UNIVERSITAT ROVIRA I VIRGILI

Master's Degree in Forensic Genetics, Physics and Chemistry

Validation of “Christmas tree” staining method for microscopic observation of spermatozoa

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ABSTRACT

Sexual aggression is a crime that affects many people all around the world. In most of the cases, males are involved and as a result, semen can be found on the victim or at the crime scene. Proper procedures can be employed to demonstrate that sexual activity has taken place through the identification of sperm cells or seminal fluid. Determining the donor of the semen samples is the next crucial step in the investigation. The aim of this work was to validate the procedure of microscopic identification of sperm using "Christmas tree" staining by considering quality control and accreditation standards for the incorporation in routine use of the lab. First, for the optimization of the study, semen swabs revealed that: i) the staining works well for both maceration and direct seminal fluid to the glass slide, and that ii) there is no significant difference on the maceration buffer (water or TE) or the maceration time. The use of DTT at 0,001 M (for a better staining) worked well in high volumes of semen but in lower ones, many cells were broken which impeded its identification according to the established standards. Second, the reproducibility of the method was assured using several substrates: underwear fabric, jeans fabric, cotton swab, panty liner and sanitary napkin. Third, the evaluation of the sensibility indicated that for sterile swabs and swabs with oral and vaginal fluid the detection limit is 1/500 and for sanitary napkin and jeans fabric is 1/1000. There was also variability in the abundance of sperm cells between semen donor and replicates. Finally, the specificity was tested to compare sperm cells with similar cells or structures that could interfere with the identification. The confirmatory method in this study was able to distinguish sperm cells from other similar structures that could be found in samples from sexual aggressions considering the described identification characteristics and the detection limit, abundance and reproducibility in several substrates was described.

INTRODUCTION

Sexual aggression is a major social and public health problem that affects people all over the world and poses a threat to the physical and mental health of the victim, the possible consequences being: eating disorders, depression and suicidality [1,2]. It has been estimated that 6 to 59% of women (globally) experience sexual violence at some point during their lifetime [3]. It is also known that in most cases the offender is known to the victim and that 99% of the offenders are males [3,4,5]. Sexual aggression is defined as behavior carried out with the intent or result of making another person engage in sexual activity or sexual communication despite his or her unwillingness to do so [6]. On the other hand, rape is most defined as nonconsensual oral, anal or vaginal penetration of the victim by body parts or objects using force, threats of bodily harm [7]. Considering the principle described by Edmond Locard: "every contact leaves a trace", it is expected that when contact between individuals involved in a crime scene occurs, evidence will be transferred to one another and to the scene to be found [8,9]. Considering the high number of cases such as the ones described above, it is important to identify semen samples and analyze them correspondingly ensuring that a well-established and reliable method of analysis is employed. The semen samples could potentially lead to the identification of the perpetrator, or at least "prove" that sexual activity has taken place.

EVIDENCES IN SEXUAL AGGRESSIONS: THE RELEVANCE OF SEMEN DETECTION

Because sexual aggression and rape are felonies, careful investigations should be made to find evidence so that the perpetrator could be found and condemned. Biological evidence is of most importance because can link the victim with the aggressor. On the other hand, physical evidence may be of less significance in this kind of investigations. For example, the most common biological evidence are semen, blood, sweat, saliva, vaginal fluid, hair, epithelial cells, among others. In cases of sexual aggression or rape, semen is considered the most important type of evidence because it can prove that sexual contact occurred and also identify the perpetrator through DNA analysis [10,11]. The semen is a fluid made of glandular secretions and cellular components. The latter includes spermatozoa, which are the characteristic cells of semen and contain the genetic information in males. However, detached epithelial cells in very low quantities could also be

found. Spermatozoa have three major structures (Fig 1.): the head, mid piece, and the tail, measuring a total length of 50-60 μm. The morphology of the head can be described as a flattened ovoid shape with an acrosome at the apical portion. An ejaculation usually measures between 1-6 mL of semen, averaging 3,5 mL and contains approximately 50-100 million spermatozoa per milliliter [12,13].

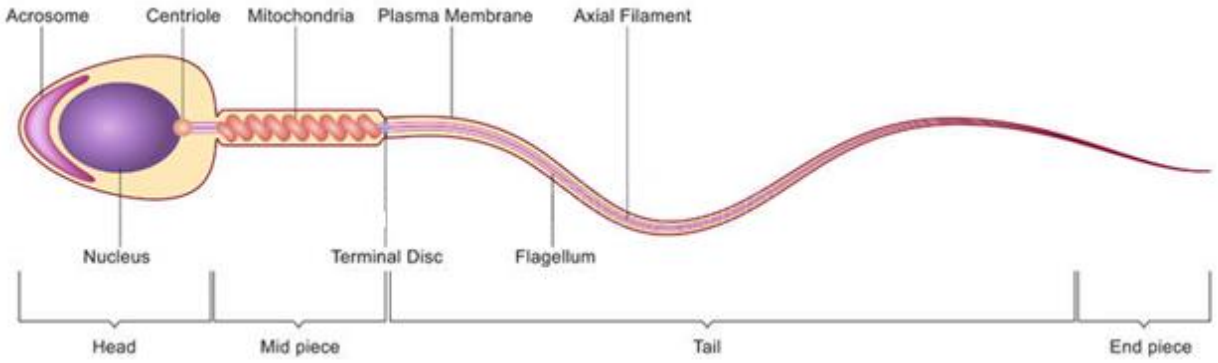

Figure 1. Sperm cell anatomy. Extracted from www.dreamstime.com/stock-illustration-sperm-cell-anatomy-vector-labelled-illustration-human.

EVIDENCES IN SEXUAL AGGRESSIONS: SAMPLE COLLECTION AND PERSISTENCE

The evidence recovered on sexual aggression cases may be from the crime scene and clothes as usual investigations [14,15], but unlike other crimes, the victim's body may be the most important place to find the evidence [16]. Because the nature of the evidence is usually time sensitive, the quicker the victim goes to get checked out by a medical examiner the better so that they gather forensic evidence and preserve it accordingly. Usually, sexual assault evidence, for example intimate samples collected with swabs, clothing, bedding and related items are collected and submitted for examination to forensic laboratories for semen detection, which may lead later on to the analysis of DNA to reveal the perpetrator genetic profile. It is expected to find better quality evidence if the samples are collected within 24 h of the crime, but in some cases, evidence may persist for a few more days. It has to be noted that sperm cells have a higher survival rate than seminal fluid because the later has protein nature which breaks down quicker [17]. Another important issue is that environmental conditions of the body have a direct relationship with the survival of sperm cells [18], and it has also been found that their survival time is longer in

postmortem body orifices [19]. In the study of Allery J. P. et al, [20] the decreased detection of spermatozoa was related first with the length of time since intercourse and the sperm volume, but there are other aspects to be taken into consideration, like the area of the body where the semen is encountered. Some studies have shown that spermatozoa may be found in the human cervix up to 7 to 10 days' post coitus [21,22] meanwhile others obtained slightly different results. For example, a recent study [23] described that in the vagina the expectation of observing sperm after 18 h from the intercourse is at 35% of the analyzed swabs; after 48 h at 20%; and after 96 h at 2% (more than 3000 swabs analyzed). The same study also analyzed 510 internal anal swabs, and they found that the expectation of observing sperm cells was lower than for vaginal samples, only observing spermatozoa in 24% of cases within the first 6 h after intercourse; 16% within 48 h and only 2,3% after 48 h, which is considered very low. Other studies obtained similar results for anal samples[24]. For oral cavity, one study found that sperm can be present up to 6 h [25] and another, on the other hand, which examined a total of 405 internal mouth swabs found that the persistence of sperm cells is very low even within the first 6 h from the intercourse, but they could be found up to 15 h [23].

THE PROCESS OF ANALYSIS OF SEMEN SUSPECTED SAMPLES

The first step for semen detection is to do a visual examination of the evidence. When it is difficult to visualize it, an alternate light source (ALS) which is a non-destructive tool may be used to screen the evidence for the presence of semen [26]. Nevertheless, ultraviolet light is not recommended for visual examination of the evidence if genetic profiles are intended because it could degrade the DNA. The wavelengths at which semen stains fluoresce is from 450 to 530 nm, typically at 455 nm [27]. It is known that ALS is not specific to semen, and other body fluids such as saliva, urine or sweat will fluoresce as well but generally not as brightly [26,28]. After a stain is suspected of being semen, further presumptive and confirmatory tests should be employed.

