tests for fluorescence, semen microscopy, and acid phosphatase were negative. However, tests for the presence of PSA and Sg showed positive results on cotton fabrics, weakly positive in some traces on mixed fabrics, and negative on synthetic fabrics.

At the same time, the difference in the amount of recovered DNA was established depending on the type of material. The amount of DNA on a T-shirt varied over a wide range, but in general, it was higher than on shirts and trousers; trace amounts of DNA found on the toes and pantyhose were unsuitable for analysis. Upon that, full DNA identification was obtained only in the study of cotton T-shirts. The results of the study depend, most likely, not only on the type of fabric but also on the qualities of donor semen. Since the calculated value is higher than the critical one, there are statistically significant ($p < 0.05$) differences in the amount of DNA depending on the number of washes and the type of fabric. Nonetheless, these data are not sufficient to describe any categorical findings, and they confirm the preliminary results available in the literature on the secondary DNA transfer (Nussbaumer et al., 2003; Kamphausen et al., 2015; Voskoboinik et al., 2017; L.T. Brown Migration Patterns of Seminal Fluid Components and Spermatozoa in Semen Stains Exposed to Water and Blood, 2016) and support previously proposed concept on "owner’s DNA" transfer between items of

clothing in the washing machine (Kulstein, Wiegand, 2018). However, this concept has not yet been confirmed and requires additional research.

Collectively, DNA profiling is the method mostly affected by the washing of the clothing samples. Differences in the amounts of DNA obtained in our and other similar studies (Brayley-Morris et al., 2015; Jobin, De Gouffe, 2003; Nussbaumer et al., 2003; Stefandou et al., 2010; Andrews, Coquoz, 1994) may be due to differences in the washing programs used (temperature, brand, and type of detergent, a washing machine brand, etc.), as well as the storage time of material evidence with traces of semen, clothing composition, and various donors. DNA transfer between pieces of clothing in a washing machine could also affect the amount of DNA extracted. Some studies show (Brayley-Morris et al., 2015; L.T. Brown Migration Patterns of Seminal Fluid Components and Spermatozoa in Semen Stains Exposed to Water and Blood, 2016) that multiple washes of cotton fabric with semen stains only lead to minimal loss of recovered DNA, which suggests that numerous washing may not have a significant effect on DNA recover. In this study, three brands of automatic washing machines with identical cycles and a washing temperature regime (30 °C) were used, and the results show that some devices can remove DNA better than others. However, the type of washing powder and washing machine, as well as the temperature of the water during washing in different regions may vary depending on the area, national characteristics, various economic factors. For example, previous studies have shown that similar amounts of DNA can be extracted from semen stains, blood or saliva, when washing at 30◦C, 40ºC and 60ºC (Brayley-Morris et al., 2015; Edler et al., 2017; Andrews, Coquoz, 1994), however, it is possible to use 90ºC during washing. It is possible that the use of such a high temperature along with longer wash cycles may more significantly affect semen stains and reduce the amount of remaining DNA more strongly.

The research results showed that there are significant differences in the ability to preserve the traces of semen subjected to washing, depending on the type of fabric of the carrier object. Thus, semen stains located on natural fabrics are preserved better, as confirmed by previous studies (Harbison, Fleming, 2016; Brayley-Morris et al., 2015; Nussbaumer et al., 2003; Crowe et al., 2003). It has been shown that washed cotton fabrics hold semen better than washed Capron or nylon (Schlagetter, Glynn, 2012; Edler et al., 2017). When semen stains are placed on synthetic fabrics, the possibility of their subsequent group and individual identification is almost wholly absent. In our study, we found that the type of fabric affects the efficiency of semen stain detection when examining them using the serological determination of blood groups and DNA analysis, which corresponds to published data.

Additionally, we analyzed the effect of enzyme-containing and non-biological detergents on the effectiveness of the examination. It is found that enzyme-containing detergents significantly reduce the possibility of identifying semen stains, thus reducing its effectiveness (Table 11).

