SPERM HY-LITER™ for the identification of spermatozoa from sexual assault evidence

Westring, Christian Gustav; Wiuf, Morten; Nielsen, S Jock; Fogleman, James C; Old, Jennifer B; Lenz, Camilla; Reich, Karl A; Morling, Niels

Published in:
Forensic science international. Genetics

DOI:
10.1016/j.fsigen.2014.06.003

Publication date:
2014

Forensic Science International: Genetics 12 (2014) 161–167

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

SPERM HY-LITER™ for the identification of spermatozoa from sexual assault evidence

Christian G. Westring [a,b,*], Morten Wiuf [a], S. Jock Nielsen [a], James C. Fogleman [d], Jennifer B. Old [b], Camilla Lenz [a], Karl A. Reich [c], Niels Morling [a]

[a] Section of Forensic Genetics, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Frederik V's Vej 11, 2100 Copenhagen, Denmark
[b] NMS Labs, Department of Criminalistics, Forensic Biology Section, 2300 Stratford Avenue, Willow Grove, PA 19090, USA
[c] Independent Forensics, 4600 Roosevelt Road Suite 201, Hillside, IL 60162, USA
[d] Department of Biological Sciences, University of Denver, 2190 East Iliff Avenue, Denver, CO 80208, USA

ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form 3 May 2014
Accepted 2 June 2014

Keywords:
Forensic science
Sexual assault evidence
Spermatozoa
Microscopy
Baecchi's staining method
SPERM HY-LITER™

ABSTRACT

Accurate microscopic identification of human spermatozoa is important in sexual assault cases. We have compared the results of examinations with (1) a fluorescent microscopy method, SPERM HY-LITER™, and (2) Baecchi's method for identification of human spermatozoa. In 35 artificial, forensic type samples, spermatozoa were identified in 45.7% with SPERM HY-LITER™ in Copenhagen, in 54.3% in the laboratory of the manufacturer of SPERM HY-LITER™, and 40.0% of the samples with Baecchi's staining method. When differences occurred between the two methods, it was significantly more often that SPERM HY-LITER™ detected spermatozoa when Baecchi's method did not ($t_s = 6.567$, $df = 1$, $P = 0.048$). This trend was also seen in selected compromised or degraded samples and in selected adjudicative samples. The reactions with spermatozoa from dog, horse, pig and bull were negative with SPERM HY-LITER™, whereas Baecchi's method was non-selective. Data from forensic casework samples in Copenhagen from two years (2008 and 2009) are presented. The samples from 2008 were investigated using Baecchi's method, while those from 2009 were investigated using SPERM HY-LITER™. The frequencies of positive results were similar between the two methods for the two years (27.9% and 32.1% respectively). Analysis of acid phosphatase (ACP) activity for the positive results obtained for these two years does not support the use of a negative ACP result as a prescreen for microscopic analysis for spermatozoa.

1. Introduction

The identification of spermatozoa and seminal fluids in sexual assault crimes is a critical aspect of forensic genetic investigations. Several chemical and cellular constituents of seminal fluid are often used in the identification of seminal fluid following sexual assault. For example, prostatic acid phosphatase (ACP), zinc, prostate specific antigen (PSA), seminogelin and MHS-5 [1–7] are often used as screening and/or presumptive tests in order to determine the location or presence of seminal fluid on various substrates. Furthermore, they are also useful in cases where the perpetrator is either oligospermatic or azoospermatic, where few or no spermatozoa can be found [8,9]. Despite significant advances in testing procedures and methodologies, these tests still lack the specificity and stringency needed to accurately portray them as confirmatory tests. Common binding motifs among higher primates and other mammalian species have been reported, more adequately portraying these tests as presumptive screening tools [10].

The positive identification of spermatozoa in sexual assault cases is a critical step in determining the investigative strategy for the laboratory analyst. Furthermore, in most cases, the identification of spermatozoa has an important impact on the outcome of a case. Therefore, forensic genetic laboratories devote a great deal of time, efforts and resources to search for spermatozoa. Until now, search for spermatozoa has been performed by microscopic analysis using traditional histological stains like Baecchi's staining method with acid fuchsin and methyl blue, hereafter referred to as Baecchi's method, Kernechtrot-Picroindigocarmine (KPIC) and Hematoxylin-eosin (H&E) [11–16]. These methods are based on the identification of morphological structures and staining patterns of spermatozoa (Fig. 1). However, traditional staining

* Corresponding author at: NMS Labs, Department of Criminalistics, Forensic Biology Section, 2300 Stratford Avenue, Willow Grove, PA 19090, USA.
Tel.: +1 215 366 1380.
E-mail address: christian.westring@NMSlabs.com (C.G. Westring).