The order in which other tests are applied depends on the organization of the lab. The biological test may be the first used and it consists in the identification of sperm cells through microscope visualization which is considered a confirmatory test for the presence of spermatozoa [20,29]. This is done through a staining technique; in this work, it has been used the Christmas Tree staining

[30]. The procedure is to add liquid extract onto a microscope slide, dry it and afterward apply a Nuclear Fast Red dye which will turn the cellular nuclei red. The nuclei of sperm cells will be intense red, and the acrosomal cap will have a pinkish/white color. This can be differentiated from the epithelial cells which will have a less intense red color probably because of the greater laxity of DNA. Then, the sample is stained with picroindigocarmine, which stains the tails of sperm cells and the cytoplasm of epithelial cells green/blue. Some factors that could difficult the microscopic examination are an excessive concentration of epithelial cells, bacterial or cellular debris as well as low spermatozoa counts. Other factors which could condition the presence of spermatozoa might be degradation over time, use of prophylactics, lack of ejaculation and vasectomy just to name some of them [31,32,33].

Lately, new microscopy related techniques have emerged for the detection of spermatozoa. First, there is the KPICS SpermFinder detection instrument (NicheVision, LLC, Akron, OH). This instrument utilizes an algorithm to identify possible spermatozoa based on the color contrast of acrosome/nuclei and size. It creates an electronic image of the slide and saves the location of the possible spermatozoa so that the scientist can verify the finding [34,35]. Other advantages are that it usually takes less time to make the analysis and detects significantly more spermatozoa [36]. Another instrument utilized to detect spermatozoa is SPERMHY-LITER™ (Independent Forensics, Lombard, IL) which uses a fluorescently labeled antibody that is specific to the head of human sperm [37,38]. This makes the detection of spermatozoa easier even when the sample has a high concentration of other cells. However, the general disadvantages of these methods are the cost and equipment requirements, that are not available for most labs.

If the presence of semen is not confirmed by the mentioned methods, a biochemical presumptive test can be carried out. One possible approach is acid phosphatase (AP), which is one of the most commonly employed test [39]. Seminal AP is an enzyme which provides energy for sperm motility and is present in semen in high quantities [40]. It should be taken into account that the concentration of AP is not correlated with the presence of spermatozoa [41]. This test consists of applying the reagent (e.g. Bertamine Fast Blue reaction) to a moistened swab collected from the evidence. If the presence of AP is detected, in the prescribed time period a pink to purple color

should be noticed [42]. It is possible to obtain false positives because AP is present also in other body fluids (vaginal and saliva) [43,44], but its presence in semen is 20-400 times higher [39,45]. The possibility to obtain high AP levels on post-pubertal girl's vagina or cervix ranges from 24 [46] to 72 h after ejaculation [43,47]. In the case of mouth samples in the same conditions the detection time is about 6 h and in rectum less than 24 h [48].

The seminal protein Prostate Specific Antigen (PSA), also known as p30, has usually been used to detect the presence of semen and it is considered one of the most sensitive methods [49]. Its concentration in semen ranges between 390 to 3000 μg/mL [50] which is considered high. With this method, also exists the possibility of obtaining false positives which could be male urine, peripheral blood (highly hemolytic samples with an intense red color), rectal secretions, breast milk [49] and sweat glands [51]. Nevertheless, the levels of p30 in semen are approximately 1000 times more concentrated than in urine. To perform this test, an antigen/antibody immunoassay card is used. In post-pubertal girl's vagina or cervix, PSA could be found up to 48 h [46]. In contrast to AP, this test can be applied to azoospermic and vasectomized individuals.

RSID-Semen test (like PSA, is a membrane strip test) is another semen preliminary test widely used, which identifies the presence of Semenolegin protein (Sg) [52]. This antigen is specific for human semen and cannot be confused with other body fluids from women or semen from other mammals except for lung carcinomas [53], skeletal muscle, kidney, colon, and retina [54,55]. But normally, tissue samples are not submitted in sexual assault cases for semen detection that is why it should not be a problem. Therefore, Sg detection is considered a confirmatory test.

DNA PROFILING IN ASSAULT CASES

Due to the scientific progress, DNA analysis of biological fluids has increased the statistical probability of linking offenders to their victims and nowadays the most important evidence for legal proof is the DNA [56,57]. When identification of spermatozoa is negative and also when the identity of the aggressor is unknown, analysis of DNA may be employed to obtain the genetic profile from the epithelial cells left on the victim's body. It has been found that during the intercourse male epithelial cells from the penis or saliva can transfer into the vagina [58]. Also, it

would be possible that epithelial cells were transferred if digital penetration had taken place. This can be very helpful in a case where a vasectomized or azoospermic male is the aggressor because semen will not be found, but epithelial cells may be present from the ejaculatory duct and urethra [59,60]. Then, the cases where the perpetrator is a male and the victim is a female, Y-STR typing may be employed to obtain the genetic profile of the Y chromosome. This is very useful because a profile could be obtained even if the amount of female DNA is several times higher than the male's [61,62]. Furthermore, differential lysis method, which is widely used in this cases, could be employed to separate sperm from epithelial cells by centrifugation. For the analysis of DNA, it was detected from 48 h to 8 days after the incident in cases where the presence of spermatozoa was negative which indicated that sensitivity of Y-STR typing is very high, even more than cytology [61].

Although the increased sensibility for the obtaining of a DNA profile has been a great development, in some cases this can be an issue. That is mainly because unrelated DNA to the crime (background DNA) or DNA originated from secondary transfer can be detected and a genetic profile could be obtained. For example, in shared living spaces, DNA from other persons may be deposited onto the victim's clothing. A study demonstrated that spermatozoa from a single pair of semen-stained panties could be transferred to all items that are laundered with[63]. Consequently, wrong conclusions could be drawn.

VALIDATION

Because forensic methods are used with the aim of obtaining results for the criminal justice system, those results must be consistent, reliable, reproducible and accurate. As a consequence, high validation and quality control requirements have been established by international organizations and it is necessary for a forensic lab to be accredited according to the International Organization for Standardization (ISO). These high standards rest on two concepts which are: quality control and quality assurance, to deliver a quality product. The first refers to the measures that are taken to ensure that a result and its interpretation meets the established standard of quality. The later has to do with monitoring, verifying and documenting the laboratory performance. Two very common practices of assuring this last quality standard are participating

in proficiency tests and laboratory auditory. Some of the benefits that accreditation brings could be the optimization of time and work, certainty in the results, credibility and reliability of the lab, high qualified personnel, evaluation of the lab limitations and identification of correction and improvement actions, among others. Another very important factor that has to do with good practice of evidence analysis is the chain of custody. This refers to the chronological documentation of the custody of the evidence that ensures that no external factor has tempered it.

Forensic genetic labs are generally accredited under the international standard EN ISO/IEC 17025 as mentioned before [64], where the validation is stated as a key requirement. According to ISO17025 "*The laboratory shall validate non-standard methods, laboratory-designed/developed methods, standard methods used outside their intended scope, and amplifications and modifications of standard methods to confirm that the methods are fit for the intended use. The validation shall be as extensive as is necessary to meet the needs of the given application or field of application. The laboratory shall record the results obtained, the procedure used for the validation, and a statement as to whether the method is fit for the intended use."* Validation then is the way of demonstrating that a method is suitable for the specific purpose intended in an objective way, the ability and conditions under which the results are reliable and by which any limitations that it could have, are understood and explained.

AIM OF THE STUDY

The aim of this study was to validate the procedure of microscopic observation of spermatozoa using "Christmas tree" staining for the incorporation in routine use of the lab taking into consideration the quality control and accreditation standards.

METHODS

1) Bibliographic review. Careful research for the published material on the subject has been conducted to take into consideration possible variables that have been described by other investigators and could help to avoid negative results and optimize the method.