4. CONCLUSIONS:

The results demonstrate that the efficiency of semen stains analysis by traditional methods vary depending on the conditions of washing. For all means, a decrease in detection efficiency depending on the number of washes performed, was observed. Among traditional morphological and immunological methods, laundry has the most significant effect on the possibility of carrying out a microscopic analysis of semen stains and conducting a reaction to the detection of acid phosphatase. Some methods retain their effectiveness regardless of the use of different types of laundry detergent (determination of fluorescence, PSA, and Sg detection), which confirms the results of previous studies. Enzyme-containing detergents affect biogenic traces and the consequences of forensic examination significantly reducing the effectiveness of some screening methods and the amount of DNA detected.

Nevertheless, this study shows that it is possible not only to identify semen stains but also to recover DNA in the quantities sufficient for further DNA profiling even after three washes. This can be achieved by the suitable sample

preparation, and the success is dependent on to differences in the washing programs used (temperature, brand and type of detergent, a washing machine brand, etc.), as well as the storage time of material evidence with traces of semen, fabric composition, and variation between donors.

Collectively it is recommend a mandatory examination of clothing after washing. At the same time, all suspicious traces with negative microscopy results (taking into account possible vasectomy or aspermia) require further testing to exclude false-negative results. Besides, when pre-wash information is available, screening methods should be chosen with care to ensure that they are appropriate.

Reliability, specificity, and objectivity in the study of traces that have been destroyed are today one of the most urgent problems. Therefore, a systematic approach and the correct choice of research strategy in combination with operational information obtained during the investigation will help to prove the facts of sexual violence. Based on the above, it can be assumed that the use of traditional research methods in combination with an appropriate screening strategy and a suitable material removal method is effective and allows a positive, informative result to get in cases of examining clothes after their washing when proving the facts of sexual violence.

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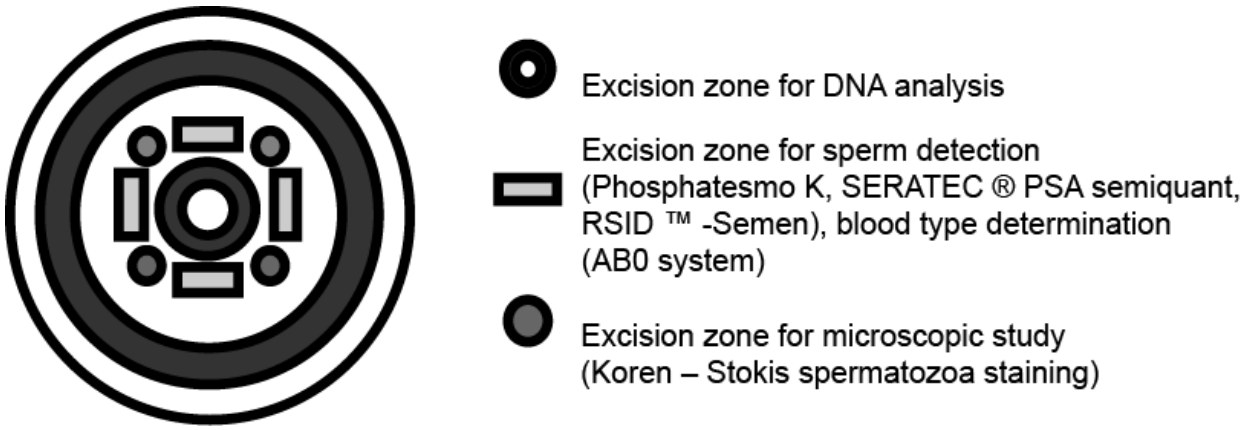

Figure 1. *The scheme of material removal from an experimental stain depending on the type of study*