http://dx.doi.org/10.1016/j.fsigen.2014.06.003
1872-4973/

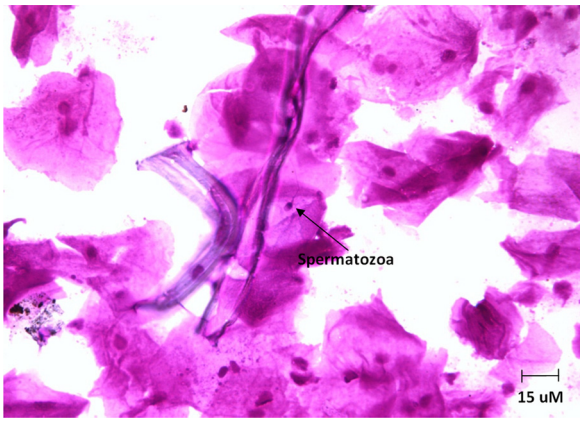

Fig. 1. Microscopic analysis of a sample stained with the Baecchi's method. Digital image was captures at 400× magnification. The scale bar was set at 15 μM. The arrow points at a sperm cell.

methods lack specificity and sensitivity, requiring extensive analyst training in order to achieve the necessary proficiency of microscopic examination of sexual assault evidence. The identification of human spermatozoa in challenging samples with low sperm count, high epithelial cell density, mixtures of cells and microorganisms from rectal samples, degradation of sperm cells with detachment of the tail from the head, etc. can be extremely difficult, often resulting in negative and/or inconclusive results. Furthermore, the examination may be very time consuming. Given the limitations of microscopic examination of samples stained with usual histological stains, improvements in specificity and effectiveness of methods for identifying human spermatozoa from sexual assault evidence would be highly advantageous.

SPERM HY-LITER™ was developed and validated for the microscopic analysis of human spermatozoa from sexual assault evidence [17]. The specificity of this method is obtained through an Alexa 488 fluorescently (green flourescein isothiocyanate – FITC) tagged monoclonal antibody, which specifically targets an antigen on the nuclear membrane of sperm cells. In conjunction with the Alexa 488 tag, the blue nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI), is also incorporated as part of the staining (Fig. 2). As a result, all cells containing DNA rich nuclei can be visualized through the selective use of a DAPI compatible fluorescent filter. This makes it possible to visualize the various cells without the need for selective degradation of epithelial/vaginal cells by proteinase K treatment prior to microscopic analysis. Due to the intense fluorescence, as few as one sperm cell can be identified among a dense sample of vaginal epithelial cells common in sexual assault type swabs.

Although the advantages of the fluorescent microscopy for identifying cellular components are widely recognized by the scientific community, its application in forensic science has been limited to date. The objective of the current study, therefore, was to compare the success of identifying human spermatozoa with the fluorescent SPERM HY-LITER™ method with that of Baecchi's method that is a traditional, histological investigation used in many laboratories. Here, we present the results of our internal validation study performed at the Section of Forensic Genetics, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Denmark.

2. Materials and methods

2.1. Sample collection procedure for the initial validation study

Sample collections from sexual assault victims were performed at the Section of Forensic Genetics, University of Copenhagen, Denmark. Sample sources were separated into two distinct categories: (1) volunteers from the Section of Forensic Genetics used to develop forensic-type samples, and (2) adjudicated sexual assault cases. Biological samples (buccal, vaginal, penile, rectal, and/or epithelial swabs) were collected using DNA-free, sterile cotton-tipped swabs from 6 volunteers with no recent history (>two weeks) of unprotected sexual activity. All sources of biological samples and/or substrate controls were screened by microscopy for the presence of human spermatozoa prior to the study. A total of 9 sexual assault-type samples were collected by a forensic pathologist from four volunteers. The time elapsed between the assault and sample collection ranged from 4 to 72 h. Duplicate samples using DNA-free, sterile cotton swabs were collected from various anatomical regions (buccal, vaginal introitus, vaginal fornix posterior, rectum, and breasts), labeled, and stored separately for independent testing.

2.2. Sample collection procedure for the cohort studies

Table 1 shows the types of samples that were analyzed. All samples from sexual assault cases in 2008 were microscopically examined with Baecchi's method and screened for ACP. All samples from sexual assault cases in 2009 were microscopically examined with the SPERM HY-LITER™ method and screened for ACP.

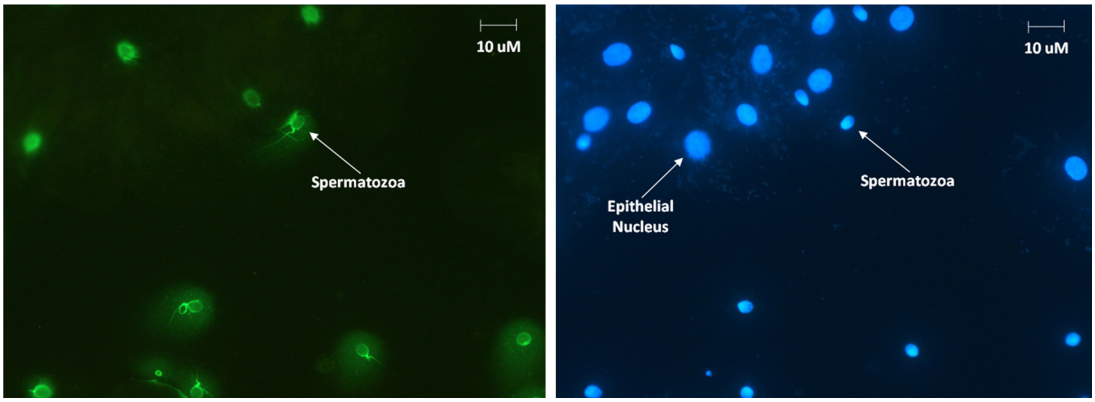

Fig. 2. Microscopic analysis of SPERM HY-LITER™ stained samples. Magnification: 400×. Green colored elements are sperm heads and a few sperm tails. Blue colored elements are nuclei. The Alexa 488 tagged spermatozoa were viewed through a selective FITC filter (left image) and characterized by a strong, green fluorescent signal. DAPI staining (right image) identify nuclei of vaginal epithelial cells and spermatozoa. The images shown are from the same field of view. The scale bar set at 10 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Categories of items investigated.