2) Optimization of the method. The optimization of the assay consisted in the comparison between some variables to see which one of them is more suitable for the obtaining of better results. A total of 22 samples were prepared in this section. The variables analyzed were:

    - Firstly, semen put directly on the glass slide was compared to macerated samples of semen deposited on swabs. All of the tests were done with two semen donors (S2 and S6) in the same conditions and 30 µL per sample was employed.
    - For the maceration, milliQ water and TE was tested to see if there is a significant difference.
    - Available literature [65] described that the use of DTT partially brakes the head of sperm and condenses the chromatin of nuclei which permits the entrance of the staining more easily. Then, considering the two maceration solutions already described, different concentrations of DTT (0,001, 0,01 and 0,1 M) were employed in the maceration liquid to see if a better staining, and eventually observation of the sperm, could be obtained.
    - Furthermore, the incubation of the sample at different times (1 h, 6 h, and 12 h) was analyzed.

    Finally, in this section the criteria for the identification of the sperm was established: to avoid confusion with other cells like bacteria, yeast… or structures that have a similar morphology and size, the distinction between the acrosome and the nuclei should be clear; the first one, as described above has a pinkish-white color in the apical part meanwhile the later has a strong red color in the lower part of the cell. When tails can be observed, the identification does not cause confusion with other cells (protocol in the annex section). In addition, a scoring system was also established

to evaluate sperm density on slides (Table 1). At least 10 fields where sperm was detected were considered to classify a sample with a determined score.

Table 1. Scoring system used for the evaluation of sperm density.

Sperm cells density	Score
Absence of sperm cells in the slide	**0**
1-5 sperm cells are observed in the slide	**1**
2-5 sperm cells are observed in at least 3 fields	**2**
More than 5 sperm cells are observed in at least 3 fields	**3**
Numerous sperm cells (+10) are observed in several fields	**4**

3) Elaboration of assay documents. It includes the establishment of the protocol that will be followed and preparation of a standardized worksheet. The protocol was adapted to the laboratory routine procedure structure, including objective and scope, references, procedure general information, equipment, assay description, results interpretation and assay records. The worksheet was elaborated following the internal registry structure (where materials, controls, reagents, equipment used have to be recorded), in order to facilitate the fulfillment when developing the assay. A copy of both protocol and worksheet is provided in the annex section.

4) Sample preparation. The material needed for sample preparation was: sterile swabs, sanitary napkin, pantie liner, panties, jeans fabric, glass slides and RSID semen kit. Most of the biological samples needed were supplied voluntarily by laboratory personnel: two semen donors (S2 and S6); two oral swabs donors (O1 and O2); two vaginal swabs donors (V1 and V2); used sanitary napkin; menstrual blood; blood; urine; and dog semen (one swab and cotton fabric). Aliquots of evidence from forensic casework were also used only when the sufficient quantity was available to not compromise the sample in case further analysis should be made. The provided samples were: vaginal wash from a recent case and two "old" vaginal washes; one from 2010 and the other from 2011, which gave positive for PSA test but were not checked by microscopy (conserved in the freezer).

5) Protocol employed. Samples of known concentration of seminal fluid were prepared on different substrates and left to air-dry at least for half an hour. The next step was to

remove the cotton carefully from the wooden stick or the piece of the substrate and put it inside an Eppendorf tube for maceration for at least 1h with 200 µL of liquid, mixing the samples by vortex occasionally. To increase the extraction efficiency, the substrate was teased with forceps. It should be considered that a balance is to be achieved in the volume of water used to ensure the optimal release of sperm cells and altogether obtain a concentrated extract. It has been described that the extraction of the whole swab with at least 200 µL of water appears to give the optimal release of sperm [66]. Then, 30 µL of extraction liquid from these samples, as well as samples of vaginal wash, direct sperm, centrifuged samples and dog + human semen, for which maceration step was not necessary, were deposited on a glass slide and dried in the oven at 56 C for half an hour and finally, the Christmas tree staining was performed. It basically consists on adding Nuclear Fast Red to the glass slide until the sample is covered, wash with milli-Q water after 15 min, add Indigocarmine for 15-20 s, wash with ethanol and finally put the cover glass and let it dry (described in more detail in the protocol attached in the annex section).

6) Validation assays. There are mainly three methods in a validation study that could be analyzed: identification assay, qualitative and/or quantitative determination. In each one of these methods, several parameters could be studied. In this case, reproducibility, sensibility (detection limit) and selectivity/specificity were considered regarding the type of validation needed for this assay.

A. Reproducibility / repeatability

It refers to the obtainment of the same result when a series of measures are studied in different conditions, for example, the analyst, the day of the analysis, etc. In this section, 50 µL (for the two semen donors) was deposited on different substrates: underwear fabric, jeans fabric, cotton swab, panty liner and sanitary napkin. For panty liner and sanitary napkin, only the first layer of fabric was used for maceration because if not the maceration solution will convert to a gel. Also, this layer, especially for the sanitary napkin it is quite pierced so maybe this probably will have an effect on the

retention of sperm cells. The assay was repeated by another analyst and by duplication, obtaining 3 replicates for each sample and donor.

B. Sensibility (detection limit)

This section alludes to the lowest dilution at which the cells of interest can be observed.

- Several volumes of semen (10, 5, 1 and 0,5 µL) and several dilutions (1:10, 1:100, 1:500 and 1:1000 with a total of 30 µL) were deposited on jeans fabric, sterile swabs (macerated with 0,001 DTT), and sanitary napkin. For the dilutions on sterile swabs, the assay was repeated by another analyst by duplication. Moreover, RSID semen kit was used to compare the results with an already validated sensibility test. First, the maceration from a 5 µL volume sample (described above) was used to compare with another sample in the same conditions but macerated in the buffer from RSID to see if macerated samples in water could be used instead of macerating them with buffer as already was described in a previous study [67]. But, it was found that the intensity of the test with water maceration was lower than in buffer so this second was chosen for the samples analysis.
- To determine the effect of other body fluids, the same was done with oral and vaginal cotton swabs which were collected by women donors the day before and processed the same day that the semen was deposited on the swabs.
- Finally, in this section of dilutions and volumes, 2 samples in which no sperm was detected were chosen to be centrifuged and see if sperm cells could be observed.
- To check the persistence of sperm cells through time, vaginal swabs were used to simulate post-coital samples depositing sperm (10 µL) at the same time on all swabs and then analyzed at 24 h, 48 h, 72 h, 1 and 2 weeks later. The samples were kept at room temperature. For each time period two sets of samples were prepared (two simulated couples).

- Also, vaginal wash from an aggression case was analyzed for the persistence of sperm cells, and every day a glass slide was prepared until the sperm could not be observed anymore. In addition, two sexual aggression vaginal washes from 2010 and 2011 that gave positive for semen with PSA method were analyzed to see if sperm cells could be found.

C. Selectivity/specificity

Is the capacity of a method to distinguish, in this case, a type of cell from a mixed sample without being confused by other components of the sample. Then, the study of this parameter consists of determining the possible components (types of cells) which could interfere with the identification of the cells of interest. To test possible false positives or cells that may difficult the distinction of sperm cells the following fluids were analyzed: menstrual blood, blood, saliva, urine and vaginal fluid. Also, used sanitary napkin and used sanitary napkin with 30 µL of semen were prepared too. Similarities between dog and human sperm have been previously reported, and for that reason, it was included in the validation process. As it was expected to be difficult to distinguish between them, first dog sperm was obtained and confirmed and later a mixture of dog and human sperm was also prepared for observation.

7) Validation report. This document should contain the steps and assays that were done and the results obtained as well as if the meted validation was successful, with its limits of detection (all this steps are part of the validation process, this report is not included in this work).

RESULTS AND DISCUSSION

1. Optimization. Several experiments were performed in order to evaluate a set of variables that could influence the results.
    a. For semen applied directly to the glass slide, a very good staining was obtained (Fig. 2), and in most of the cases, the whole sperm cells could be observed very well, so both macerated semen and liquid semen can be equally used.
    b. A comparison between water and TE was done (Fig. 2). No difference was detected and for that reason, the use of water was chosen over TE.
    c. Regarding the effect of DTT addition, in samples S6-TE with DTT the staining was less intense in comparison to the same samples with water and samples from S2-TE DTT, that is why this cannot be attributed to the use of TE neither DTT (Fig. 2). Besides, the use of DTT seemed to increase the quality of the staining when 0,001 dilutions were employed, so it was chosen to continue using it for the first samples of sensibility part (sterile swabs + semen). Regarding the staining of sperm cells in other DTT dilutions, a considerable number of broken sperm nuclei (the acrosome could not be differentiated from the nuclei having round bloated irregular shape) was detected, especially in 0,1 DTT samples. The abundance of sperm cells was high, scoring almost all samples 4 except for two, in which maybe the extraction did not work so well or the semen was not homogeneous so with the same amount of semen fewer sperm cells were caught (Table 2). The result of obtaining a high number of cells was expected because a considerable amount of semen was deposited on the swabs.

