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TECHNICAL NOTE

Immunohistochemical staining of human sperm cells in smears from sexual assault cases

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Abstract In the routine clinical examination of sexual assault victims, apart from documenting physical evidence of abuse, securing evidence, typically DNA from blood, semen, or saliva, is an important part of the process. Often the presence of semen is considered a most interesting piece of evidence. Not only does it often contain enough DNA for DNA profiling, but it also strongly indicates that an actual sexual act has taken place. The examination of smear slides obtained in sexual assault cases is a time-consuming task especially for the less trained and in cases where the smear only contains few sperm cells. In this work the goal was to develop a procedure to rapidly visualize human sperm cells in smear slides with the use of bright-field microscopy. Using SPERM HY-LITER™ by Independent Forensics, human sperm cells are visualized using a fluorescently labeled mouse antibody which significantly decreases the time used to screen smears. By further using an EnVision+ (DAKO) protocol, it was possible to create an immunohistochemical staining method to visualize sperm cells in bright-field microscopy with the same level of certainty as when using SPERM HY-LITER™.

Keywords Sexual assault evidence · Human sperm cells · Bright-field microscopy · SPERM HY-LITER™ · Immunohistochemical staining method

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Introduction

In the routine clinical examination of sexual assault victims, apart from documenting physical evidence of abuse, such as bruising, scratches, and stains, securing evidence, typically DNA from blood, semen, or saliva, is an important part of the process. Often the presence of semen is considered a most interesting piece of evidence. Not only does if often contain enough DNA for DNA profiling but it also strongly indicates that an actual sexual act has taken place.

At this institute the routine forensic examination includes sampling the introitus and the fornix posterior of the vagina with a cotton swab. Other areas of interest due to the statements of victim and witnesses or injuries are also wiped down. Smear slides of the cotton swabs are made immediately before the cotton swabs are packed and sent to the Institute of Forensic Genetics in Copenhagen. The slides are then normally kernechtrot picroindigocarmine (KPIC) stained and examined for sperm cells using bright-field microscopy. The microscopy provides a rapid answer to whether or not the sample is likely to contain enough DNA for profiling and is one of the traditionally used methods together with prostate specific antigen, seminal acid phosphatase and semenogelin measurements for indication of semen or seminal fluids in the sample [1–5].

The examination of smear slides is a time-consuming task especially for the less experienced and in cases where the smear only contains few sperm cells. Using SPERM HY-LITER™ by Independent Forensics, human sperm cells are visualized using a fluorescently labeled mouse antibody which significantly decreases the time used to screen smears. SPERM HY-LITER™ specifically stains human sperm cells and is validated by several laboratories, including the Danish Institute of Forensic Genetics, however unpublished [6]. The kit is used by the FBI and is now used as standard

examination by the Danish institute of forensic genetics on all sexual assault evidence (SAE). Unfortunately fluorescence microscopes are not a standard piece of equipment at the Danish institutes of forensic medicine.

In this work the goal was to develop a procedure to visualize human sperm cells in smear slides, in an equally time-saving procedure as when using SPERM HY-LITER™ but with the use of bright-field microscopy. The obvious choice would be to use immunohistochemical staining as often used in routine histological pathological examinations. The commercially available antibodies are however usually targeting premature sperm cells and used in the diagnostics of infertility and other spermiogenesis defects [7–10].

Materials and methods

As SPERM HY-LITER™ is a tested product produced to visualize ejaculated sperm cells, we decided to use this as the base for our study. Since the epitope of the fluorescently labeled mouse antibody is not commonly available, it would be time consuming or impossible to try to identify it. Instead we decided to try to target the mouse antibody.

For this study 12 vaginal smears, four oral smears, and 15 smears from sperm samples were investigated (Tables 1 and 2). Sperm samples, containing living normal sperm cells, were obtained from the fertility clinic of the University Hospital of Odense. Vaginal smear slides were collected from sexual assault cases, examined at the Institute of Forensic Medicine, University of Southern Denmark, Odense.

SPERM HY-LITER™ does, according to the manufacturer, specifically stain human sperm heads, with a fluorescently labeled mouse antibody which can be visualized using a fluorescein or Alexa 488 filter. It furthermore incorporates a nucleic acid stain making it possible to visualize both sperm cell nuclei and nuclei from epithelial cells, using a DAPI filter [11]. All information on the SPERM HY-LITER™ kit including technical information and validation report can be found online (http://www.ifi-test.com/shl.php).