	Baecchi's method (2008)		SPERM HY-LITER (2009)	
	N = 1019	%	*N* = 1426	%
Cotton swabs	891	87.4	1147	80.4
Cloth (clothing)	87	8.5	138	9.7
Cloth (other)	13	1.3	55	3.9
Condom	15	1.5	43	3.0
Paper	2	0.2	13	0.9
Other	11	1.1	30	2.1

2.3. Sample preparation procedure

All simulated forensic-type samples were prepared in duplicate. Samples were prepared using sterile swabs and/or various substrates (cotton, denim, tissue paper, white linens, etc.). In order to test the effects of external contaminants and cellular degradation, several sample sets were also subject to chemical and/or environmental insult (10% bleach, detergent (whitening), soil, concrete, warm/moist environment at 37 °C, latex condom (nonoxynol-9 lubricated), mixed source body fluids, etc.). Each sample was inoculated with known quantities of sperm ranging from ≤10 to ≥50 sperm and left to air dry at room temperature (unless samples were subject to further environmental/chemical insult). Samples containing non-human spermatozoa were also analyzed in order to determine morphological differences and specificity of each staining method. Samples were analyzed both as single source and mixed (human/non-human sperm/buccal cell) source samples. In order to achieve low copy number samples, several serial dilutions of human semen in phosphate buffered saline (PBS) were prepared. A total of 100 μl of serially diluted semen samples ranging from 1×10^1 to 1×10^4 spermatozoa/μl were heat fixed on a Poly-L-Lysine coated slide, stained with Baecchi's stain and microscopically counted in order to achieve serially diluted standards of known concentration.

Samples were divided into various categories depending on the sample source, type, and analytical difficulty (Table 2). In all cases, each sample was assigned a unique and random alphanumeric identifier for the duration of the study. The unique identifier

Table 2
Results of microscopic examinations of 35 simulated forensic type samples.

Sample	Sample type (*N* = 35)	Quantity of sperm present	Results		
			Baecchi's method Copenhagen	SPERM HY-LITER Copenhagen	SPERM HY-LITER Illinois
090122F-1	Buccal Swab	Low (≤10 sperm cells)	(−)	(−)	(−)
090122F-2	Vaginal Introitus	Low (≤10 sperm cells)	(−)	(−)	+
090122F-3	Buccal Swab	*Negative control*	(−)	(−)	(−)
090122F-4	Vaginal Fornix	Low (≤10 sperm cells)	(−)	(−)	(−)
090122F-5	Rectum	Low (≤10 sperm cells)	(−)	(−)	(−)
090122F-6	Epitheleal	Low (≤10 sperm cells)	+	+	(−)
090122F-7	Vaginal Introitus	*Negative control*	(−)	(−)	(−)
090122F-8	Buccal Swab	Standard (≤50 sperm cells)	+	+	+
090122F-9	Vaginal Introitus	Standard (≤50 sperm cells)	(−)	(−)	+
090122F-10	Vaginal Fornix	Standard (≤50 sperm cells)	(−)	(−)	+
090122F-11	Rectum	Standard (≤50 sperm cells)	(−)	+	(−)
090122F-12	Epitheleal	Standard (≤50 sperm cells)	+	+	+
090122F-13	Vaginal Fornix	*Negative control*	(−)	(−)	(−)
090122F-14	Degraded Sample, Buccal Swab	Standard (≤50 sperm cells)	+	(−)	+
090122F-15	Degraded sample, Vaginal Introitus	Standard (≤50 sperm cells)	(−)	+	+
090122F-16	Degraded Sample, Vaginal Fornix	Standard (≤50 sperm cells)	(−)	+	(−)
090122F-17	Degraded Sample, Rectum	Standard (≤50 sperm cells)	(−)	(−)	(−)
090122F-18	Degraded Sample, Epitheleal	Standard (≤50 sperm cells)	+	+	+
090122F-19	Semen stain on denim with 10% bleach	Neat semen	+	+	(−)
090122F-20	Rectum	*Negative control*	(−)	(−)	(−)
090122F-21	Semen stain on white paper towel	Neat semen	+	+	+
090122F-22	Semen stain on white toilet paper	Neat semen	+	+	+
090122F-23	Semen mixed with soiled applicator	Standard (≤50 sperm cells)	(−)	(−)	(−)
090122F-24	Semen swab from concrete	Standard (≤50 sperm cells)	+	(−)	+
090122F-25	Semen stain on denim fabric	Neat semen	+	+	+
090122F-26	Semen stain on cotton applicator with blood	Neat semen	+	+	+
090122F-27	Semen stain on cotton applicator with menstrual blood	Standard (≤50 sperm cells)	(−)	(−)	+
090122F-28	Rectum swab with semen and blood	Standard (≤50 sperm cells)	(−)	(−)	+
090122F-29	Epitheleal	Low (≤10 sperm cells)	+	+	(−)
090122F-30	Vaginal	*Negative control*	(−)	(−)	(−)
090122F-31	Semen in condom	Neat semen	+	+	+
090122F-41	Vaginal with blood	Low (≤10 sperm cells)	(−)	(−)	(−)
090122F-42	Vaginal with blood	Standard (≤50 sperm cells)	(−)	(−)	+
090122F-43	Degraded Vaginal with blood	Standard (≤50 sperm cells)	(−)	+	+
090122F-46	Gray cotton cloth	Neat semen	+	+	+
	Positive results		14	16	19
	Success rate (%)		40.0	45.7	54.3