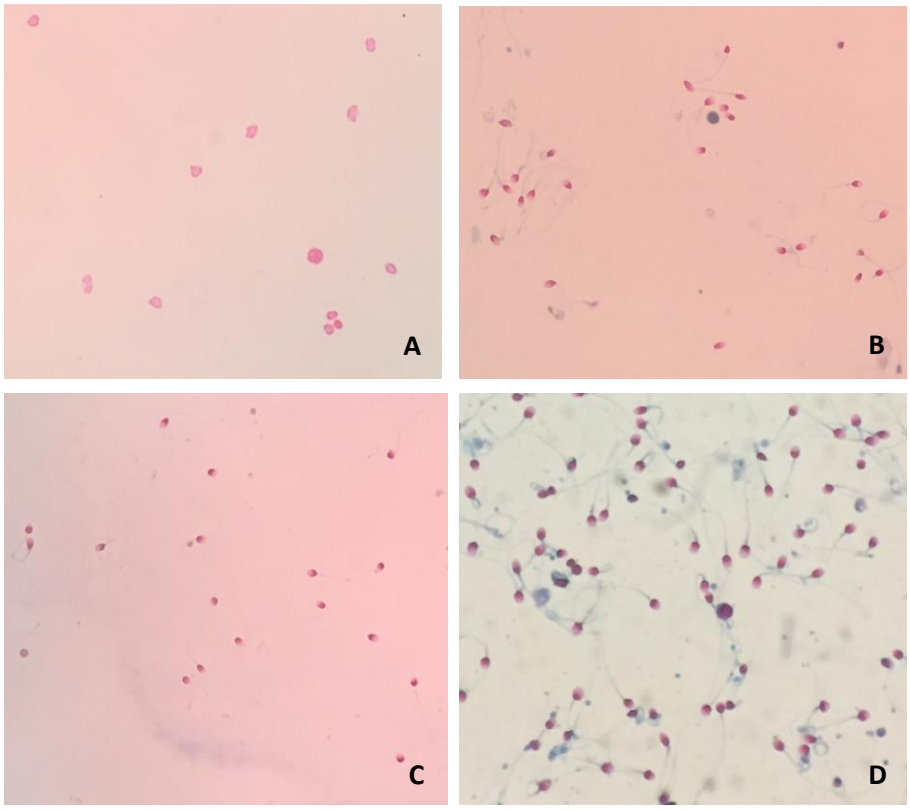

Figure 2. Microscopy observed sperm cells at 40x except D that is 60x. The 40x samples were zoomed in meanwhile D is original format of the picture. A is S6-TE-0,1, B is S2-TE-0,001, C is S6-H2O-0,01 and D is no macerated sperm.

d. About the maceration time, all samples were classified with score 4 and no significant difference between them was noticed (Fig. 3; Table 2).

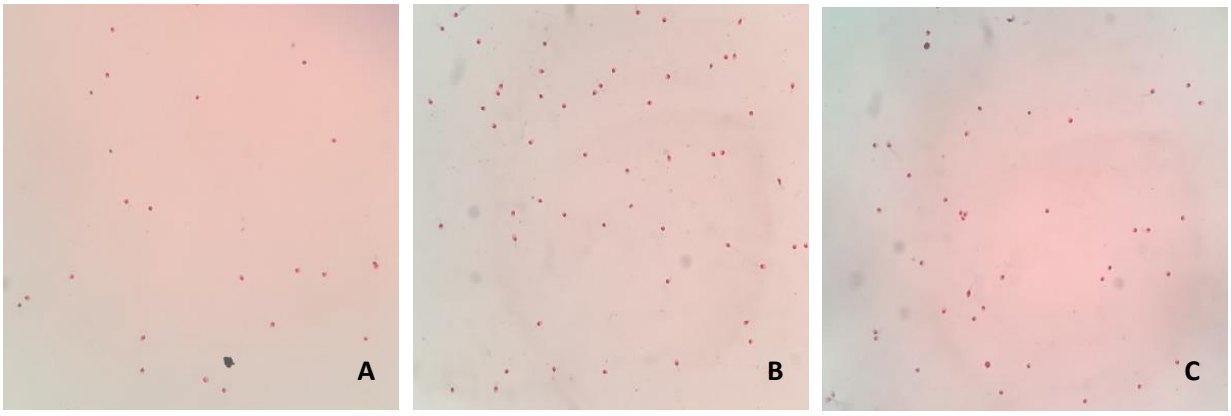

Figure 3. Samples at 40x, all at the same scale. A is S6-H2O-1h, B is S6-6h and C is S6-12h.

Table 2. Sperm density for the optimization samples.

Samples	No maceration	Water	Water 0,1 DTT	Water 0,01 DTT	Water 0,001 DTT	TE	TE 0,1 DTT	TE 0,01 DTT	TE 0,001 DTT
Score S2	4	4	4	4	4	4	4	4	4
Score S6	4	4	4	4	4	2	4	4	3

2. **Reproducibility**. A series of repetitions of semen additions on several substrates done by different analysts was performed to assure the reproducibility of the method. As it can be seen in table 3, for all samples the maximum score was obtained (4). This means that reproducibility of the method is assured when considerable amount of sperm (around 50 µL) is employed.

*Table 3. Sperm density scoring system for reproducibility samples. *The series of samples from 1st analysis were done by one analyst and the 2nd and 3rd by another one.*

	S2 (1st)	S2 (2nd)	S2 (3rd)	S6 (1st)	S6 (2nd)	S6 (3rd)
Jeans fabric	4	4	4	4	4	4
Sanitary napkin	4	4	4	4	4	4
Pantie liner	4	4	4	4	4	4
Panties	4	4	4	4	4	4
Sterile swab	4	4	4	4	4	4

3. Sensibility. For the detection limit of sperm cells, several substrates were used with different volumes and dilutions.
    a. For the sterile swabs, as mentioned before, 0,001 DTT solution was employed and in all dilutions (1/10, 1/100, 1/500 and 1/1000 if sperm was detected) and some of the lower volume samples (5 µL, 1 µL and 0,5 µL), many sperm cells were found to have broken nuclei, being the higher volumes the less affected (Fig. 4). Maybe that is why samples from optimization were well stained because high volume of semen was deposited on the swab (30 µL) meanwhile, in this case, being fewer cells of sperm in the sample the effect of DTT was greater. Because this could difficult the identification of sperm cells, possibly confusing them with other structures that are stained in the same color and shape, it was decided to stop

using DTT. It was also observed that nearly in all samples Nuclear Fast Red did not work well resulting in less intense red staining. This happened also in some of the samples from optimization and no reasonable explanation was found. It may be related to the use of DTT, maybe the reagent was degraded, but if this was the case it should have affected all the samples, that is probably why neither of this explanations are accurate. Another thing that could have happened is that in the process of the staining, not sufficient reagent was proportionated to the glass slide, but again, it does not seem to be the case.

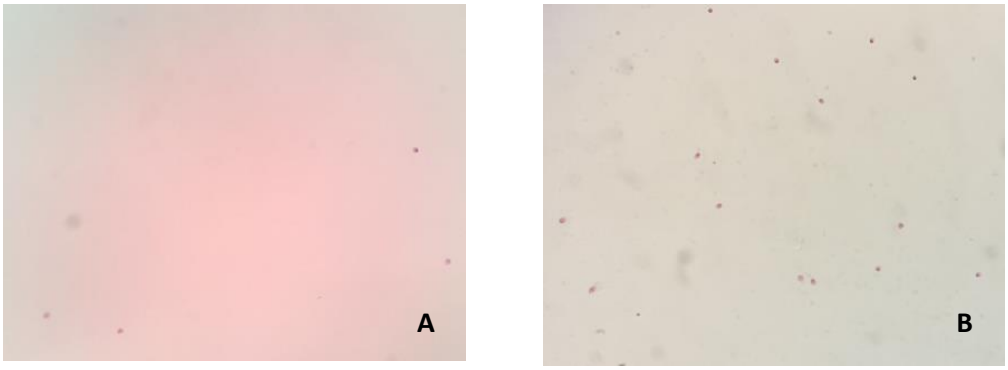

Figure 4. Microscopy observations at 40x. A is sample S6-1.10 and B S6-1 µL. In the first picture broken sperm cells can be observed

On one hand, the samples which contained 10 and 5 µL volumes were classified with score 4 except one sample which was 3, and 1 and 0,5 µL samples were all in the same category with score 2. On the other hand, the dilutions of 1/10 had score of 3 and no significant difference between 1/100 and 1/500 was found, which were all 2. The other dilutions, S2-1/1000 and S6-1/1000, two structures similar to sperm cells were seen in the second but with no clear distinction between acrosome and nuclei that is why the confirmation was not positive, but, taking into consideration the effect of DTT it was possible that those cells were sperm.