Following the product protocol with the manufacturer's suggested modification of adding 10 µl dithiotreitol (DDT) solution per drop of sample preparation solution, sperm sample smears and vaginal smear slides were stained with the SPERM HY-LITER™ kit [12, 13]. After the staining the unmounted slides were controlled using fluorescence microscopy and the sperm cells counted. The slides were then stained with EnVision+, from the Danish-based company DAKO, a one-step protocol targeting primary mouse antibody, using the protocol from the Department of Clinical Pathology, University Hospital of Odense. Different incubation periods and washing methods were tested, resulting in the following optimized protocol for EnVision+:

1. A 3×2-min wash in TNT buffer (0.10 M Tris pH 7.5—0.10 M NaCl and 0.05% Tween20),
2. Fifteen minutes incubation with “Ready-to-use” EnVision+ polymer (K4001),
3. A 4×2-min wash in TNT buffer, and
4. Five minutes incubation with DAB+ (DakoCytomation K3468—1 ml buffered substrate/drop of DAB Cromogen).

Table 1 Comparison of number of sperm cells found in vaginal smears, using only SPERM HY-LITER™ and with SPERM HY-LITER™ and EnVision+ staining

Slide no.	Smear type and quantum of epithelial cells	No. of sperm cells found, SPERM HY-LITER™ staining using fluorescence microscopy	No. of sperm cells found, SPERM HY-LITER™ and EnVision+ staining
1	Vaginal smear, some epithelial cells	0	0
2	Vaginal smear, a lot of epithelial cells	0	0
3	Vaginal smear, a few epithelial cells	0	3
4	Vaginal smear, some epithelial cells	>40	36 and 8 imprints in halo
5	Vaginal smear, a lot of epithelial cells	>100	>100
6	Vaginal smear, some epithelial cells	0	0
7	Vaginal smear, a lot of epithelial cells	0	0
8	Vaginal smear, some epithelial cells	0	0
9	Vaginal smear, some epithelial cells	>100	>100
10	Vaginal smear, some epithelial cells	5	3
11	Vaginal smear, some epithelial cells	>200	>200
12	Vaginal smear, no epithelial cells	0	0
13	Oral smear, a few epithelial cells	0	0
14	Oral smear, a few epithelial cells	0	0
15	Oral smear, a few epithelial cells	0	0
16	Oral smear, a few epithelial cells	0	0

Table 2 Comparison of number of sperm cells found in sperm sample smears using only SPERM HY-LITER™ and with SPERM HY-LITER™ and EnVision+ staining

Slide no.	No. of sperm cells found, SPERM HY-LITER™ staining using fluorescence microscopy	No. of sperm cells found, SPERM HY-LITER™ and EnVision+ staining
1	>500	>500
2	>500	>500
3	>500	>500
4	>500	>500
5	>500	>500
6	>500	>500
7	>500	>500
8	>300	>300
9	>200	>200
10	>500	>500
11	>100	>100
12	>500	>500
13	>300	>300
14	>500	>500
15	>300	>300

The EnVision+ kit includes the "Ready-to-use" EnVision+ polymer (K4001) and the DAB+ components. After the protocol was optimized, further testing was done on smears from mouth swabs.

Results and discussion

After the staining with SPERM HY-LITER™, the slides were examined using fluorescence microscopy, sperm cells were counted, and the results compared to the number of sperm cells identified after the following EnVision+ staining. In the SAE smears, there were no differences in the number of sperm cells found (Table 1), and the sperm sample smears also showed identical results (Table 2). Strongly indicating that the EnVision+ protocol did stain all the previously SPERM HY-LITER™ labeled sperm cells.

The optimized EnVision+ protocol induces an intense brown color to the previously SPERM HY-LITER™-stained sperm cells (Fig. 1). In all but one smear, it was possible to indentify the vaginal cells, however the faint staining in some cases demanded a higher magnification to do so. The background coloring of epithelial cells in the smears seems to be more intense in the slides which contain sperm cells and more so especially in epithelial cells overlaying sperm cells. This could indicate a "leaking" of proteins from the sperm cells being caught in the folds of the epithelial cells, a "leaking" which could also be suggested by the halo surrounding the heads of the sperm cells in most of the slides examined. As the DDT added in the SPERM HY-LITER™ staining induces permeability in the sperm cells, this seems a logical explanation.