(−): Negative results.

ensured complete anonymity of known individuals and eliminated any potential bias based on known sample source/ID. All procedures for testing and applied experimental research on human samples were performed in accordance with the standard operating procedures used for accredited forensic casework by the Section of Forensic Genetics, the agreement with the Danish Ministry of Justice concerning development of new methods for forensic genetic case work material, and the approval by the Danish ethical committee (KF-01-037/03 and H-1-2011-081).

2.4. Sample extraction and staining

The extraction of biological material from swabs/stains from simulated and/or sexual assault cases was done in accordance with the standard operating procedures of the serology laboratory. In short, a small (approximately 0.5–1 cm^2 piece of fabric, or 25% of a cotton swab) piece of sampling material (cotton swab, fabric, etc.) was placed in 500 μl of double deionized water (ddH_2O) and sonicated for 30 min in a 1.5 ml Eppendorf tube. The suspended cellular debris was transferred to a new 1.5 ml Eppendorf tube and pelleted by centrifugation. A total 400 μl of supernatant was removed and the pellet was resuspended in the remaining 100 μl of ddH_2O. Two aliquots of 50 μl from each sample were then transferred to the sample window of a labeled microscope slide. For consistency, both Baecchi's and SPERM HY-LITER™ stained samples were analyzed on hydrophobic masked 11 mm circular well microscope slides (Part Number 9111-25, IFI, Hillside, IL).

Samples were stained with Baecchi's stain according to the standard operating procedures of the serology laboratory. In short, 50 μl of cellular extract was aliquoted onto hydrophobic masked 11 mm circular well microscope slides. Samples were heat fixed at 37 °C until dry and fixed with 96% ethanol. The fixed cellular extracts were stained for 1 min using 2–3 drops of Baecchi's stain (2.5 ml 1% methyl blue, 7.5 ml 1% acid fuchsin, 2.7 ml 12 M HCl, 87.3 ml ddH_2O). Excess stain was removed with a gentle stream of 96% ethanol and air dried prior to final fixation with Permount mounting medium and cover slip (Fisher Scientific, catalog number SP15-100 and 12-544A).

The staining procedure for the SPERM HY-LITER™ kit (Part Number 7250, IFI, Hillside, IL) was followed according to the manufacturer's suggested protocol (see SPERM HY-LITER *PLUS*™ Staining Protocol). In short, 50 μl of cellular extract was aliquoted onto hydrophobic-masked 11 mm circular single-well microscope slides. Samples were dried at 37 °C and fixed with 96% ethanol. Slides were stained using: 2 drops of Fixative (white cap) – incubate 10 – wash with 1X Wash buffer – add 2 drops of Sample Preparation solution (yellow cap) with 2 μl 1 M 1,4-Dithiothreitol (DTT) (10 μl for rectal samples) – incubate 30 min – wash with 1X Wash Buffer – add 2 drops of Blocking solution (red cap) – incubate 30 min – wash with 1× Wash buffer – add 2 drop Sperm Head Stain solution (green cap) – incubate 30 min – wash with 1× Wash buffer. Samples were air dried for 30 min prior to final fixation with 1 drop of mounting media (blue cap) and a cover slip. SPERM HY-LITER™ stained samples were stored in dark storage boxes until analysis.

2.5. Microscopic examination, data imaging and storage

The microscopic analysis of sexual assault type samples (Baecchi's and SPERM HY-LITER™) was performed by two analysts from the Section for Forensic Genetics using a Leica DM6000B fluorescent/light transmission microscope (Leica Microsystems, Germany). The complete sample set was first analyzed by Baecchi's method and light transmission microscopy under 100–400× magnification. All results and digital images of positive/negative slides were recorded in accordance with the standard operating procedures of the Section for Forensic Genetics. The SPERM HY-LITER™ stained slides were analyzed by fluorescent microscopy under 100–400× magnification using FITC and DAPI filters [17]. Each slide was scanned from side to side, and digital images of positive/negative slides were taken and stored for further analysis. All microscopic analyses on forensic-type samples at the Section of Forensic Genetics were performed in sequential order without previous knowledge of sample source, content and/or results.

A duplicate set of samples was microscopically analyzed by staff members at Independent Forensics, Hillside, IL. The results from both laboratories were kept confidential until the final comparison or the results of the study.