In addition, the same dilutions (without DTT) done by another analyst to evaluate reproducibility at lower volumes of sperm, show that for volumes samples, only 4 out of 8 the same result was obtained. Moreover, usually the second analyst found less sperm density in 3 out of the 4 samples. For the dilutions, the staining did not

work well for some of the samples (the ones with "-" in table 4), and neither the 1st replicate because in most of the samples, which a considerable number of sperm cells should be found, were scored with 0. The results of the 1st analyst and the 2nd replicate of the 2nd analyst are more alike. The differences are that for the 1/100 dilution one obtained a score of 3 and the other one of 2, and also, that the 2nd analyst found sperm cells at both 1/1000 dilutions. The detection limit could not be established at 1/1000 because not in all samples (1st analyst and 2nd analyst 2nd replicate) the presence of sperm cells was confirmed, so the limit is at 1/500. Therefore, as these results show, reproducibility at lower volumes of sperm is not that accurate and maybe this is because of the extraction from the swabs, which varies, or that the semen is not homogenous and depending on the pipetting more or less sperm cells are caught having a more obvious effect on lower volumes than in higher.

Table 4. Sperm density of sterile swabs for sensitivity reproducibility evaluation.

	S2 10µL	**S6 10µL**	**S2 5µL**	**S6 5µL**	**S2 1µL**	**S6 1µL**	**S2 0,5µL**	**S6 0,5µL**
1st analyst	4	4	3	4	2	2	2	2
2nd analyst	3	3	3	3	3	2	2	2
	S2 1/10	**S6 1/10**	**S2 1/100**	**S6 1/100**	**S2 1/500**	**S6 1/500**	**S2 1/1000**	**S6 1/1000**
1st analyst	3	3	2	2	2	2	0	0
2nd analyst 1st replicate	2	-	1	0	0	0	0	0
2nd analyst 2nd replicate	-	-	3	2	2	2	1	1

RSID results that were done for 1/100, 1/500 and 1/1000 dilutions, for semen 2 all were positive and for semen 6 the first one was positive and the second (1/500), the test line could barely be seen. It can be deduced from these findings that depending on the semen, maybe the time that has passed since the sample was obtained (degradation of the semenolegin) or the content of this protein which

maybe varies depending on the person, different detection limits can be found. Then, for this test the detection limit is the same as for sterile swabs, at 1/500.

b. For oral swabs, in comparison with sterile swabs for the dilutions category, 1/10 for both semen donors has 1 score less (2) and 1/100 and 1/500 are all 1* indicating that could have score 2 as the sterile swabs. The only sample in this assay that was sperm negative is S6-O1-1/1000. The detection limit is 1/500 because in both samples sperm could be observed. For volume category, 10 and 5 µL S6 samples have 1 score less (2) than sterile swabs (Fig. 5). The rest of the volumes (1 and 0,5 µL), fewer sperm cells could be found than in sterile swabs; 3 out of 4 samples were classified with score 1* meanwhile the sterile swabs were all 2. What these results show is that in general, the oral swabs had a lower number of sperm cells, the detection limit is the same as before (1/500) but sperm cells were also seen at 1/1000 dilution.

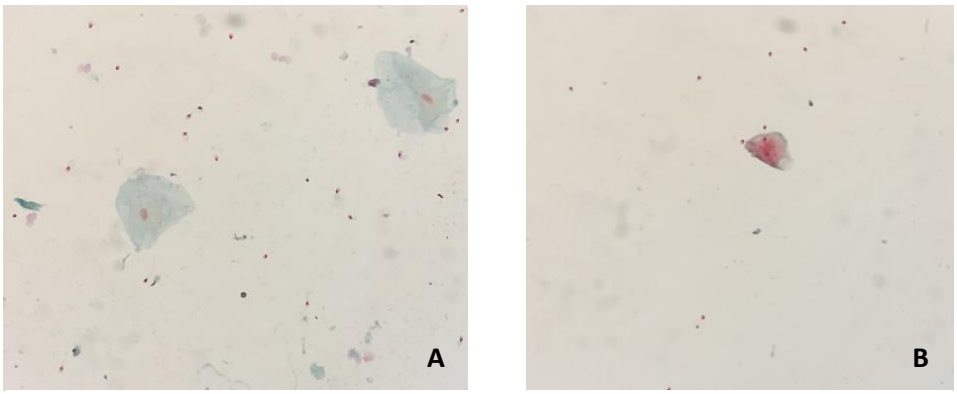

Figure 5. Microscopy observations at 40x. Example of the two pairs of donors. A is sample O2-S2-10 µL and B is O1-S6-10µL.

The dilutions in the vaginal swabs gave similar results to oral swabs except for both 1/500 dilutions in which fewer sperm cells were detected. Sperm was found in all samples, except for S6 – V1- 1/1000 that was not analyzed because it had a high number of similar structures to sperm so the finding of sperm was difficult, so detection limit was established at 1/500 but more samples should be analyzed to confirm it or to see if it is at 1/1000 (Fig. 6). For volume category in some of the

samples, the amount of sperm cells is different than oral swabs, but no relevant or obvious pattern could be established. Something that should be considered, derived from microscopy observation, is that all V1 samples had more cellular material than V2, where epithelial cells were better preserved and observed without much debris. This fact can interfere with sperm detection so in some of the lowest dilutions was very difficult to distinguish sperm from other nuclei or structures because they were very abundant.

In general, both oral and vaginal samples had lower scores than sterile swabs. This could be attributed to variability of the number of sperm cells (explained in the previous section) or maybe these fluids could have affected somehow the extraction from the swabs retaining more sperm cells.

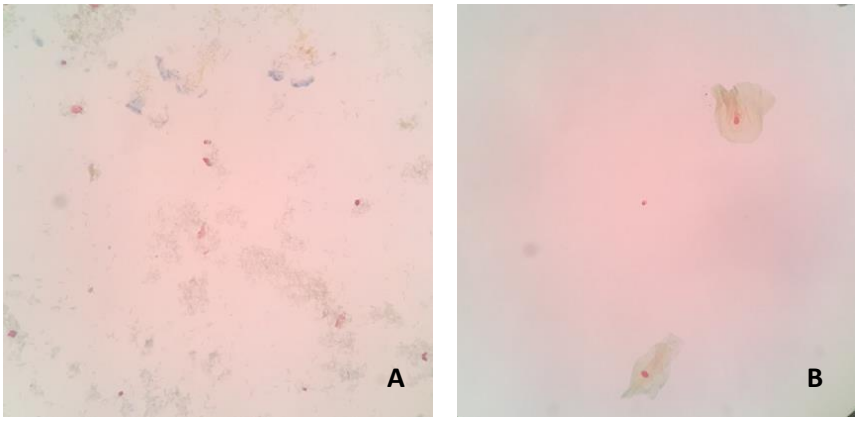

Figure 6. Microscopy observations at 40x. A is samples V1-S6-1.1000 and B is V2-S2-1.1000. The differentiation of vaginal cellular material can be noticed.

c. Sperm cells were observed on all sample preparations of jeans fabric. In comparison to sterile swabs, for the dilutions category, the number of sperm cells was higher in 1/10 samples (score 4) (Fig. 7). For the rest of the dilutions, it seemed that there was no significant difference, all having a score of 2. This shows that for jeans fabric the detection limit is 1/1000 or lower. For volumes category, all samples gave a score of 2. The ones containing more volume of semen (10 µL and 5 µL) seemed that had fewer cells than other corresponding samples on different surfaces. It is interesting that the volume of semen is not related to the abundance

of sperm cells, for both semen donors, for this substrate. The same is true for the dilutions except for 1/10. It is odd that 1/10 dilution had a much higher abundance of semen than the sample of 10 µL because this last one has a higher quantity of semen. To confirm this results, first a replicate should be made for all samples and if the results are the same, higher volumes of sperm and lower dilutions should be made to see until when this pattern is held, and also find the detection limit.

