F. Samuel Baechtel,[1] *Ph.D.; John Brown,*[2] *B.S.; and Linda D. Terrell,*[3] *M.A.*

Presumptive Screening of Suspected Semen Stain In Situ Using Cotton Swabs and Bromochloroindolyl Phosphate to Detect Prostatic Acid Phosphatase Activity

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ABSTRACT: A novel approach to the presumptive screening of questioned semen stains has been developed which enables the rapid identification of stains which are devoid of semen. Questioned semen stains can be swabbed with a moist cotton swab, and the prostatic acid phosphatase (SAP) activity transferred to the swab identified through assay with 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Controlled laboratory studies revealed that the BCIP swab procedure was as sensitive as the semiquantitative SAP test currently employed in the FBI Laboratory for the presumptive screening of semen stains. A validation study of the BCIP swab procedure in parallel with the current procedure using 4305 case evidence stains indicated that the BCIP swab procedure was as effective as the current procedure in identifying those questioned stains which lack semen. The advantage of the BCIP swab procedure is that it can be performed on questioned stains in situ and thereby avoids the requirement of removing and extracting the stain before assay of SAP activity.

KEYWORDS: pathology and biology, semen, phosphatases, screening procedures, presumptive semen testing, prostatic acid phosphatase

The analysis of stains suspected of containing semen usually is initiated by carrying out presumptive biochemical tests for substances characteristically, but not uniquely, found in this body fluid. Stains that yield positive presumptive test results are subjected to further testing to authenticate the presence of semen. The advantage of performing a presumptive test for semen presence is that the number of questioned stains that require extensive additional testing can be significantly reduced, thereby increasing the forensic science laboratory's productivity.

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[1]Research chemist, Forensic Science Research and Training Center, FBI Laboratory, FBI Academy, Quantico, VA.

[2]Special agent, Serology Unit, FBI Laboratory, Washington, DC.

[3]Formerly, Forensic Science Research and Training Center, FBI Laboratory, FBI Academy, Quantico, VA; now, Eastern Virginia Medical School, Norfolk, VA.

Perhaps the most common presumptive test employed in the identification of semen is the assay for seminal fluid prostatic acid phosphatase (SAP) activity. This test is used widely for several reasons: SAP is present in semen at high concentrations compared with other body fluids [*1,2*]; it can be assayed readily using a variety of substrates [*3,4*]; the enzyme is stable for long periods of time in dried semen stains [*5*]; and, as this paper will demonstrate, its absence from a stain strongly suggests the absence of semen.

Two technical approaches are in use to test for SAP in suspected semen stains. Some laboratories employ a spot test for SAP [*6*] in which the enzyme is transferred directly from the suspected semen stain onto moist filter paper by blotting. The filter paper is then assayed for the presence of SAP activity using a coupled assay system which terminates in the deposition of an insoluble dye product at the site of enzyme activity. Other laboratories identify possible semen stains visually; remove the stain from the item and extract its components in a buffer solution and assay for SAP activity in the extract using *p*-nitrophenylphosphate or sodium thymolphthalein monophosphate (STMP) as the substrate. Although both approaches are suitable for the detection of SAP activity, neither technique is conducive to the rapid identification of stains likely to be devoid of semen. Thus, an alternative approach was developed which reduces the sampling handling requirements associated with the detection of SAP activity. This approach involves the absorption of the chemical constituents present in a small area of a suspected semen stain onto a moist cotton swab, followed by placement of the swab into a buffered solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) [*7*]. The appearance of an aqua color indicates that phosphatase activity is present in the stain. This paper describes the results of evaluations conducted on laboratory prepared stains as well as stains identified on various items of evidentiary value using the BCIP-swab method.

Materials and Methods

Reagents

For the assay of SAP using STMP as a substrate, the components supplied in the "acid phosphatase reagent assay kit," manufactured by Worthington Diagnostic Systems, Freehold, New Jersey, were used after reconstitution according to the directions enclosed with the kit. BCIP toluidine salt and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Company, St. Louis, Missouri. Dimethyl sulfoxide, ACS grade, was obtained from Fisher Scientific Company, Silver Spring, Maryland. Polystyrene, 96-well, microplates were purchased from Dynatech Laboratories, Alexandria, Virginia.