2.6. Examination of acid phosphatase activity (ACP)

A modification of the test of Huggins and Talalay (1945) was used [18]. The test is based on the colorless sodium phenolphthalein phosphate as a substrate that is cleaved into phenolphthalein producing redness at alkaline pH. The investigation was performed by applying filter paper saturated with the indicator substrate buffer to the objects for 2 min and spraying with alkaline buffer and determining the color of the filter paper.

3. Results

3.1. Microscopic analysis of simulated forensic type samples

Figs. 1 and 2 show representative results obtained with the Baecchi's method and SPERM HY-LITER™, respectively. Negative control swabs from six volunteers were negative for the presence of human spermatozoa with both staining methods. Intimate swabs were used to develop a comprehensive sample set of negative controls (5 samples), low sperm count, i.e. ≤10 spermatozoa, standard count, i.e. ≤50 spermatozoa, and other routine/challenging forensic-type samples containing varying quantities of semen (35 samples total). Table 2 shows the results of microscopic examinations of the 35 simulated forensic-type samples with Baecchi's method and SPERM HY-LITER™ performed in both Copenhagen and Illinois. Whole spermatozoa and spermatozoan heads were characterized by size, morphological shape, and the staining of the cell (acrosome, plasma membrane, nucleus, tail, etc.). A binary scoring system ("+" one or more sperm cells confirmed (with or without heads), "−" no sperm cells confirmed) was used in the assessment of each sample. No effort was made to quantify the number of sperm observed in different samples as this is not typically done for actual forensic samples. Sperm cells were identified in 40.0% of the samples with Baecchi's method, 45.7% with SPERM HY-LITER™ in Copenhagen and in 54.3% with SPERM HY-LITER™ in Illinois. Excluding the negative controls, there were six differences between the results obtained with Baecchi's method and SPERM HY-LITER™ in Copenhagen and 11 differences between Baecchi's method and SPERM HY-LITER™ in Illinois, and the differences were not correlated with sperm quantity. These two comparisons are replicates since the same samples were analyzed in Copenhagen and in Illinois. If differences were random with respect to detection, then one would expect half of the differences to be in the +/- direction (detection by Baecchi's method and no detection using SPERM HY-LITER™) and half the differences to be in the +/- direction (no detection by Baecchi's method and positive detection using SPERM HY-LITER™). The results in Table 2 show that the differences are predominately in the +/- direction (4/6 for the comparison performed in Copenhagen and 8/11 for the comparison done in Illinois). A one-tailed *t*-test of the mean of the two replicates (average +/- = 0.697) versus an expected value of 0.500 was performed. A one-tailed test was used because the overall success rate was higher using SPERM

Table 3
Species specificity: microscopic examination of human and non-human spermatozoa mixed with human epithelial vaginal cells.

Sample	Sample type ($N = 10$)	Baecchi's method Copenhagen	SPERM HY-LITER Copenhagen
090122F-47	Epithelial cells without human sperm	(−)	(−)
090122F-48	Epithelial cells and human sperm	+	+
090122F-49	Epithelial cells and horse sperm	+	(−)
090122F-50	Epithelial cells and pig sperm	+	(−)
090122F-51	Epithelial cells and bull sperm	+	(−)
090122F-52	Epithelial cells and horse plus human sperm	+	+
090122F-53	Epithelial cells and pig plus human sperm	+	+
090122F-54	Epithelial cells and bull plus human sperm	+	+
090122F-55	Epithelial cells and dog sperm	+	(−)
090122F-56	Epithelial cells and dog plus human sperm	+	+
	Correct results	6	10
	Incorrect results	4	0

(−): Negative results.
+: Positive results.

HY-LITER™ (Table 2), so we were specifically interested in testing whether that method was more effective than Baecchi's method. The results support the contention that, when differences between the two techniques occur, the differences are more often that SPERM HY-LITER™ detects spermatozoa when Baecchi's method does not ($t_s = 6.567$, $df = 1$, $P = 0.048$).

3.2. Specificity of the SPERM HY-LITER™ examinations in simulated forensic type samples

Table 3 shows the results of 10 samples containing human vagina epithelial cells mixed with either human sperm or animal sperm and analyzed with Baecchi's method and SPERM HY-LITER™. Baecchi's method returned a positive result whenever sperm were present regardless of the species contributor. In all cases, SPERM HY-LITER™ correctly identified the presence of human spermatozoa and was negative when the only spermatozoa present were from a nonhuman source. Thus, Baecchi's method was nonspecific whereas SPERM HY-LITER™ successfully discriminated between human and nonhuman spermatozoa.

3.3. Comparison of SPERM HY-LITER™ and Baecchi's method in selected sexual assault samples

Table 4 shows that in 9 samples that were taken from adjudicated sexual assault cases, Baecchi's identified spermatozoa in 44.4% of the samples while the SPERM HY-LITER™ method was positive in 55.6% (Copenhagen) and 66.7% (Illinois) of the samples, respectively. The small sample size does not allow a statistical analysis. However, all three of the differences between Baecchi's method and either of the replicates using the SPERM HY-LITER™ method were in the +/- direction. That is, samples in which SPERM HY-LITER™ identified multiple spermatozoa whereas no sperm cells could be demonstrated with the Baecchi's method.

