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Vaginal swab specimen processing methods influence performance of rapid semen detection tests: a cautionary tale☆

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Abstract

Background: Detection of semen biomarkers in vaginal fluid can be used to assess women's recent exposure to semen. Quantitative tests for detection of prostate-specific antigen (PSA) perform well, but are expensive and require specialized equipment. We assessed two rapid immunochromatographic strip tests for identification of semen in vaginal swabs.

Study Design: We tested 581 vaginal swabs collected from 492 women. Vaginal secretions were eluted into saline, and PSA was measured using the quantitative IMx (Abbott Laboratories, Abbott Park, IL, USA) assay. Specimens were also tested using the ABAcard p30 test (Abacus Diagnostics, West Hills, CA, USA) for detection of PSA and RSID-Semen test (Independent Forensics, Hillside, IL, USA) for detection of semenogelin (Sg).

Results: Vaginal swab extraction using saline was compatible with direct assessment of vaginal swab eluates using ABAcard for PSA detection, but not for Sg detection using RSID. The rapid PSA test detected 91% of specimens containing semen compared to 74% by the rapid Sg test.

Conclusion: Investigators are urged to optimize vaginal swab specimen preparation methods for performance of RSID or other tests to detect semen components other than PSA. Previously described methods for PSA testing are not uniformly applicable to other tests.

Keywords: Semen; Prostate-specific antigen; Human semenogelin protein; Vaginal swabs; Rapid test

1. Introduction

Measurement of objective markers of semen exposure, rather than reliance on self-reported behavior or mechanical failure of barrier methods, can improve the accuracy of studies designed to evaluate contraceptive efficacy. Detection of seminal biomarkers in vaginal secretions provides objective evidence of a woman's recent exposure to semen. Prostate-specific antigen (PSA, also known as p30) has been validated as a reliable marker of semen exposure in studies of vaginal specimens obtained after vaginal insemination with different volumes of semen [1,2]. Seminal biomarkers have long been used in forensic detection of semen in vaginal specimens in sexual assault cases [3,4]. Both PSA, secreted by the prostate [5], and semenogelin (Sg), the major seminal vesicle secreted protein in human semen [6], are useful markers for forensic identification of semen [7–11]. In

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addition, Y-chromosome DNA contained within sperm cells can be detected by polymerase chain reaction [12,13]. Rapid, immunochromatographic strip tests for PSA and Sg are available commercially.

We recently reported good performance of the rapid ABAcard p30 test (Abacus Diagnostics, West Hills, CA, USA) compared to a quantitative assay (IMx PSA, Abbott Laboratories, Abbott Park, IL, USA) for detection of PSA in vaginal swabs [14]. In the present study, we compared and contrasted ABAcard for detection of PSA and Rapid Stain Identification test (RSID-Semen, Independent Forensics, Hillside, IL, USA) for detection of Sg to identify semen in vaginal swab specimens that had been specifically processed using previously described methods for quantitative PSA detection [1,14–16].

2. Materials and methods

2.1. Vaginal swab specimens

Vaginal swab specimens were obtained from women participating in two different research studies that are described in detail elsewhere [14]. Briefly, 402 vaginal swabs were from 313 women who participated in a study comparing two methods of STI prevention and control among sex workers in Dhaka, Bangladesh, conducted from February 2005 through September 2006, and 179 vaginal swabs were from women who participated in a study comparing two interviewing techniques to obtain reports of sexual behaviors among sexually active women in Zimbabwe from November 2006 through January 2007. Women were not recruited based on the timing of recent intercourse. However, most women (82%) reported vaginal intercourse (with or without a condom) in the previous 48 h. Women were recruited into the studies only after providing written informed consent according to Good Clinical Practice Guidelines. The Bangladesh study was approved by the Biomedical Institutional Review Board (IRB) of the University of North Carolina at Chapel Hill and by the IRB of the International Center for Diarrhoeal Disease Research, Bangladesh. The Zimbabwe study was approved by IRBs of Family Health International, the University of North Carolina at Chapel Hill, the University of California at San Francisco and by the ethics review committees of the Medical Research Council of Zimbabwe and the Medicines Control Authority of Zimbabwe.

Vaginal specimens were collected on cotton-tipped swabs (Falcon™ Screw Cap Single SWUBE™ applicator, Becton Dickinson and Co., Sparks, MD, USA). Immediately after collection, swabs were air dried, stored in screw-capped tubes and shipped at ambient temperatures to the research laboratory at the University of North Carolina at Chapel Hill.

