Science and Justice 55 (2015) 118–123

Contents lists available at ScienceDirect

Science and Justice

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

Semen searching when sperm is absent

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ARTICLE INFO

Article history:
Received 28 August 2013
Received in revised form 8 January 2015
Accepted 29 January 2015

Keywords:
Semenogelin
Sperm
Sexual assault
Y-chromosome
Azoospermy

ABSTRACT

Sexual assault cases have varying factors that may mask semen findings when analysing evidence at the forensic laboratory. Semenogelin (Sg) is a potential marker for the identification of semen even at azoospermy or when few sperm cells are found. The current study examined Sg in normospermic and azoospermic donors as an internal evaluation of sensitivity, specificity and interference. The impact of a historical review of 53 judicial sexual assault cases over a five-year period was also analysed. The use of varying tests was of importance to prioritize certain samples within cases. Semen findings by Sg were then compared to prostate-specific antigen (PSA), phosphatase enzyme (AP) and Y-chromosome presence, the latter being used in an attempt to link semen fluid identification with obtaining a male DNA profile. Test findings were the highest ever registered for Sg (1:400,000), PSA (1:800,000), AP (1:25,000) and sperm cytology (SC) (1:50,000). Our results demonstrated the usefulness of using the Sg marker to avoid a false semen-negative result (6% cases), particularly in cases where sperm was absent or scarce (11% spermatozoa positive cases). Results were expressed in categories according to the set: Sg–PSA–AP. Thus, categories I (full positive, 46%), VI (full negative, 27%) and III (Sg/PSA positive; 11%) were the most frequent and Y-chromosome was obtained in 59%, 12% and 12% ratios, respectively. In conclusion, Sg was recommended for the workflow procedure of semen investigation when sperm absence is expected either from azoospermic/oligospermic or normospermic semen, especially before/after ejaculation.

1. Introduction

Some sexual assault cases are difficult to solve in the forensic laboratory, due to the scarcity or absence of spermatozoa in the semen samples. Best practices incorporate a combination of techniques to assess the samples under investigation prior to DNA typing. For example, in some assault cases, there is a lack of spermatozoa identified in a sample. This absence may be explained by several factors, not just the aggressor's azoospermy or oligospermy, but also the potentially long period after intercourse or penetrations without ejaculation, which are common [1]. The value of short-tandem repeats from the Y-chromosome (Y-STRs) in identifying men through studying the semen of azoospermic samples demonstrates the possibility of identification of this aggressor type; however, if the biological fluid is not preliminarily characterized, the case might pass as a false negative. The use of multiple tests is important, in order to prioritize certain samples. For instance, acid phosphatase (AP) is considered a non-diagnostic test, yet, in spite of that, is highly sensitive and is the primary method to locate items for semen investigation, sometimes misused for semen searching purposes; current forensic laboratories use it to avoid underestimation of semen presence [2]. Indeed, AP is present in seminal plasma at concentrations of 400 times greater than are found in other body fluids, so its presence can and should be detected when selecting of evidence for microscopy. Other recently-developed methodologies based on RNA have been identified for forensic purposes and offer the potential benefit of higher specificity than traditional biochemical techniques; nevertheless, the latter are still used due to the lower stability of RNA in casework samples [3].

Sperm cytology (SC) is the confirmatory test used in forensic cases, however, other techniques are valid in the absence of sperm cells to guide subsequent analyses. Today, both AP and the prostate-specific antigen (PSA) are not confirmatory tests, but can help with semen research in many laboratories [4]; PSA is an antigen present in both male and female biological fluids and therefore should only be used after more diagnostic methods of analysis.

Semenogelin (Sg) I and semenogelin II are the dominating protein components of the coagulum formed by freshly ejaculated human semen. The primary source of these proteins is the seminal vesicle, though Sg I and II can also be detected in the vas deferens, prostate and epididymis, proving Sg as a biomarker for the identification of semen [5]. Sg cleavage products are detectable by two monoclonal antibodies against human Sg in the RSID™-Semen test [6,7]. The Sg concentration may be higher than PSA in the seminal fluid; in this way, evidence containing semen but not PSA can be common.

The main aims of the current study were to 1) evaluate the implementation of the Sg biomarker when spermatic cells are likely to be in low quantity or absent, and 2) to show which factors in casework are more effective in semen-searching with this type of evidence. An

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http://dx.doi.org/10.1016/j.scijus.2015.01.008
1355-0306/

internal evaluation of sensitivity, specificity and interference of semen with other biological fluids from a variety of normospermic and azoospermic donors was carried out. Additionally, case-history records spanning a five-year period of judicial cases were assessed to compare difficult forensic cases where spermatozoa were scarce or absent. Resulting comparisons among Sg and PSA and SC, AP, and prostatic acid phosphatase (PAP), as well as Y-chromosome presence, were followed when possible.

2. Materials and methods

2.1. Control samples

Raw semen coming from different subjects was analysed to test sensitivity, specificity and interference (Table 1). Donor C1 was an anonymous normospermic; subject samples C2 and C3 and C4 and C5 were donated by IVI (*International Semen Bank*), as normospermic and azoospermic donors, respectively (Table 1). Donated semen liquid samples were from healthy 18–25 year-old individuals. Samples shipped from IVI to the National Institute of Toxicology & Forensic Science (INTCF, Ministry of Justice) were packed in dry ice and stored at −80 °C. Direct aliquots of liquid semen were pipetted for analysis.