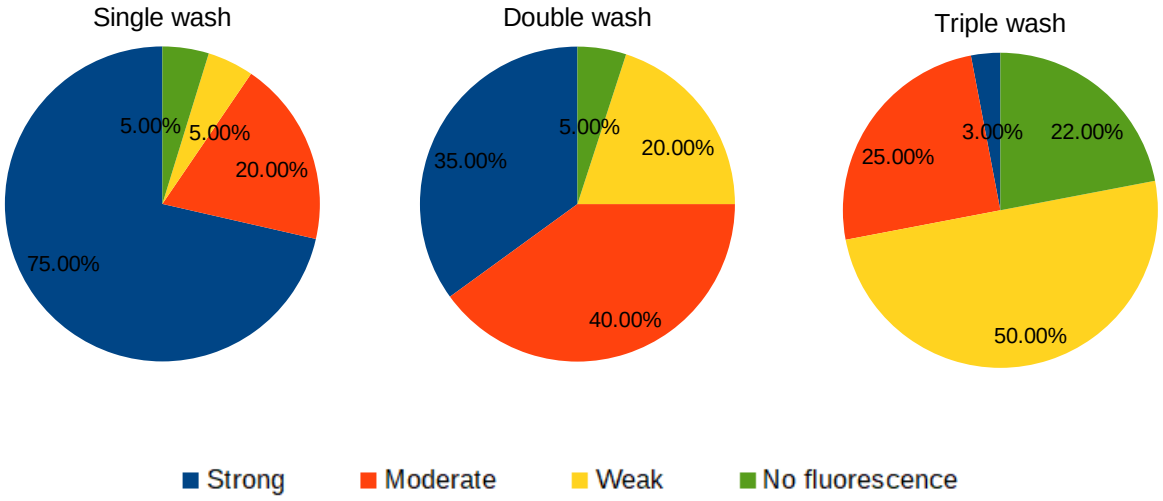

Figure 2. *Evaluation of the fluorescence degree for semen stains using an alternative light source (Forensic Light Source - Mini-Crimescope Advance) depending on the number of washes*

Table 1a. *Summary table of the main parameters used in the study (a type of clothing, semen donors (A–M), a brand of a washing machine, type of washing powder, washing conditions, number of washes) (n = 430)*

Type of clothes	Donors	Number of stains	Washing machine, brand, type of loading	Detergent	Parameters (T°C)	Number of washes
T-shirt 1	A, D, H, L	8	Whirlpool TDLR 70220, top	Persil ® Power-Liquid ®	30º C	3
	A: D	1				
	H: L	1				
T-shirt 2	B,C,G,I	8	BoschWLG 2416 M, frontal	Laundry detergent Tide Original	30º C	3
	B: I	1				
	C: G	1				
	E,F,K,M					

T-shirt 3	K: M	1	SiemensWS12T540OE, frontal	Washing powder ECOVER ZERO NON BIO	30º C	3
	E: F	1				
Blouse 4	A, D, H, L	10	WhirlpoolTDLR 70220, top	Persil ® Power-Liquid ®	30º C	3
Blouse 5	B, C, G, I	10	BoschWLG 2416 M, frontal	washing powder TIDE ORIGINAL	30º C	3
Blouse 6	E, F, K, M	10	SiemensWS12T540OE, Frontal	washing powder ECOVER ZERO NON BIO	30º C	3
Tights 7	A, D, H, L	10	WhirlpoolTDLR 70220, top	Persil ® Power-Liquid ®	30º C	3
Tights 8	B, C, G, I	10	BoschWLG 2416 M, frontal	washing powder Tide original	30º C	3
Tights 9	E, F, K, M	10	SiemensWS12T540OE, frontal	washing powder ECOVER ZERO NON BIO	30º C	3
Trousers 10	A, D, H, L	8	WhirlpoolTDLR 70220, top	Persil ® Power-Liquid ®	30º C	3
	A: L	1				
	D: H	1				
Trousers 11	B, C, G, I	8	BoschWLG 2416 M, frontal	washing powder TIDE ORIGINAL	30º C	3
	G: C	1				
	B: I	1				
Trousers 12	E, F, K, M	10	SiemensWS12T540OE, frontal	washing powder ECOVER ZERO NON BIO	30º C	3
	E: F	1				
	K: M	1				
A pair of socks 13	A, D, H, L	1	WhirlpoolTDLR 70220, top	Persil ® Power-Liquid ®	30º C	3
A pair of socks 14	B, C, G, I	10	BoschWLG 2416 M, frontal	washing powder TIDE ORIGINAL	30º C	3
A pair of socks 15	E, F, K, M	10	SiemensWS12T540OE, frontal	washing powder ECOVER ZERO NON BIO	30º C	3
Shirt 16	A, D, H, L	8	WhirlpoolTDLR 70220, top	Persil ® Power-Liquid ®	30º C	3
	D: H	1				
	A: L	1				
Shirt 17	B, C, G, I	8	BoschWLG 2416 M, frontal	washing powder TIDE ORIGINAL	30º C	3
	B: G	1				
	C: I	1				
Shirt 18	E, F, K, M	8	SiemensWS12T540OE, frontal	washing powder ECOVER ZERO NON BIO	30º C	3
	E: K	1				
	F: M	1				