For recovery of vaginal secretions, each swab was placed into 3.0 mL phosphate-buffered saline, incubated at room temperature for 15–30 min, agitated and pressed against the side of the tube to elute the sample. Vaginal specimens were centrifuged at 250×*g* for 10 min, and the supernatants were removed from cell pellets and stored at −80°C until testing. These conditions were chosen specifically for PSA detection using a quantitative assay (IMx PSA; Abbott Laboratories) to take advantage of previously established criteria for detecting recent semen exposure based on PSA concentrations, using these vaginal swab extraction methods [1,15–17]. Vaginal swab eluates were stored frozen for variable periods of time (up to 2 years) before testing; however, all tests reported here were performed within a 6-week period. Thus, comparisons between rates of semen detection using different testing methods are valid; however, we cannot rule out that semen components may have degraded during frozen storage.

2.2. Quantitative PSA testing

Supernatants (0.20 mL) from vaginal swab eluates were tested using the IMx PSA assay (Abbott Laboratories). The enzyme immunoassay measures PSA concentrations from 0.04 to 50 ng/mL; samples with initial test results >50 ng/mL were diluted 1:100 with buffered saline and retested to obtain PSA concentrations. Vaginal swab eluates containing ≤1.0 ng PSA/mL were considered negative; those containing >1.0 ng PSA/mL were considered positive for detection of semen, as previously described [1].

2.3. Rapid PSA testing

The vaginal swab extraction procedure described above using buffered saline was consistent with the manufacturer's instructions for testing using ABAcard (Abacus Diagnostics). We loaded 0.20 mL of vaginal swab eluate directly into the sample well of the immunochromatographic strip test cassette according to the instructions. After a 10-min incubation at room temperature to allow sample migration throughout the test strip, a positive result was indicated by pink lines in both test and control areas. A negative result was indicated by a line in the control area only. A control line was visible in all tests with vaginal swab eluates, documenting valid ABAcard results. Buffered saline alone produced a visible line in the control area only. Buffered saline containing 1 ng purified human PSA/mL produced a faint, but consistently visible line in the test area, and lines from solutions containing ≥5 ng PSA/mL were markedly darker. For this analysis, specimens that produced any visible line in the test area plus a line in the control area of the strip were considered positive by the rapid PSA test. A negative rapid PSA result was indicated by a line in the control area only.

Like all immunoassays that depend on antigen–antibody interactions, the ABAcard test and the RSID test (described below) are subject to potential interference in the presence of excess antigen, which impairs immune complex formation. As a result of this so-called high-dose hook effect, high concentrations of the analyte can give false-negative results. The threshold concentrations at which rapid semen test

results may be subject to the high-dose hook effect have not been established by the manufacturers. However, our unpublished observations suggest that this threshold may be between 2000 and 5000 ng PSA/mL for the ABAcard.

2.4. Rapid semenogelin testing

It is important to note that the vaginal swab extraction procedure described above using buffered saline does not conform with the manufacturer's instructions for testing with the Rapid Stain Identification test (RSID-Semen, Independent Forensics), which specifies the use of RSID-Semen Extraction Buffer. Nevertheless, we wished to determine whether the test could be used for detection of Sg in these specimens, as extraction with buffered saline is commonly used for PSA testing from vaginal swabs. In initial experiments, buffered saline alone or buffered saline diluted 1:5 in RSID Semen Running Buffer according to the manufacturer's instructions was applied to the sample well of the rapid test strip as directed. Samples that produced a visible line in test and control areas of the strip were considered positive by the rapid Sg test; those with a line in the control area only were considered negative. Buffered saline alone produced uniform false-positive results with visible lines in control and test areas of the strip, whereas buffered saline diluted 1:5 in running buffer was negative. Accordingly, the vaginal specimens, which had already been extracted in buffered saline, were diluted 1:5 in running buffer for RSID testing. A control line was visible in all tests with diluted vaginal swab eluates.

2.5. Calculations and statistical analyses

As a result of the requirement for specimen dilution and the loading characteristics of the RSID test device, the amount of vaginal swab eluate tested by RSID was 10-fold lower (fivefold dilution and half the volume loaded into the sample well) than the corresponding specimen tested using the ABAcard. To account for the additional dilution of specimens required for RSID testing, PSA concentrations in specimens were adjusted to 1/10 the values determined by the quantitative assay in the undiluted vaginal swab eluates for analysis of RSID results.