Semen specificity and interference with other biological fluids including blood, urine, saliva, and vaginal and anal fluids were evaluated for Sg determinations. Biological fluids were taken from eight anonymous volunteers at the INTCF: four men and four women. Fluids were collected on sterile cotton swabs, left to air-dry, and stored at −80 °C until processing. Informed consent was obtained from healthy subjects who were notified that they could withdraw from the study at any point, in accordance with standard human subject protection policies.

2.2. Case history

Samples from 53 sexual assault cases at the INTCF were identified from a five-year period for Sg evaluation, following comparison with the different methodologies of semen analysis (Table 1). To test the quality of the Sg marker to solve sexual assault cases, a number of difficult-to-resolve evidence pieces were selected as samples, due to their absence or low-quantity of sperm. Evidence from swab and clothes (1 cm^2) samples were incubated in 300 μL of distilled water for 1 h at room temperature before analysis.

2.3. Techniques analysis

Semenogelin results were compared to those obtained by AP, PSA and SC in control samples. This battery was repeated on cases, with the addition of PAP and Y-chromosome analyses when possible.

AP activity was identified with the Brentamine Fast Blue test. The purple colour of a positive reaction and time taken for the colour to develop were recorded. A total of 10 μL of eluted sample was used for the enzyme AP analysis. A low-level standard allows for 20 units/mL to produce a suitable colour change in 10 min; however, in our laboratory, we limited colour change to a maximum time of 5 min to assure AP quality. The activity range considered was categorized as: strong, weak, or null activity. An aliquot of 20 μL of eluted sample was analysed for PAP when possible. Separation of PAP with respect to vaginal phosphatase was in acrylamide gel at pH 4.0 and finally stained with naphthyl phosphate coupled to Fast Blue.

For semen controls, analysis of phosphatase, PSA detection, and sperm cytology samples were eluted in distilled water at room temperature. Swab portions for specificity and interference studies on Sg were incubated in a 350 μL volume of 1:1 RSID™ universal buffer (Tris, NaCl, KCl, EDTA, Triton X-100 m, Tween 20, BSA and sodium azide) and distilled water, as well as in a 350 μL volume of 1:1 RSID™ universal buffer and 1/100 semen (from C3) in water dilution, respectively. After 1 h of incubation at room temperature, the same enzymatic tests and immunoassays were followed on the swabs.

For Sg determinations, serial semen dilutions of up to 1:800,000 were prepared with 1:1 RSID™ universal buffer and distilled water. Immunoassay membrane strips for Sg and PSA were Rapid Stain Identification RSID™-Semen test (Independent Forensics *IF*, Hillside, Illinois, USA) and Seratec® Semiquant test (GmbH Goettingen, Germany), respectively. Volumes 200 μL and 100 μL of eluted samples were used for PSA and Sg detection, respectively.

The Kernechtrot–Picroindigocarmine KPIC or "Christmas Tree" staining technique was used to identify spermatic cells or spermatozoa. This method is considered a discerning method of sperm against the nuclei of epithelial and yeast cells currently present in the analysed samples. The volume of 10 μL centrifuged sediment (pellet) was selected for microscopic staining in three diluted series of 1:2500, 1:10,000 up to 1:50,000 from C1, C2 and C3 semen controls, as well as for all casework samples. A result was considered positive when two observers identified at least 2 spermatozoa heads.

The procedure for developing a Y-chromosome haplotype used differential lysis using proteinase-K, phenol–chloroform extraction and Amicon Ultra-30/Centricon-100 purification; DNA quantification by a Quantifiler Duo (Applied Biosystems, AB); and PCR amplification and detection by a Kit ABIPRISM Y Filer (AB), ABI 3130 and Genemapper.

2.4. Data analysis

Data on the victim's age, the victim–perpetrator relationship, location of the assault, medical examinations performed, particular vulnerability, and intake of alcohol/drugs were documented when possible. Differences for semen results were assessed using Sigma Stat for Windows version 3.5 with *alpha = 0.05* (Systat Software, Inc.). Power calculations for contingency tables were performed. Yates correction was used in calculating chi-square tests.

3. Results

3.1. Semen controls

3.1.1. Sensitivity

Table 2 shows semen sensitivity from five subjects according to AP, PSA, SC and Sg techniques. The semen donor C3 reached the highest Sg sensitivity—up to 1:400,000 semen dilution and further up to 1:800,000 for PSA semen test dilutions. Donor C1 obtained a Sg positive result up to 1:5000 semen dilutions, although PSA-positive results were obtained up to 1:100,000. Both C2 and C4 donors tested positive for Sg and PSA with 1:100,000 and 1:200,000 dilutions, respectively. Finally, donor C5 obtained a sensitivity of 1:200,000 and 1:400,000 for Sg and PSA, respectively. AP sensitivity reached semen dilutions of 1:2500 (C2, C3, C4), 1:10,000 (C5), and 1:25,000 being the maximum dilution with AP presence (C1 subject). All controls confirmed that for Sg determination with the RSID™-Semen kit, the proportion of 1:1 of RSID™ universal buffer dilution and water was satisfactory to allow a proper

Table 1
Number of donors (n) and evidence (N) for semen investigation at *Controls* analysis on semen sensitivity, specificity and interference of semen with other biological fluids (blood, urine, saliva, vaginal and anal fluids) are shown, as well as *casework* number (n) and analysed evidence (N) within samples (swabs, clothes).

Subjects/evidence	n	N
Controls analysis		
C1, C2, C3 (normospermic donors)	3	–
C4, C5 (azoospermic donors)	2	–
Sensitivity	5	114
Specificity and interference	8	76
Casework analysis		
Cases/evidence	53	133