Table 1b. *Summary table of the main results of semen stain detection (including stain detection methods) (spots, n = 430)*

Type of clothes	**Semen detection**					**Blood type determination using the AB0 system**
	Fluorescence (Forensic light source)	**Microscopy (Koren - Stockis)**	**AP (Phosphatesmo K)**	**SERATEC ® Psa semi quant**	**RSID ™ - Semen**	
T-shirt 1	30	20	30	30	30	27
T-shirt 2	30	20	30	30	30	21
T-shirt 3	30	20	30	30	30	24

Blouse 4	25	20	25	25	25	14
Blouse 5	25	20	25	25	25	15
Blouse 6	25	20	25	25	25	15
Tights 7	19	16	19	19	19	9
Tights 8	19	16	19	19	19	9
Tights 9	21	17	21	21	21	11
Trousers 10	28	20	28	28	28	18
Trousers 11	26	20	26	26	26	16
Trousers 12	24	20	24	24	24	14
A pair of socks 13	21	20	21	21	21	11
A pair of socks 14	20	20	20	20	20	10
A pair of socks 15	20	20	20	20	20	10
Shirt 16	23	20	23	23	23	13
Shirt 17	21	20	21	21	21	11
Shirt 18	23	20	23	23	23	14
Total	**430**	**349**	**430**	**430**	**430**	**262**

Table 1c. *Summary table of the main results of DNA detection in semen stains*

Type of clothes	**Donors**	**DNA analysis**			
		Flush, number of stains	**DNA profiles**	**Cutting, number of stains**	**DNA profiles**
T-shirt 1	A, D, H, L	4	100% one source with a matching donor profile	4	100% one source with a matching donor profile
		1	A mixture of 1: 1A1 (15): D2 (15)	-	-
		-	-	1	1: 1 mixture H1 (15): L2 (15)
T-shirt 2	B, C, G, I	4	100% one source with a matching donor profile	4	100% one source with a matching donor profile
		1	A mixture of 1: 1B1 (15): I2 (15)	-	-
		-	-	one	A mixture of 1: 1 C1 (15): G2 (15)
T-shirt 3	E, F, K, M	4	100% one source with a matching donor profile	4	100% one source with a matching donor profile
		1	1: 1 mixture	-	-

			K1 (15): M2 (15)		
		-	-	1	1: 1 mixture E1 (15): F2 (15)
Blouse 4	A, D, H, L	5	-	5	100% one source with a matching donor profile
Blouse 5	B, C, G, I	5	-	5	100% one source with a matching donor profile
Blouse 6	E, F, K, M	5	-	5	100% one source with a matching donor profile
Tights 7	A, D, H, L	5	-	5	-
Tights 8	B, C, G, I	5	-	5	-
Tights 9	E, F, K, M	5	-	5	-
Trousers 10	A, D, H, L	4	-	4	100% one source only with donors A, H, D
		1	-		
				1	Major D1 (15) and minor (1)
Trousers 11	B, C, G, I	4	-	4	100% one source only with donors B, I
		1	-		
				1	Major I2 (15) and minor (1)
Trousers 12	E, F, K, M	4	-	4	100% one source only with donors K, F, M
		1	-		
				1	Major K1 (15) and minor (1)
A pair of socks 13	A, D, H, L	5	-	5	-
A pair of socks 14	B, C, G, I	5	-	5	-
A pair of socks 15	E, F, K, M	5	-	5	-
Shirt 16	A, D, H, L	4	-	4	100% one source only with donors A, D, L
		1	-		
				1	Major A1 (15) and minor (1)
Shirt 17	B, C, G, I	4	-	4	100% one source only with donors B, I, C
		1	-		
				1	Major C1 (15) and minor (1)
Shirt 18	E, F, K, M	4	-	4	100% one source only with donors F, K, M
		1	-		
				1	Major F1 (15) and minor (1)
Total		**90**		**90**	

Table 2. *Standard criteria for evaluating results concerning AP, PSA and Sg*

Category	Phosphatesmo K (AP)	SERATEC ® PSA Semiquant	RSID ™ -Semen
	Colouring appearance, time	Test line appearance	Test line appearance
Strong	1 - 30 seconds	1-3 minutes	1-3 minutes
Moderate	31 - 60 seconds	4–7 minutes	4–7 minutes
Weak	61 - 120 seconds	8–10 minutes	8–10 minutes
Negative	Colouring is absent for 2 minutes	The line is absent for 10 minutes.	The line is absent for 10 minutes.