Fig. 1 Sperm cell stained with SPERM HY-LITER™ and EnVision+ at ×60 magnification

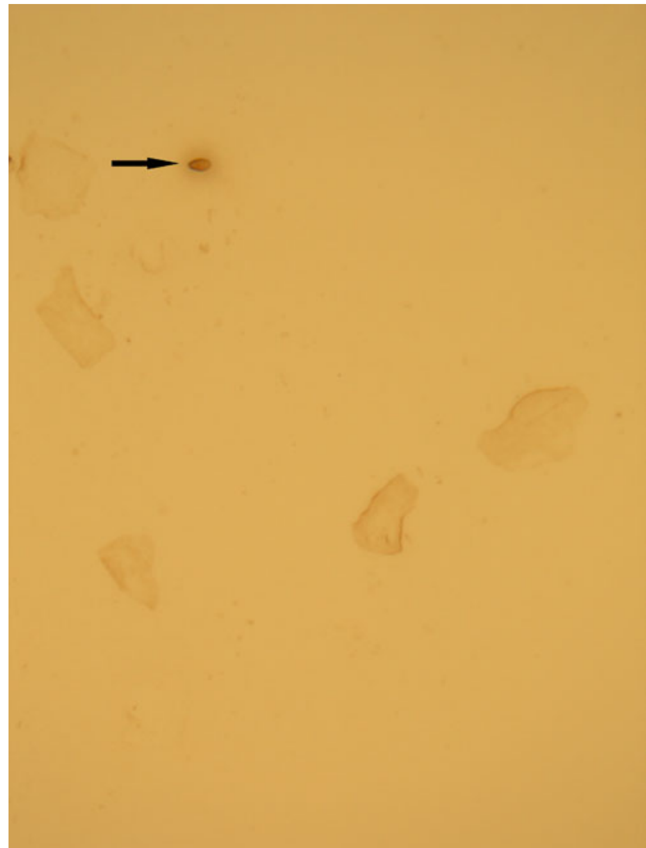

Fig. 2 A typical vaginal smear from a sexual assault case, showing a dark brown sperm cell (*arrow*) and the outline of several epithelial cells, ×20 magnification

In the vaginal smears where no sperm cells were detected, only very little and in one case no background coloring at all was observed. In most cases showing a faint outline of the epithelial cells, no nuclei were stained. This was also the case in the slides containing epithelial cells from the mouth. This would indicate that the EnVision+ combined with SPERM HY-LITER™ does not stain epithelial cells and gives the same level of certainty as the staining with only SPERM HY-LITER™ (Fig. 2).

Furthermore crystals visible in fluorescence microscopy were not visible in the bright-field microscope yielding an even more clean background. These crystals might be removed though by further rinsing in pure water as suggested by Vandewoestyne et al. [14]. In several of the test slides done with excessive TNT washing, sperm cells were washed away, leaving only the described halo with a central imprint of the sperm cell.

The time used for the SPERM HY-LITER™ staining procedure is approximately 110 min, depending on the number of slides processed per batch, as washing and drying times may vary. The SPERM HY-LITER™ protocol contains three 30-min incubation periods. The optimized EnVision+ protocol uses approximately 35 min, resulting in a total time consumption of 145 min. Screening could in most slides be done at ×5 or ×10 magnification and in cases with only a few or slightly putrefied sperm cells at ×20 magnification.

Conclusion

The combined staining with SPERM HY-LITER™ and EnVision+ is a fairly rapid process, containing several 30-min incubation periods where laboratory personnel would be able to perform other tasks. Using bright-field microscopy to screen the slides stained with SPERM HY-LITER™ and EnVision+ is a straightforward process with an obvious time reduction compared to the KPIC-stained smear slides.

The study presents the results of a pilot study, with too low a number of examined smears to do a statistical analysis. However due to the strong indication that the EnVision+ protocol only stains cells already marked with SPERM HY-LITER™ and the lack of background coloring, nothing seems to indicate that the combined staining should not have the same sensitivity and specificity as SPERM HY-LITER™ used as a monostaining method. It is my opinion that, despite the low number of slides examined in this limited study and no testing with samples from rectum or anal canal, the method can be used for everyday use in laboratories who wish to carry out examination of smear slides from sexual assault cases with a high level of sensitivity and who do not have access to fluorescence microscopy. Further investigation on a larger material is necessary before final recommendation.

Ethical standards All sampling was done according to Danish laws and ethical standards.

Conflict of interest The author declares that there is no conflict of interest.

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