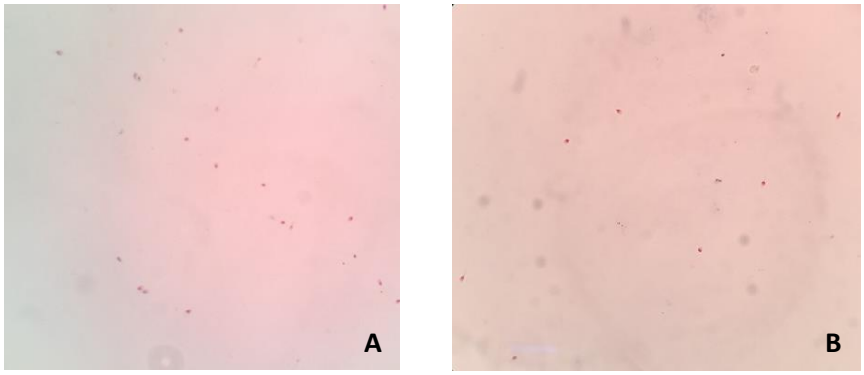

Figure 7. Microscopy observations at 40x. A is sample vq-S2-1.10 and B is vq-S2-10 µL.

d. In the case of the sanitary napkin, sperm cells were observed in all samples as well, saving dilution S6 – CP - 1/500 where only 1 cell that looked like sperm but could not be confirmed was found. This shows that maybe the extraction from the substrate in some occasions could be more efficient than others because sperm was found on 1/1000 dilution and not in 1/500. Or probably, because of the pierced substrate sperm cells were not retained for this sample. A maximum score of 2 was obtained in dilutions category which for some of the samples is lower than sterile swabs and jeans fabric. The detection limit for sanitary napkin is 1/1000 or lower. For the category of volumes, it is interesting that in spite of the many holes that this substrate has, and that only the first layer is analyzed, it had higher abundance of sperm cells than almost all the other substrates for 10, 5 and 1 µL (this last one only for S2) with a score of 4 (Fig. 8). This reflects that maybe the extraction is better than other substrates, having considered as well the loss of sperm that was absorbed by lower layers of the sanitary napkin and was not

retained in the first layer.

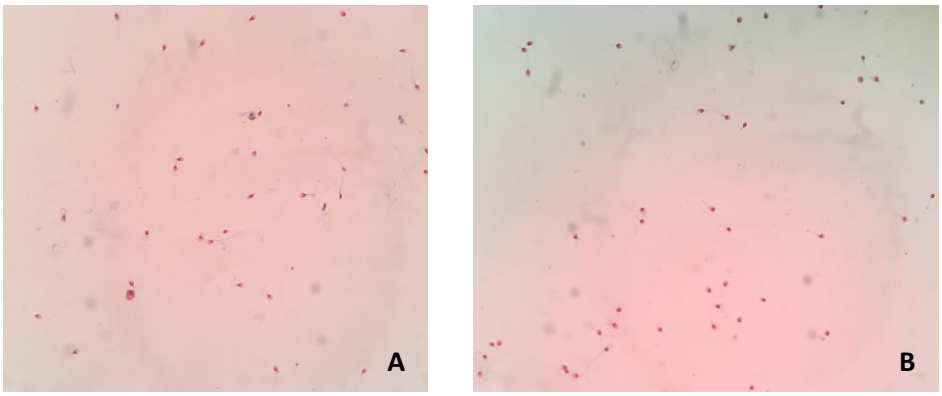

Figure 9. Microscopy observation at 40x. Sample A is cp-S2-10µL and B cp-S6-10 µL.

Table 5. Sperm density for sensitivity evaluation for every substrate used.

	Oral swabs	Vaginal swabs	Jeans fabric	Sanitary napkin	Sterile swabs
S2 – 10 µL	4	4	2	4	4
S6 – 10 µL	3	2-3	2	4	4
S2 – 5 µL	3	3	2	4	3
S6 – 5 µL	3	2	2	4	4
S2 – 1 µL	2	2	2	4	2
S6 – 1 µL	1*	1*	2	2	2
S2 –0,5 µL	1*	2	2	2	2
S6 – 0,5 µL	1*	1*	2	2	2
S2 – 1/10	2	2	4	2	3
S6 – 1/10	2	2	4	2	3
S2 – 1/100	1*	1*	2	2	2
S6 – 1/100	1*	1*	2	2	2
S2 – 1/500	**1***	**1**	2	2	**2**
S6 – 1/500	**1***	**1**	2	1?	**2**
S2 – 1/1000	1	1*	**2**	**1**	0
S6 – 1/1000	0	-	**2**	**1**	0

**Means that not all the slide was analyzed and more than 5 sperm could be found and if it is so, the sample would correspond to the 2 category. The detection limit samples are in bold.*

e. The samples S2-1/1000 (swab with semen dilution) and S6 O1 – 1/1000 (oral swab with semen dilution), which gave a negative result for the presence of sperm cells, were centrifuged (only the liquid obtained from the maceration, without the swab

in this case), to check if sperm cells remained in the liquid and could be detected. In both samples, sperm cells were found. For the S2-1/1000 sample, more than 8 sperm cells were observed in only ¼ of the analyzed glass slide, and for S6 O1 – 1/1000, only 1 cell was observed. These results lead to think that when very few sperm cells are present in the sample, the extracted volume to deposit on the glass slide may not contain sperm but some of it could have remained in the maceration water left. In addition, these results also show that, as it is expected, the extraction of sperm from the swab is not absolute but many sperm cells can be retained and, the probability of findings sperm cells in centrifuged samples is higher.

f. The swabs with vaginal fluid and semen for the persistence test, all were prepared at the same time simulating the sexual act and then analyzed at 24 h, 48 h, 72 h, 1 week and 2 weeks. Sperm cells were detected in all 10 samples, showing that once the swab is dry, sperm cells can persist during a considerable period of time (more than 2 weeks). To find out for how long sperm can persist, more tests should be done covering a wider period of time. The scores obtained from microscopy observation show that 3 samples until 72 h had score 2 and all the others 3, having higher density the samples from 1 and 2 weeks (all score 3). This has most probably to do with the extraction in the maceration process or homogeneity of semen because in all swabs was deposited the same amount of semen and because the vaginal mucous was already dry could not have affected much the sperm survival rate.

Table 6. Sperm density for samples prepared to simulate the sexual act and the time of the analysis.

Samples	S2 V2 24h	S6 V1 24h	S2 V2 48h	S6 V1 48h	S2 V2 72h	S6 V1 72h	S2 V2 1w	S6 V1 1w	S2 V2 2w	S6 V1 2w
Score	3	2	2	3	2	3	3	3	3	3

g. The persistence of sperm cells in vaginal wash was eventually not determined. Sperm cells could be observed with a score of 4 during 21 days and because the

sample was limited for it was provided from an ongoing case it was decided to conclude with this assay. The cells could be observed easily and good distinction could be made from other types of structures because the acrosome and nuclei could be observed clearly in most of the cells. Then, looks like a vaginal wash kept at room temperature for 21 days does not affect sperm cells density. Another test considering longer time period should be done to find out for how many days' sperm cells persist in the vaginal wash and see when degradation occurs. It should be noted that during the first two weekends the sample was frozen, to preserve the sperm cells because the analysis could not be made. This could have affected the cell braking it but no significant effect was noticed. For the old vaginal washes, it was found that the sample from 2011 was sperm positive and the one from 2010 was not (Fig. 9). In both samples well stained epithelial cells could be observed. The 2010 sample could be that no sperm cells were found because the donor was azoospermic or vasectomized, that the quantity of cells was so low that no sperm was caught in the 30 µL used, or less likely, that PSA test was a false positive. The conservation of frozen samples should not be a problem in only 7-8 years.