Table 3. *The results of studies on the detection of acid phosphatase in semen stains depending on the number of washings (n = 430).*

Number of washes	Acid Phosphatase (AP)			
	Negative result	Positive result		
		Appearance coloring		
		weak	moderate	intense
Single wash (n = 180)	84%	5%	8%	3%
Double wash (n = 169)	95%	5%	0%	0%
Triple wash (n = 81)	100%	0%	0%	0%

Table 4. *The results of studies on the detection of spermatozoa in semen stains depending on the number of washings (n = 430).*

Number of washes	Microscopy	
	Negative result	Positive result
Single wash (n = 180)	98%	2%
Double wash (n = 169)	100%	0%
Triple wash (n = 81)	-	-

Table 5. *The results of studies on the detection of PSA in semen stains depending on the number of washings (n = 430).*

Number of washes	SERATEC ® PSA			
	Negative result	Positive result		
		Test line colouring		
		weak	moderate	intense
Single wash (n = 180)	8%	2%	60%	30%
Double wash (n = 169)	57%	10%	30%	3%
Triple wash (n = 81)	89%	9%	2%	0%

Table 6. *The results of studies on the detection of Sg in semen stains depending on the number of washings (n = 430).*

Number of washes	RSID ™ -Semen			
	Negative result	Positive result		
		Test line colouring		
		weak	moderate	intense
Single wash (n = 180)	6%	5%	55%	34%
Double wash (n = 169)	52%	8%	35%	5%
Triple wash (n = 81)	85%	10%	5%	0%

Table 7. *DNA extraction efficiency in samples obtained by flushing*

	Washout (DNA concentration), ng / µl (+ CO)					
# of washes	**Cotton n = 30**	**Semi-synthetic n = 30**	**Synthetic n = 30**	**Wool n = 30**	**Wool blend n = 30**	**Mixed n = 30**
1	0.021 ± 0.003	0.01 ± 0.005	-	0.018 ± 0.001	0.016 ± 0.001	0.018 ± 0.003
2	0.005 ± 0.001	0.010 ± 0.001	-	0.015 ± 0.001	0.015 ± 0.001	0.015 ± 0.001
3	0.0002 ± 0.001	-	-	-	-	-

Table 8. *DNA extraction efficiency in samples obtained by cutting*

	Cutting (DNA concentration), ng / µl (+ CO)					
# of washes	**Cotton n = 30**	**Semi-synthetic n = 30**	**Synthetic n = 30**	**Wool n = 30**	**Wool blend n = 30**	**Mixed n = 30**
1	3.9 ± 0.1	2.5 ± 0.1	0.021 ± 0.003	2.1 ± 0.01	1.9 ± 0.01	2.5 ± 0.1
2	2.5 ± 0.1	1.6 ± 0.1	-	1.7 ± 0.01	1.5 ± 0.01	1.8 ± 0.01
3	2.07 ± 0.1	0.016 ± 0.001	-	0.016 ± 0.001	0.015 ± 0.001	0.021 ± 0.001

Table 9a. *DNA extraction efficiency after enzyme-containing washing means*

	Enzyme-containing washing means (DNA concentration), ng / µl					
# of washes	**Cotton n = 30**	**Semi-synthetic n = 30**	**Synthetic n = 30**	**Wool n = 30**	**Wool blend n = 30**	**Mixed n = 30**
1	3.5 ± 0.1	2.5 ± 0.1	0.021 ± 0.003	1.8 ± 0.1	1.9 ± 0.01	2.5 ± 0.1
2	2.0 ± 0.1	1.6 ± 0.1	-	1.1 ± 0.1	1.5 ± 0.01	1.1 ± 0.1
3	1.5 ± 0.1	0.016 ± 0.001		0.8 ± 0.1	0.015 ± 0.001	0.8 ± 0.1