Focusing on challenging samples (rectal or degraded samples in Tables 2 and 4), we again looked at differences between Baecchi's method and the two replicates of SPERM HY-LITER™. There were 7 differences between Baecchi's method and either the Copenhagen or the Illinois replicates. Of these, 3 out of 4 differences for samples done in Copenhagen and 2 out of 3 differences for samples done in Illinois were in the +/- direction (positive detection using SPERM HY-LITER™ when no spermatozoa were detected with Baecchi's method). Using the same test and assumptions as in Section 2.1 above, we statistically tested the hypothesis that the SPERM HY-LITER™ method was more effective at detecting spermatozoa in these degraded or otherwise compromised samples. The average +/- proportion for the two samples was 0.710 and this was tested against a presumed 0.500 proportion that would be expected if differences were random. The results were suggestive but not statistically significant at the 0.05 level ($t_s = 5.250$, $df = 1$, $P = 0.06$).

3.4. Evaluation of the methods in forensic routine casework

The results obtained in Copenhagen in 2008 with Baecchi's method were compared with those obtained in 2009 with the SPERM HY-LITER™ test. There was no difference in strategy, etc., except for the change from Baecchi's method to SPERM HY-LITER™. The presence or absence of spermatozoa detected

Table 4
Microscopic analysis of 9 sexual assault type samples (adjudicated cases).

Sample	Sample type ($N = 9$)	Baecchi's method Copenhagen	SPERM HY-LITER Copenhagen	SPERM HY-LITER Illinois
090122F-32	Vaginal Fornix	+	+	+
090122F-33	Rectum	+	+	+
090122F-34	Vaginal Fornix	+	+	+
090122F-35	Vaginal Introitus	+	+	+
090122F-36	Buccal Swab	(−)	(−)	+
090122F-37	Vaginal Fornix	(−)	+	+
090122F-38	Vaginal Introitus	(−)	(−)	(−)
090122F-39	Vaginal Fornix	(−)	(−)	(−)
090122F-40	Rectum	(−)	(−)	(−)
	Positive results	4	5	6
	Success rate (%)	44.4	55.6	66.7

(−): Negative results.

Table 5
Success rates of analyses with microscopy, serology and DNA profiling of forensic casework samples.

	Investigations in 2008 Copenhagen				Investigations in 2009 Copenhagen			
Test parameters	Positive	Negative	Total	Success rate (%)	Positive	Negative	Total	Success rate (%)
Baecchi's stain	296	765	1061	27.9%	–	–	–	–
SPERM HY-LITER™	–	–	–	–	456	966	1422	32.1%
Acid phosphatase	220	820	1040	21.2%	339	1068	1407	24.1%
Acid phosphatase if Baecchi's stain positive	176	109	285	61.8%	–	–	–	–
Acid phosphatase if SPERM HY-LITER™ positive	–	–	–	–	270	183	453	59.6%
Male DNA profile if Baecchi's stain positive	244	51	295	82.7%	–	–	–	–
Male DNA profile if SPERM HY-LITER™ positive	–	–	–	–	398	58	456	87.3%

with the two methods are compared in Table 5 along with the success rates of developing male STR-profiles for samples showing positive for spermatozoa. Spermatozoa were detected with Baecchi's method in 27.9% of the samples and in 32.1% of the samples with SPERM HY-LITER™. However, these percentages cannot be compared directly since the actual percentage of samples containing spermatozoa for the two years is not known and different methods were used in different years. The success rates of obtaining a male DNA STR profile following differential extraction after positive microscopy results were 82.7% with Baecchi's method and 87.3% with the SPERM HY-LITER™ method. A Chi Square contingency analysis of positive/negative results versus Baecchi's method/SPERM HY-LITER™ was performed. The hypothesis being tested here is whether the probability of successfully obtaining a male DNA STR profile is independent of method. Although the success rate at obtaining a male DNA STR profile is higher with SPERM HY-LITER™, the difference was not significant at the 0.05 level ($X^2 = 3.014$, $df = 1$, $P = 0.083$).

3.5. Correlation between acid phosphatase reaction and demonstration of spermatozoa in routine forensic casework

Acid phosphatase activity was demonstrated in 21.2% of the 2008 samples and in 24.1% of the 2009 samples (Table 5). Of the samples that were positive for spermatozoa with Baecchi's method and SPERM HY-LITER™, 61.8% and 59.6%, respectively, were positive for ACP. A Chi square contingency test of positive/negative results for ACP versus method did not indicate any significant difference ($X^2 = 0.339$, $df = 1$, $P = 0.561$).