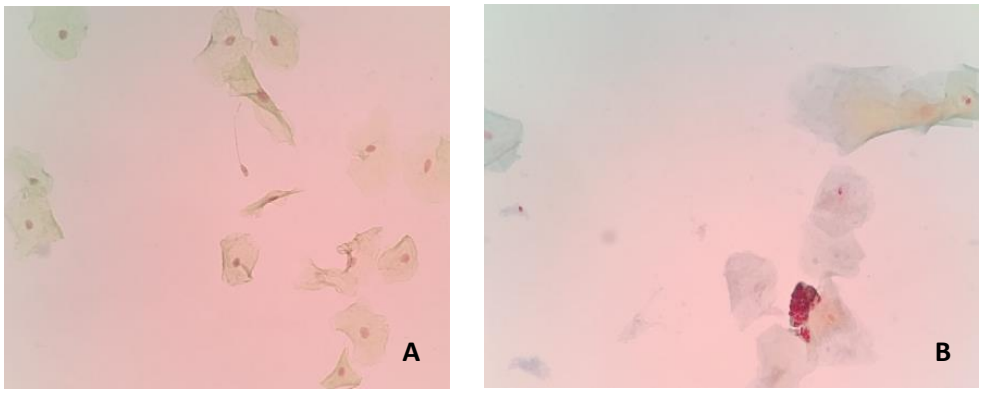

Figure 10. Microscopy observation at 40x. The A sample is vaginal wash 2010 and B is 2011.

Usually, in all samples that contained vaginal fluid cell structures similar to sperm cells were found, making sometimes hard the distinction between them except when differentiation between acrosome and nuclei could be noticed. It has also to be taken into account that in samples where only sperm was present (without DTT) for some of the cells the differentiation

between acrosome and nuclei could not be seen. This means, that maybe the lowest dilutions where only structures similar to sperm were found, could be in fact sperm with the nuclei not separated from the acrosome. So when the distinction between acrosome and nuclei cannot be noticed, in fact, it could be possible that the structure is a sperm cell (false negative).

A clearly distinctive structure of sperm cells is the tail. Some of the preparations tails could be seen very easily in many cells, but in others prepared in the same day and similar conditions, not. No clear pattern or an explanation for what could affect the presence of the tails was found.

4. Selectivity/specificity. Several fluids and cell types that could be sperm cells false positives were analyzed.
    a. The difference of sperm cells from other cell types was well seen and no very similar cells that could be confused with sperm were found (Fig. 10).
    b. Comparing the dog sperm cells with human's, small differences were found; the ones from the dog are bigger and they have quite a larger acrosome portion. This characteristic allowed to make a good distinction between the two (Fig. 10).

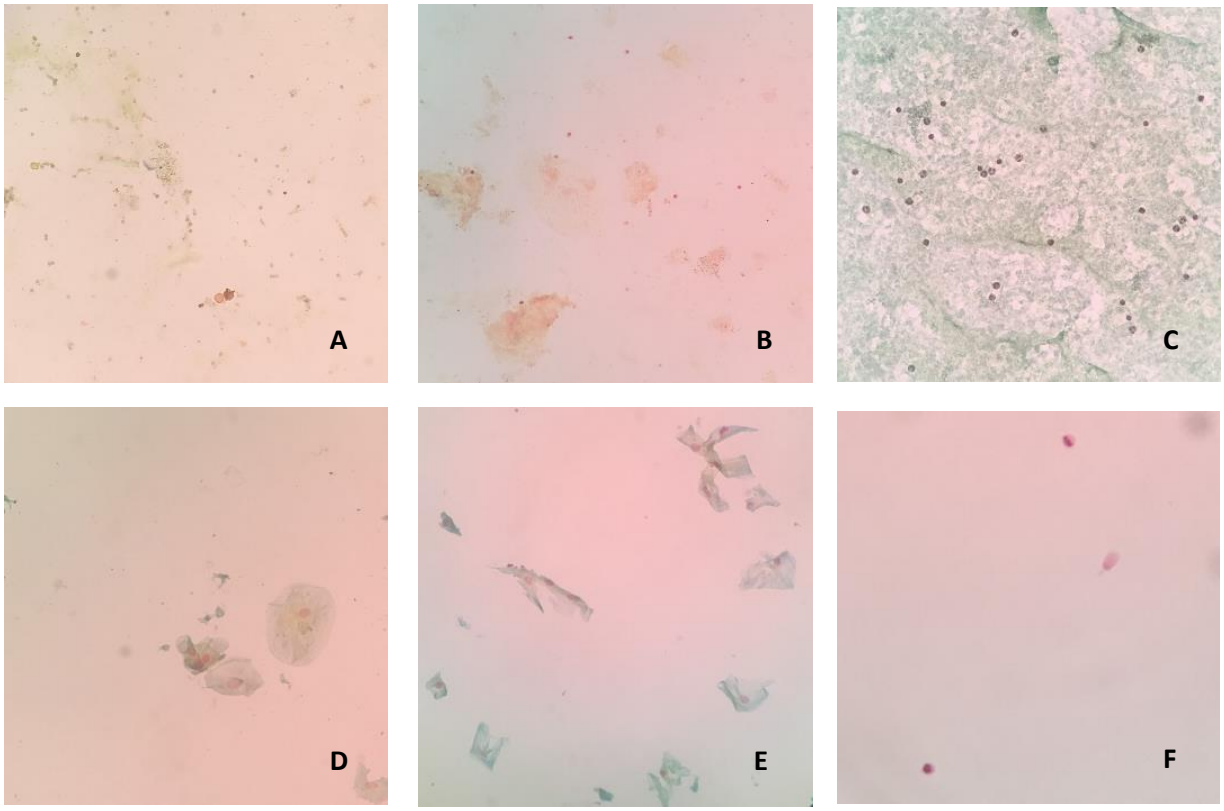

Figure 11. Microscopy slides observed at 40x. A is sanitary napkin, B is sanitary napkin + semen, C is blood, D is oral fluid, E is vaginal fluid and F is human + dog sperm. All samples except F are at the same scale. Zoom was made to see better sperm cells in F.

CONCLUSION

The first step in this validation study of microscopy observation of sperm cells was an optimization step. It has been found that semen can be applied directly to the slide, and there was no apparent difference between maceration of the swab with water or TE in the staining. The use of DTT appeared to increase the staining quality in high volume semen samples (30 µL) but in lower volumes, many sperm cells were poorly stained and no difference between acrosome and nuclei could be detected resulting in a negative identification. Also, considering the maceration time of the swabs, there was no clear difference between 1, 6 and 12 h.

The reproducibility of the method on several substrates (jeans fabric, sanitary napkin, pantie liner, panties and sterile swabs) using 50 µL of semen volume was assured obtaining a score of 4 for all samples.

The sensibility has been evaluated for sterile (for which the reproducibility was also tested), oral and vaginal swabs, jeans fabric and sanitary napkin. For all swabs types, the detection limit was dilution 1/500, although some of the samples 1/1000 were sperm positive. On the other hand, jeans fabric and sanitary napkin the detection limit was 1/1000 because in all samples from this dilution sperm cells were found. For these samples more dilutions could be made to find if the detection limit is lower. It is worthy to highlight that no clear pattern can be established in terms of the abundance of sperm cells in the preparations. For example, for the samples of 10 and 5 µL (in contrast to higher volumes like the samples from reproducibility section), we found a high variability between them having scores ranging from 2 to 4 in the type of substrate and in some cases in the semen donor as well. Also, different results were obtained when another analyst prepared the samples in some cases. Given this variability, if deeper studies were performed, it should be considered to increase the replicates of the samples and semen donors. Knowing that sperm density in semen depends on the donor, it is expected to found some differences.

RSID Sg test also gave different results depending on the semen donor. One gave positive in a dilution of 1/1000 and the other one only until 1/500 but with low intensity so the detection limit found for these samples is the same as for the swabs dilutions (1/500).

It has been proven that the use of a centrifuge to concentrate sperm cells it is an effective way to proceed when sperm cells could not be detected if there is abundant maceration liquid left.

Sperm cells in swabs with vaginal fluid (simulating the collection of the sample) can persist for a considerable amount of time. At least for 2 weeks, high sperm density was found. Similarly, high sperm density was also found to endure during 21 days in the vaginal wash. Therefore, for both types of assays, further tests could be done considering a larger period of time. Two PSA positive samples from 2010 and 2011 were tested and only one was sperm positive, the other one could be from an azoospermic or vasectomized donor.