The kappa statistic for multiple raters was calculated using the MAGREE macro from SAS/STAT Software (release 6.11 TS020). Among four independent evaluators, interreader reliability was substantial for both rapid tests with a kappa score of 0.97 (SE, 0.02) for ABAcard with a subset of 402 vaginal swab specimens and 0.78 (SE, 0.03) for RSID with a subset of 207 specimens.

Ninety-five percent confidence intervals (95% CI) for proportions were calculated according to Wilson [18]. Differences between proportions were assessed by the *z*-test using Sigma Stat for Windows version 3.5 (Systat Software, Inc.); p values <.05 were considered statistically significant.

3. Results

With the use of the quantitative IMx assay for PSA detection, 194 (33.4%) of 581 vaginal swab specimens extracted in buffered saline contained >1 ng PSA/mL (Table 1). Both rapid tests identified positives among specimens containing ≤1 ng PSA/mL; Sg detection by RSID was higher among specimens with ≤1 ng PSA/mL than PSA detection by ABAcard. Among specimens with >1.0 ng PSA/mL, PSA detection by ABAcard was significantly higher than Sg detection by RSID. For vaginal swab specimens with >1.0 ng PSA/mL, we also compared the proportion of rapid test positives among those with low, moderate and high semen exposure categories as previously defined [15,16] (Table 2). Both ABAcard and RSID detected semen in all specimens containing high levels of PSA. All low and moderate PSA positives were also positive by ABAcard, whereas RSID detected Sg in significantly fewer specimens with low PSA concentrations.

Because detection of either Sg or PSA is consistent with the presence of semen, we conducted a secondary analysis in which any vaginal swab specimen containing Sg detected by a positive RSID test or >1.0 ng PSA/mL detected by the quantitative assay was considered to be positive for semen. ABAcard detected 92% of all positives (194/212) compared to 74% detected by RSID (113/152, p<.001, *z*-ratio for proportions).

4. Discussion

We compared the qualitative detection of two different semen biomarkers, PSA and Sg, in vaginal swab specimens

Table 1
Rapid test results for qualitative detection of semen biomarkers in vaginal swab specimens eluted into phosphate-buffered saline

Qualitative semen result[a]	PSA concentration (ng/mL)[b]	PSA detected with ABAcard		Sg detected with RSID		p value[d]
		Total	Number positive (%, 95% CI)	Total[c]	Number positive (%, 95% CI)	
Negative	≤1.0	387	16 (4%, 3–7%)	483	53 (11%, 9–15%)	<.001
Positive	>1.0	194	194 (100%, 98–100%)	98	59 (60%, 50–69%)	<.001

[a] Cutoff for dichotomous PSA results to define semen exposure, as previously described [2].
[b] Determined using the quantitative IMx PSA assay.
[c] Specimen totals in qualitative categories based on PSA concentration in vaginal swab eluates diluted 1:10 for RSID testing.
[d] Probability of *z*-ratio for proportion of specimens positive with ABAcard vs. RSID.

Table 2
Rapid test results for detection of semen biomarkers in vaginal swab specimens eluted into phosphate-buffered saline, stratified by PSA concentration

Qualitative semen result[a]	PSA concentration (ng/mL)[b]	ABAcard		RSID		p value[d]
		Total	Number positive (%, 95% CI)	Total[c]	Number positive (%, 95% CI)	
Low	1.1–21.9	129	129 (100%, 97–100%)	78	42 (54%, 43–64%)	<.001
Moderate	22.0–99.9	28	28 (100%, 88–100%)	16	13 (81%, 57–93%)	NS
High	>100	37	37 (100%, 91–100%)	4	4 (100%, 51–100%)	NS

NS, Not significant.
[a] Semen exposure categories among specimens containing >1 ng PSA/mL vaginal swab eluate, as previously described [16,17].
[b] Determined using the quantitative IMx PSA assay.
[c] Specimen totals in qualitative categories based on PSA concentration in vaginal swab eluates diluted 1:10 for RSID testing.
[d] Probability of *z*-ratio for proportion of specimens positive with ABAcard vs. RSID.

extracted in buffered saline using commercially available rapid immunochromatographic strip tests. In addition, PSA concentrations in these specimens were determined using a quantitative enzyme immunoassay. Using these very same vaginal swab specimens, we recently reported that the ABAcard rapid test is 100% sensitive (95% CI, 98–100%) and 96% specific (95% CI, 93–97%) compared to the quantitative test in detecting >1.0 ng PSA/mL vaginal swab eluate [14]. Although the quantitative IMx PSA assay may be considered the 'gold standard' for detection of PSA, it is not a valid comparator for the RSID test, which detects Sg, a biochemically distinct analyte. Without a reference test for detection of Sg, and with acknowledged deviations from the manufacturer's directions for specimen processing, we did not attempt to define the performance characteristics of the RSID test for Sg. Rather, we elected to describe the percentage of specimens with positive ABAcard or RSID test results and stratify those observations based on PSA concentrations in the specimens.