4. Discussion

Identification of spermatozoa is crucial in most sexual assault cases. Several methods are used to identify spermatozoa, including microscopic examination of preparations stained with e.g. Baecchi's method. Histological staining methods are reliable for the identification of spermatozoa when the slides are scrutinized by experienced investigators, but the process is time-consuming and error prone for inexperienced investigators. With Baecchi's method, the head and tail of spermatozoa are bright crimson red. Morphologically, the staining pattern of the sperm head is characterized by a two-tone coloring of the acrosome (see Fig. 1). The light tip progressively darkens toward the centriole, where the tail connects to the head. Based upon the known size of human spermatozoa (approx. 7 μm × 5 μm, 50 μm incl. tail) and morphological staining, a trained analyst can determine the presence of human spermatozoa from a mixed pool of biological material. However, due to the presence of several species of yeast and/or microorganisms, "sperm-like" elements may be misjudged especially in vaginal and rectal swabs. The success rates of identification of spermatozoa of the mock samples were slightly better in Copenhagen and Illinois with the SPERM HY-LITER™ method than with Baecchi's method (Table 2). More importantly however, when differences occur between Baecchi's method and SPERM HY-LITER™, they are predominantly that SPERM HY-LITER™ yields a positive result when no spermatozoa are detected by Baecchi's method, and this trend is statistically significant ($t_s = 6.567$, $df = 1$, $P = 0.048$). While not statistically overwhelming, it does provide support for the statement that SPERM HY-LITER™ was more effective at detecting spermatozoa in these experiments than Baecchi's method.

The examinations of artificial, forensically relevant, 'mock' samples showed that SPERM HY-LITER™ could discriminate between (1) human and (2) dog, horse, pig or bull spermatozoa (Table 3). This can, in theory, also be achieved with Baecchi's method by a trained investigator – but it was not successful in our laboratory (Table 3). Miller et al. [17] showed that the SPERM HY-LITER™ antibody does not react with semen from common species or with other human tissues likely to be encountered in sexual assault evidence. These results support the contention that SPERM HY-LITER™ exhibits higher specificity than Baecchi's method. It should be noted that, if it were necessary to distinguish between human and non-human spermatozoa in an investigation, techniques in addition to SPERM HY-LITER™ would have to be employed.

The results of examinations of a small number of selected adjudicated sexual assault cases (Table 4) were consistent with the data presented in Table 2, in that all differences between the two methods were that spermatozoa could be detected by SPERM HY-LITER™ in samples that show negative results by the Baecchi's technique. In some samples, the presence of vaginal, rectal, and/or epithelial cells was excessive making microscopic analysis by Baecchi's method difficult and time consuming.

The comparison between results obtained in a one year period (2009) with the SPERM HY-LITER™ method and the previous year (2008) with the Baecchi's method showed that the rate of positive results from the SPERM HY-LITER™ method were slightly higher than Baecchi's method (32.1% versus 27.9%). As previously mentioned, however, these two values cannot be statistically compared. The identification of spermatozoa was associated with a slightly higher success rate of full autosomal STR profiles (SEfiler Plus) with SPERM HYLITER positive samples (87.3% of the cases) than that obtained with samples that were positive with Baecchi's method (82.7%, $P = 0.083$), but this result is not statistically significant.

SPERM HY-LITER™ also seemed to facilitate the identification of spermatozoa in challenging samples. Degraded and otherwise compromised samples, e.g. from Tables 2 and 4, were readily examined and the results were more often that the SPERM HY-LITER™ method detected sperm when Baecchi's method did not. Again, the marginal probability of the statistical test ($t_s = 5.250$, $df = 1$, $P = 0.06$) was suggestive but not significant at the 0.05 level,

and further experimentation is necessary before a definitive conclusion can be made. The biological material from these types of samples is often associated with an abundant flora of yeast and bacteria that may be a challenge to less experienced investigators when Baecchi's method is used.

The selective use of FITC and DAPI filters increased sensitivity and specificity of the SPERM HY-LITER™ method while demonstrating the ability to differentiate stained spermatozoa and other cells like vaginal cells, bacteria, yeast, etc. Non-specific binding of fluorophore-conjugated antibodies was not observed and the favorable signal-to-noise ratio made it easy to distinguish the spermatozoa in routine cases.

The comparison between microscopic results and ACP activity demonstrated a limited sensitivity and specificity of ACP testing. Only about 60% of the samples shown in Table 5 that were positive by either method were also positive for ACP, and there was no significant association between ACP +/- results and method ($X^2 = 0.339$, $df = 1$, $P = 0.561$. However, acid phosphatase activity decreases in vaginal samples over time [16] and ACP assays are not generally reliable beyond 48 h. The adjudicated sexual assault samples reported in Table 5 were collected over a variety of time spans and include samples that were collected well beyond 48 h after the alleged assault. ACP has traditionally been utilized as a screening tool for determining location or presence of seminal fluid on larger items and intimate swabs from sexual assault cases prior to microscopic analysis [7,19–22]. Given the results from this sample set, it would not be advisable to use negative ACP screening results as a "stop point" in sexual assault investigations, since about 40% of our ACP assays were negative even though spermatozoa were present. Realizing that more sensitive screening methods for seminal fluid exist, such as tests for prostate specific antigen (PSA), we have taken this into consideration and substituted a commercial test for PSA for the ACP investigation of vaginal swabs and selected items, while we still use testing for ACP of larger textiles, etc.

Our department has used Baecchi's method for years. However, the SPERM HY-LITER™ method obviously offers advantages due to the specific staining of spermatozoa. Therefore, we have decided to introduce the SPERM HY-LITER™ method in routine examinations of sexual assault evidence.