Furthermore, sperm cells were compared with other cells or structures that could cause confusion in the identification. Sperm cells can be confirmed when the acrosome and nuclei can be well observed, or tails are present. Nevertheless, as it was observed, in some cases the acrosome is not differentiated from the nuclei and false negatives can be made.

Eventually, in this work it has been achieved to tune the Christmas tree staining method for the identification of sperm cells for laboratory routine use thanks to the analysis of reproducibility, sensibility and specificity.

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ANNEX

1. Protocol:

Instruction 7: Christmas Tree test (confirmatory method to detect the presence of semen by visualizing sperm cells)

Method basis:

Semen is composed of sperm and seminal fluid. The distinguished cell element is sperm, but other types of cells are also found, such as epithelial cells, leukocytes, prostate cells, testicular cylinders and bacteria. Normally, an ejaculation has between 1 and 6 mL of semen, with an average of 3,5 mL and contains within a range of 50 and 100 million sperm per milliliter. Spermatozoa are constituted by three characteristic structures: the head, the mid piece and the tail with a total length of 50-60 µm and the head has an oval shape when it is seen from the front. The survival of the sperm depends on the medium to which they are subjected and the type of surface, the tails being the most susceptible to degradation and therefore more difficult to see.

Christmas tree satining, also known as Kernechtrot-picroindigocarmine, can detect sperm and other cells by combining two dyes which stain the cells structures red and green, that is why it is usually named "Christmas tree". The Nuclear Fast Red is a water-soluble acid aminoacnthraquinone that reacts with cell nuclei resulting in red coloration. The result is usually a less intense coloration in the nucleus of the epithelial cells due to a greater laxity of the DNA, and intense red in the nucleus of the spermatozoa because it is usually more condensed and on the other hand, pinkish/white in the acrosome (small deposit located at the apical end of the sperm head). The other dye is Picroindigocarmine, formed by picric acid (or trinitrophenol) and indigo carmine (or indigodisulfonic acid sodium salt). This is also water-soluble and it is used for the staining of collagen fibers of connective tissues and cytoplasm, which will allow to see the tails of the sperm and cytoplasm of mostly epithelial cells that may be present in the sample.

7.1 Assay preparation

a. Materials

- Bucket
- Microscope glass slides
- 56 ºC oven
- Optical microscope
- Whatman filter paper
- Precision analytical balance
- Beaker
- Volumetric flask
- Funnel
- Magnetic stirrer
- Refrigerator

b. Reagents

The necessary solutions are:

- Water grade I (milli-Q)
- 96% Etanol
- Aluminium suphate ($Al_2(SO_4)_3*16H_2O$) ≥ 95%
- Nuclear Fast Red ($C_{14}H_8NNaO_7S$)
- Indigocarmine

- Picric acid (saturated solution)
- Coverslip sealant

c. Sample preparation

For fabric stains that could contain semen, 5mm x 5mm piece is cut out and left macerating in 200 µL water in an Eppendorf tube for a minimum of 2 h. The fabric piece has to be covered in water and vortex it occasionally.

For samples collected with swabs, these are cut out and it is left to macerate in water in an Eppendorf tube a minimum of 2 h. The swab has to be covered in water and vortex it occasionally.

For liquid samples, both vaginal wash and semen can be applied directly to the glass slide.

d. Precautions:

- Use of gloves and lab coat: the seminal fluid and all the materials that have been in contact with it must be handled and disposed of as potentially capable of transmitting infection.
- Nuclear Fast Red: irritant, wear suitable clothing, mask, goggles and protective gloves.
- Picroindigocarmine: toxic by inhalation, by contact with skin, and by ingestion. Explosive when it is allowed to dry. Wear suitable clothing, mask, goggles and gloves.

a. Preparation of Nuclear Fast Red reagents:
    - Reagents
        - Aluminum sulfate
        - Nuclear Fast Red
        - Millli-Q water
    - Preparation steps:
        - Heat 250 ml of Millli-Q water until boiling
        - Dissolve 6,25 g of Aluminum Sulfate
        - Add 125 mg of Nuclear Fast Red
        - Stir until total dissolution
        - Let the solution cool down
        - Filter with filter paper
        - Make up to 250 ml with Milli-Q water
        - Keep ii refrigerated at 2-8 ºC (Reagent expires in 6 months)

b. Preparation of Picroindigocarmine reagents:
    - Reagents:
        - Indigocarmine ($C_{14}H_8N_2NaO_8S_2$)
        - Picric acid ($C_6H_3N_3O_7$)
        - Milli-Q water
    - Preparation steps:
        - Weigh 0,823 g of indigo carmine and dissolve in 250 ml of saturated picric acid solution
        - Stir at room temperature during the night
        - Filter with filter paper
        - Keep ii refrigerated at 2-8 ºC (Reagent expires in 6 months)

Observations:

- Do not let dry, add to the bottle the same volume of picric acid as the one extracted with milli-Q water.

e. Controls to make before starting to work

Check the expiration date of the reagents.

7.2 Assay steps

1. Clean the Surface of the slide with ethanol to remove any dust and grease particles.
2. Make an extension on the slide from a sample volume or a mash obtained from a tissue fragment or a swab (10 µL in case of differential lysis or direct vaginal wash or 30 µL for macerated samples).
3. Fix the cells to the slide by incubating in the oven at 56 ° C for at least 30 min, or until the water evaporates.
4. Add 1 or 2 drops of Fast Red Nuclear dye to cover the entire extension and incubate for 15 minutes (do not exceed the time indicated because the dye could dry on the sample).
5. Gently wash with milli-Q water until the Nuclear Fast Red is removed (approximately 5 s). Do not throw the water directly upon the cells because the pressure could drag the material.
6. Add 1 or 2 drops of picroindigocarmine dye to cover the entire extension and leave it between 15 and 20 seconds.
7. Wash gently with 96% ethanol and allow to dry at room temperature.
8. Place the cover on the slide using a fixative material, allow to dry and observe under a microscope.
9. If no spermatozoa are detected in the sample, you can try to centrifuge the mash, partially remove the supernatant to concentrate the sample and repeat the staining.

Results interpretation:

- Positive (+): it is considered a positive identification of sperm cells when i) cells with different coloration between the acrosome and the nucleus are observed, ii) when the tail of the spermatozoa is observed and / or iii) when the sperm cells (oval shape) are well differentiated from other cell types with nucleus and cytoplasm (usually larger and less intensely stained). If sperm cells are observed, the presence of semen is confirmed. In the worksheet it has to be specified the category observed (from 0 to 4 at 40X). If it is possible a photograph of the results should be made to be included in the case folder. The categories of the results are:
    - 0: absence of sperm in the preparation
    - 1: 1-5 spermatozoa observed in the preparation
    - 2: 2-5 spermatozoa observed in at least 3 fields or more than 5 sperm in the whole preparation, but very dispersed (not assignable to later categories)
    - 3: more than 5 spermatozoa observed in at least 3 fields
    - 4: numerous sperm observed in several fields

- Negative (-): If sperm cells are not observed, the presence of semen cannot be confirmed.
- Inconclusive: the presence of cellular structures that are also detected with this staining can make it difficult to identify sperm. Some of these structures could be: yeasts, bacteria, free cell nuclei, leukocytes or even sperm from another animal (dog). In the latter case, it is recommended to compare with control samples (from semen and cell structures different from human sperm) and see if the morphological characteristics allow their differentiation.

2. Worksheet

Worksheet: **Preliminary tests**
Code: FT/XF/01-07
Reference procedure: PE-XF-01
Instruction 7

Analyst: Date:

Samples:

_____
_____
_____

Description of the samples:

_____
_____
_____

Preparation date of the reagents
Picroindigocarmine:
Nuclear Fast red:
Coverslip sealant:

Volume used for the maceration:
Time of maceration:

Variations of the instruction:

_____
_____

Observations:_____
_____
_____
_____

Results:

_____

_____

_____

_____

_____

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Results categories:

0: absence of sperm in the preparation

1: 1-5 spermatozoa observed in the preparation

2: 2-5 spermatozoa observed in at least 3 fields or more than 5 sperm in the whole preparation, but very dispersed (not assignable to later categories)

3: more than 5 spermatozoa observed in at least 3 fields

4: numerous sperm observed in several fields