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Optimization of spermatozoa detection using immunofluorescent staining and laser micro-dissection

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ABSTRACT

The present study evaluated the use of an immunofluorescence-based assay for the microscopic detection of human spermatozoa, following which the fluorescence-labelled spermatozoa could be excised with a laser micro-dissection system. The Sperm Hy-Liter™ PI kit was able to detect spermatozoa from as little as 20 nL of semen. No interference or non-specificity were observed when the kit was used on semen mixed with various body fluids such as blood and urine, as well as when semen was spiked onto different types of fabric. Good results could also be obtained with rectal samples which contain auto-fluorescent fecal materials through the use of dual FITC/PI filters. We also developed a method for concurrent testing of two protein biomarkers of semen (semenogelin and prostate-specific antigen) and detection of spermatozoa. This approach would maximize the evidential value from a single piece of sexual assault exhibit. The results also showed that staining by Sperm Hy-Liter™ PI does not interfere with DNA recovery, facilitating the generation of clear male DNA profiles from dissected spermatozoa, thereby making profile interpretation less complex. In summary, Sperm Hy-Liter™ PI staining was demonstrated to be sensitive, robust and specific.

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1. Introduction

Chemical and cellular constituents of seminal fluid are often used in the identification of seminal fluid. For example, prostatic acid phosphatase (ACP), zinc, prostate-specific antigen (PSA), semenogelin and MHS-5 [1–6] are often used as presumptive tests for the determination of the presence of seminal fluid. Furthermore, these biochemical assays are also useful in cases involving oligospermic or azoospermic perpetrators where detection of spermatozoa may be impossible [7]. Our laboratory currently utilizes a combination of both PSA and semenogelin as biomarkers of semen [8]. However, recent publications have suggested these tests to be of insufficient specificity to be used as confirmatory tests in forensic casework. PSA and semenogelin have been reported to be found at low quantities in several types of body tissues and also in the serum of individuals diagnosed with cancer [9–13]. For these reasons, detection of seminal fluid markers is often considered suitable only as preliminary screening tools for the presence of semen. In contrast, the visualization of spermatozoa is definitive for confirming the presence of semen. Additionally, spermatozoa were demonstrated to persist longer within the vaginal vault and can be detected on post-coital samples at longer time intervals compared to these seminal fluid markers [14,15]. Current methods for visualization of spermatozoa in many forensic laboratories are based on histological staining. Commonly used stains include hematoxylin/eosin and nuclear fast red/picroindigocarmine (Christmas Tree staining) [16,17], but these are not specific to spermatozoa. Furthermore, these staining methods are also not amenable to automation or computer-aided searching because of the low levels of color contrast between the cell types of interest in these preparations [16]. The search for spermatozoa can be further complicated by the presence of cell debris or large numbers of other cells such as yeasts. Recently, fluorescence *in situ* hybridisation-based (FISH) methods have been combined with laser micro-dissection to simultaneously identify and isolate male (sperm and non-sperm) cells [18–21]. Two groups have reported using the Sperm Hy-Liter™ (Independent Forensics, Lombard, IL) [22] to specifically stain human sperm heads using a fluorescence-tagged antibody prior to their capture by laser micro-dissection [23,24]

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The Sperm Hy-Liter™ PI kit uses the Alexa 488 fluorescence-tagged (green flourescein isothiocyanate–FITC) monoclonal antibody, which is specific for an antigen on the nuclear membrane of sperm cells. A second fluorescent dye, propidium iodide (PI), is used for concurrent viewing of all cell nuclei in the sample. This makes it possible to visualize various cells within the sample without having to selectively degrade epithelial cells by proteinase K treatment prior to microscopic analysis.

The Leica® LMD6500 system (Leica Microsystems Pte Ltd, Wetzlar, Germany) consists of a fluorescence microscope coupled with a laser micro-dissection (LMD) module. After spermatozoa identification, the solid-state UV laser can dissect the detected spermatozoa into collector tubes via gravity assistance. This contact-free cell collection method would minimize the risk of sample contamination in forensic casework. The ability to dissect individual spermatozoa for DNA analysis would reduce the need to interpret mixed autosomal STR profiles resulting from incomplete separation of spermatozoa and female epithelial cell fractions by differential DNA extraction [25].

In the present study, the Sperm Hy-Liter™ PI kit was evaluated for its sensitivity, robustness, specificity and compatibility with downstream DNA extraction.

2. Materials and methods

2.1. Optimization of Sperm Hy-Liter™ PI staining

2.1.1. Sample preparation

In the current study, buccal cells were used as a proxy for vaginal epithelial cells as the same type of epithelial cells, i.e. simple squamous, is found in both oral and vaginal cavities. Buccal samples were obtained by asking volunteers to gargle 20 mL of water, which was subsequently concentrated to 3 mL to obtain a buccal suspension. For spiking of swabs, human semen was diluted accordingly with the buccal suspension. For spiking of fabrics and tissue paper, human semen was diluted accordingly with PBS (phosphate buffer saline, Sigma–Aldrich, St. Louis, MO).