5. Conclusion

The results of the internal validation of the SPERM HY-LITER™ kit for microscopic identification of human sperm demonstrated that SPERM HY-LITER™ was generally more effective than traditional histological staining by Baecchi's method. SPERM HY-LITER™ was also shown to be specific for human spermatozoa whereas Baecchi's method did not distinguish between human and non-human sperm contributors. The higher specificity and effectiveness of SPERM HY-LITER™ allows for a faster and more reliable visual screening of evidentiary material for spermatozoa.

Acknowledgements

The work was financed by the Section of Forensic Genetics, Department of Forensic Medicine, Faculty of Health and Medical Sciences, University of Copenhagen. We thank Dr. Nikolaj Friis Hansen for his assistance in sample collections, the sample donors, Ms. Helle Byrgesen, Ms. Rikke Larsen, Ms. Susanne Solstad and Ms. Marisa Fahrner for technical assistance.

References

[1] J.P. Simich, S.L. Morris, R.L. Klick, K. Rittenhouse-Diakun, Validation of the use of a commercially available kit for the identification of prostate specific antigen (PSA) in semen stains, J. Forensic Sci. 44 (1999) 1229–1231.
[2] T.J. Wang, H.G. Rittenhouse, R.L. Wolfert, C.M. Lynne, N.L. Brackett, PSA concentrations in seminal plasma, Clin. Chem. 44 (1998) 895–896.
[3] M.N. Hochmeister, B. Budowle, O. Rudin, C. Gehrig, U. Borer, M. Thali, et al., Evaluation of prostate-specific antigen (PSA) membrane test assays for the forensic identification of seminal fluid, J. Forensic Sci. 44 (1999) 1057–1060.
[4] R. Saferstein, Forensic Science Handbook, II, 1988, pp. 348–369.
[5] L.R. Ricci, S.A. Hoffman, Prostatic acid phosphatase and sperm in the post-coital vagina, Ann. Emerg. Med. 11 (1982) 530–534.
[6] W. Keil, J. Bachus, H.D. Troger, Evaluation of MHS-5 in detecting seminal fluid in vaginal swabs, Int. J. Legal Med. 108 (1996) 186–190.
[7] P.J. Hooft, H.P. van de Voorde, The zinc test as an alternative for acid phosphatase spot tests in the primary identification of seminal traces, Forensic Sci. Int. 47 (1990) 269–275.
[8] N. Khaldi, A. Miras, K. Botti, L. Benali, S. Gromb, Evaluation of three rapid detection methods for the forensic identification of seminal fluid in rape cases, J. Forensic Sci. 49 (2004) 749–753.
[9] P.H. Whitehead, A.E. Kipps, The significance of amylase in forensic investigations of body fluids, Forensic Sci. 6 (1975) 137–144.
[10] S. Johnston, J. Newman, Validation study of the Abacus Diagnostics ABAcard® HemaTrace® membrane test for the forensic identification of human blood, Can. Soc. Forensic Sci. 36 (2003).
[11] B. Baecchi, Neue Methode zum Nachweis der Spermatozoen in Zeugflecken, Dtsch. Med. Wochenschr. 35 (1909) 1105–1106.
[12] H. Evers, F. Heidorn, C. Gruber, G. Lasczkowski, M. Risse, R. Dettmeyer, et al., Investigative strategy for the forensic detection of sperm traces, Forensic Sci. Med. Pathol. 5 (2009) 182–188.
[13] J.P. Allery, N. Telmon, A. Blanc, R. Mieusset, D. Rouge, Rapid detection of sperm: comparison of two methods, J. Clin. Forensic Med. 10 (2003) 5–7.
[14] J.P. Allery, N. Telmon, R. Mieusset, A. Blanc, D. Rouge, Cytological detection of spermatozoa: comparison of three staining methods, J. Forensic Sci. 46 (2001) 349–351.
[15] R.P.M. Celli, The demonstration of seminal fluid stains on paper. II. The treatment of laboratory studies dealing with rape, Minerva Ginecol. 46 (1994) 351–352.
[16] R.E. Gaensslen, Sourcebook in Forensic Serology, Immunology, and Biochemistry, 1983.
[17] K.W. Miller, J. Old, B.R. Fischer, B. Schweers, S. Stipinaite, K. Reich, Developmental validation of the SPERM HY-LITER(TM) kit for the identification of human spermatozoa in forensic samples, J. Forensic Sci. 56 (2011) 853–865.
[18] C. Huggins, P. Talalay, Sodium phenolphthalein phosphate as a substrate for phosphatase tests, J. Biol. Chem. 159 (1945) 399–410.
[19] F.S. Baechtel, J. Brown, L.D. Terrell, Presumptive screening of suspected semen stain in situ using cotton swabs and bromochloroindolyl phosphate to detect prostatic acid phosphatase activity, J. Forensic Sci. 32 (1987) 880–887.
[20] P. Hooft, H. van de Voorde, Comparative study of the sensitivity and specificity of the zinc and acid phosphatase spot tests for the detection of seminal stains, Z. Rechtsmed. 103 (1990) 581–586.
[21] P. Hooft, H. van de Voorde, Evaluation of the modified zinc test and the acid phosphatase test as preliminary screening methods in sexual assault case material, Forensic Sci. Int. 53 (1992) 135–141.
[22] E.W. Kivela, On finding spermatozoa in suspected seminal stains, J. Forensic Sci. 9 (1964) 138–139.