Table 9b. *DNA extraction efficiency after non-biological washing means*

	Non-biological washing means (DNA concentration), ng / µl					
# of washes	**Cotton n = 30**	**Semi-synthetic n = 30**	**Synthetic n = 30**	**Wool n = 30**	**Wool blend n = 30**	**Mixed n = 30**
1	3.9 ± 0.1	2.8 ± 0.1	0.05 ± 0.01	2.1 ± 0.1	2.0 ± 0.01	2.5 ± 0.1
2	2.5 ± 0.1	1.8 ± 0.1	0.021 ± 0.003	1.5 ± 0.1	1.7 ± 0.01	1.7 ± 0.1
3	2.07 ± 0.1	0.021 ± 0.001	0.015 ± 0.001	1.0 ± 0.1	0.018 ± 0.001	1.2 ± 0.1

Table 10a. *The parameters of the study on semen transfer inside a washing machine (a type of clothing, semen donors, a brand of a washing machine, type of washing powder, washing conditions, number of washes)*

Type of clothes	Donors	Number of stains	Washing machine, brand, type of loading	Detergent	Parameters (T°C)	Number of washes
T-shirt 19	A,D,H,L	0	WhirlpoolTDLR 70220, top	Persil ® Power-Liquid ®	30ºC	2
Tights 20	A, D,H,L	0	BoschWLG 2416 M, frontal	washing powder TIDE ORIGINAL	30ºC	1
Trousers 21	A,D,H,L	0	SiemensWS12T540OE, frontal	washing powder ECOVER ZERO NON BIO	30ºC	1
Socks 22	A,D,H,L	0	BoschWLG 2416 M, frontal	washing powder tide original	30ºC	1
Shirt 23	A,D,H,L	0	SiemensWS12T540OE, frontal	washing powder ECOVER ZERO NON BIO	30ºC	1

Table 10b. *Summary table of the main results of semen stain and DNA detection.*

Type of clothes	Semen detection					Blood type (AB0)	DNA analysis	
	Fluorescence (Forensic light source)	**Microscopy (Koren - Stockis)**	**AP (Phosphates-mo K)**	**SERATEC ® PSA semiquant**	**RSID ™ -Semen**		**Type of material withdrawal - cutting**	**DNA profiles obtained**
T-shirt 19	-	-	-	+	+	+	5 places	Mixture A 1 (15), D2 (15), H 3 (15), L4 (15)
Tights 20	-	-	-	-	-	-	-	-
Trousers 21	-	-	-	+	+	+	5 places	- Major D2 (15), H3 (15) and minor L (2), A (2)
Socks 22	-	-	-	-	+	±	-	-

Shirt 23	-	-	-	+	+	+	5 places	Major D1 (15) A3 (15) and minor L (2), H (2)

Table 11. *The results of semen trace identification after washing, depending on the type and consistency of synthetic detergents.*

Type of fabric	Type and consistency of synthetic detergents		
	Non-biological (without enzymes)	Biological (with enzymes)	
	Powder	Powder	Liquid
	ECOVER ZERO NON BIO.	Tide Original	Persil® Power-Liquid®
One wash			
Cotton (100% cotton)	+++	++	++
Semisynthetic (gabardine)	+++	++	++
Synthetic (100% nylon)	++	+	+
Wool (wool 90%, viscose 10%)	+++	++	++
Wool blend (70% wool, 20% cotton, 10% coolmax)	+++	++	++
Mixed (65% cotton, 30% polyester, 5% elastane).	+++	++	++
Two washes			
Cotton (100% cotton)	++	+	+
Semisynthetic (gabardine)	++	+	+
Synthetic (100% nylon)	+	±	±
Wool (wool 90%, viscose 10%)	++	+	+
Wool blend (70% wool, 20% cotton, 10% coolmax)	++	+	+
Mixed (65% cotton, 30% polyester, 5% elastane).	++	+	+
Three washes			
Cotton (100% cotton)	+	±	±
Semisynthetic (gabardine)	+	±	±
Synthetic (100% nylon)	±	-	-
Wool (wool 90%, viscose 10%)	+	±	±
Wool blend (70% wool, 20% cotton, 10% coolmax)	±	-	-
Mixed (65% cotton, 30% polyester, 5% elastane).	+	±	±

+ - weak possibility of identifying results

++ - moderate (medium) possibility of identifying results

+++ good (strong) possibility of identifying results