In this comparison of two rapid immunochromatographic strip tests for detection of semen in vaginal swab specimens, PSA was detected by ABAcard significantly more frequently than Sg was detected by RSID. However, the study has important limitations that must be considered in interpreting these results. Specimen preparation was compatible with direct assessment of vaginal swab eluates using ABAcard for PSA detection, but for Sg detection using RSID, acceptable test performance required fivefold dilution of samples with running buffer supplied with the test. The imperfect specimen preparation for use with RSID likely reduced the sensitivity of the test. To compensate for specimen dilution and lower test volume (necessitated by a smaller sample well in the RSID test device compared to the ABAcard), we adjusted PSA concentrations to 1/10 the actual values determined by the quantitative assay for analysis of RSID test results. However, this adjustment could not compensate for impaired RSID sensitivity that may have resulted from the use of a different extraction buffer. Thus, the apparent greater sensitivity of the rapid PSA test compared to the Sg test for detection of semen in vaginal swabs must be considered to be preliminary and specific for the specimen preparation methods used in our study. However, these methods are used commonly, and our experience may benefit others considering the use of RSID for detection of semen in vaginal fluid.

This study was further complicated by comparing different individual semen components as markers of this complex biological specimen. Although both Sg and PSA are invariably present in human semen, individual semen specimens with a detectable level of one component may not necessarily contain a similarly detectable concentration of the other. Sg is a natural substrate for cleavage by the serine protease PSA [6], and the concentration of Sg is inversely correlated with PSA concentration in seminal plasma, independent of the level of PSA protease activity [19]. Sg cleavage products are detectable by monoclonal antibodies against Sg [10,19], and the capture antibody in the RSID test does detect cleaved Sg (K. Reich, Independent Forensics, personal communication). Thus, Sg that had been cleaved by PSA in a specimen would still theoretically be detectable by RSID, and specimens in this study with high PSA concentrations were positive with RSID. Sg concentrations (4–68 mg Sg/mL) [20] are generally higher than PSA concentrations (0.2–5.5 mg PSA/mL) in seminal fluid [3,21]. Therefore, specimens containing semen in which Sg but not PSA can be detected are to be expected. Indeed, such specimens were identified in our study and accounted for approximately 10% of all positive vaginal swab samples in which either Sg or >1.0 ng PSA/mL was detected. However, PSA was detected in the absence of Sg in 25% of positive vaginal swab specimens. Suboptimal specimen preparation for the RSID test, as discussed above, may account for the failure to detect Sg in these samples. We did not dilute vaginal swab eluates beyond 1:5 with the running buffer supplied with the test kits for fear of compromising RSID sensitivity even further; however, it is possible that specimens with high concentrations of Sg could have produced false-negative results. According to the RSID product insert, 20-fold dilution of samples containing large amounts (3–50 μL) of pure semen eliminates false negatives resulting from the high-dose hook effect. We estimate that the dilution of semen in secretions in the vagina and further into the buffered saline used for extraction of material from vaginal swabs resulted in a 500- to 5000-fold dilution of semen in samples tested by RSID. Thus, it is unlikely that the high-dose hook effect affected the RSID results. However,

we cannot rule out that some false-negative results may have occurred if specimens contained unusually high concentrations of Sg.

There are several advantages to using PSA as a marker of semen exposure. The use of PSA allows investigators to capitalize on previous work characterizing the kinetics of PSA clearance from vaginal swab specimens prepared in the same way that was used in our study. We do not know the kinetics of Sg clearance or degradation in the vagina or whether the presence of PSA and Sg in vaginal fluid from women with recent semen exposure is correlated. Studies are needed to provide the necessary context for interpreting Sg detection in vaginal swab specimens: How long after semen exposure can Sg be detected in vaginal fluid? How soon does Sg disappear compared to PSA? What concentrations of Sg correspond to semen exposure resulting from problems with condom use? We urge investigators to optimize vaginal swab specimen preparation methods for the performance of RSID or other semen detection tests and not rely on previously described methods for PSA testing. Research needs include comparison of specimens collected in buffered saline and RSID or other buffers for detection of the two semen markers. For rapid PSA detection, ABAcard is an appropriate, simple and relatively inexpensive test to identify a biological marker of recent semen exposure in vaginal swabs [14].

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