Fingers of volunteers were pricked with a lancet and blood swabs were obtained by using sterile cotton swabs to sample the blood. Urine swabs were obtained by soaking sterile cotton swabs into 100 μL of human urine and dried overnight. Condom swabs were obtained by rubbing sterile cotton swabs 10–15 times against the outside and inside of unused lubricated Durex® Fetherlite condoms (Durex, Trafford Park, Manchester). For optimization and validation studies, all swabs and substrate cuttings (1 cm × 1 cm) were spiked with 100 μL and 40 μL of diluted human semen, respectively and dried overnight.

All dried samples were cut and incubated in PBS for 1 h at room temperature. Cells were pelleted by centrifuging at 10,000 × *g* for 5 min. The supernatant was removed and the cell pellet re-suspended in 50 μL of PBS. For rectal swabs, an additional washing step was performed with 400 μL of PBS to remove fecal material. Using ImmEdge™ Pen (Vector Laboratories, Burlingame, CA), a 10 mm diameter hydrophobic barrier circle was drawn on the membrane slide onto which 10 μL of cell suspension was spread.

2.1.2. Fixation method

Cell suspension from cotton swabs spiked with 1:20 human semen dilutions were either added to membrane slides and heat fixed at 50 °C for 10 min, or added to membrane slides coated with 0.1% (w/v) poly-L-lysine (Sigma–Aldrich, St. Louis, MO) and air dried for 25 min at room temperature before Sperm Hy-Liter™ PI staining. For rectal samples, rectal swabs were spiked with 1:20 human semen dilutions before being processed. The resulting cell suspensions were either added to membrane slides and heat fixed at 37 °C or 50 °C for 10 min, or added to membrane slides coated with 0.1% (w/v) poly-L-lysine and air dried for 25 min at room temperature before Sperm Hy-Liter™ PI staining.

2.1.3. Membrane slides selection

Frame PPS (poly-p-phenylene sulphide), PPS and PEN (polyethylene naphthalate) membrane slides (Leica Microsystems Pte Ltd, Germany) were evaluated for use with the Sperm Hy-Liter™ PI kit. Briefly, cotton swabs spiked with 1:20 human semen dilutions were processed and the resulting cell suspensions were heat fixed onto each type of membrane slides and subjected to Sperm Hy-Liter™ PI staining.

2.1.4. Dithiothreitol (DTT) concentration

Cell suspension from cotton swabs spiked with 1:20 human semen dilutions (three donors, in duplicates, $N = 6$) were heat fixed and stained with Sperm Hy-Liter™ PI. A separate set of cell suspensions from rectal swabs spiked with 1:20 human semen dilutions (one donor, in quadruplicates, $N = 4$) was also prepared. Independent Forensics (IFI) had recommended a final concentration of 120 mM and 240 mM DTT for general samples and rectal samples, respectively [26]. In the present study, DTT concentrations of 100, 200, 300 and 400 mM were tested for buccal samples while DTT concentrations of 200, 300 400 and 500 mM were tested for rectal samples. For staining of buccal samples, 4.5, 10, 17 and 27 μL DTT (1 M stock from Sigma–Aldrich, St. Louis, MO, pH 8.0; adjusted using 1 N potassium hydroxide) were added to 40 μL of IFI Sample Preparation Solution. For staining of rectal samples, 10, 17, 27 and 40 μL DTT were added to 40 μL of IFI Sample Preparation Solution. All membrane slides were then stained with Sperm Hy-Liter™ PI using the specific DTT volume and concentration.

2.1.5. Sperm Hy-Liter™ PI staining

Staining of the slides was carried out in accordance to the Sperm Hy-Liter™ PI kit protocol with some modifications. For each slide, 10 μL of 1 M DTT was added to 40 μL of sample preparation solution. Instead of the recommended two drops, one drop of Fixative Solution, Blocking Solution and Sperm Head Staining Solution were used for each slide. Mounting media was omitted as coverslips were not required for the membrane slides. Slides were washed between each step using 100 μL of the 1× wash buffer. An additional final wash using 1 mL of ultrapure deionized water was added to remove residual salt crystals originating from the wash buffer. This was necessary because the salt crystals were highly auto-fluorescent in the green fluorescence channel and will interfere with detection of the stained spermatozoa.

2.2. Laser Micro-Dissection system

The membrane slides were viewed on the Leica® LMD6500 using PI and FITC filters. Photographs were taken using a Leica DFC310 FX digital color camera mounted over the ocular piece. Sperm heads are identified by green fluorescence under the FITC filter while all cell nuclei are identified by red fluorescence under the PI filter. Stained spermatozoa were laser micro-dissected (laser settings: power 14/60, aperture 2/50 and speed 21/100) into the cap of a 0.5 mL microfuge tube containing approximately 20 μL of Promega Casework Extraction Buffer (Promega, Madison, WI).

2.3. Sensitivity studies

Cotton swabs were each spiked with human semen dilutions as follows: 1:10, 1:20, 1:50, 1:100, 1:300, 1:700 and 1:1000 before

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