Semen Specimens and Case Evidence Stains

Semen samples were obtained in sterile containers from healthy donors at the FBI Academy and stored at −70°C until used for research studies. Dilutions of semen were made with HEPES-buffered saline (HBS) (0.01*M* HEPES-0.144*M* NaCl, pH 7.2).

Questioned semen stains were evaluated from 144 cases of alleged sexual assaults submitted to the FBI Laboratory for examination. Stains were screened for SAP activity by the BCIP-swab technique and by the semiquantitative STMP procedure currently employed by the FBI Laboratory. Vaginal swabs submitted for examination were not tested by the BCIP-swab technique.

BCIP-Swab Technique

The BCIP substrate solution composition was 0.5-mg BCIP/mL 0.01*M* acetate buffer at pH 5.5. It was necessary to dissolve the BCIP in a few drops of dimethyl sulfoxide before

bringing the solution to volume with acetate buffer. The BCIP solution was stable for at least four weeks when stored at 4°C.

Each questioned stain was stroked lightly with a cotton tipped applicator swab that had been moistened to dampness with deionized water. SAP activity transferred to a swab was detected by placing the swab into a 12-by 75-mm glass test tube which contained 200 μL of BCIP substrate solution. Two control swabs were used as references for control purposes. First, an unstained, dampened cotton swab was introduced to the BCIP substrate solution. This unstained swab remained at room temperature until all the questioned stains being tested at that time had been swabbed and the swabs placed into substrate. In addition, a known semen stain control was stroked with a dampened swab and put into the substrate. Finally, all test tubes were placed into a 37°C water bath for a 15-min incubation period. The appearance of any blue color on the swab was recorded as a positive test result. Because the blue color of hydrolyzed BCIP was insoluble and did not fade, the test results could be read at any time following the 37°C incubation phase of the test.

Semiquantitative STMP Test Procedure

The semiquantitative assay of SAP activity was carried out in microplates by adding 5 μL of questioned stain extract to 20 μL of STMP substrate solution. The assay was run for 30 min at 37°C when the hydrolysis was terminated by the addition of 100 μL of alkali reagent. The level of SAP catalytic activity was judged by visual inspection of the intensity of color in the reaction wells. Laboratory protocol requires that any sample which yields as little as a trace (slightly green color) of activity be subjected to confirmatory tests for the presence of semen. Control tests were included on each microplate and included a known semen stain standard, an unstained standard, and an unstained standard to which no STMP substrate had been added. All controls were extracted concurrently with the questioned stains.

Quantitative Measurements of SAP Activity

Quantitative measurements of SAP catalytic activity were carried out by adding 10 μL of sample to 50 μL of STMP substrate solution in a microplate. After 30 min at 37°C, the reactions were terminated by adding 140 μL of alkali reagent. The absorbancies of the reaction products were measured at 590 nm on an automated microplate reader. A standard curve was prepared with thymolphthalein which was linear up to 15-nmoles thymolphthalein/200-μL assay volume. To ensure that the SAP activity of specimens did not exceed the assay limits, each extract was tested at three dilutions, 1:10; 1:100; and 1:500. An international unit (IU) of SAP activity was taken as the quantity of enzyme that produced 1 μmole of thymolphthalein per minute at 37°C.

Presumptive and Confirmatory Testing of Questioned Semen Stains

A total of 4305 questioned stains was tested for the presence of SAP activity by both the BCIP-swab method and by the semiquantitative STMP method. The BCIP-swab tests were performed first on the stains in situ. Subsequently, a 1-cm^2 area of each stain was cut out and extracted in 250-μL HBS in a 1.5-mL polypropylene centrifuge tube for 1 h at room temperature or overnight at 4°C. When the cutting was taken, a deliberate effort was made to avoid the contiguous area of the stain which had been swabbed for the BCIP-swab test. After extraction, the cutting was transferred to a 0.5-mL polypropylene centrifuge tube which had had a 1.0-mm hole formed in its bottom. The 0.5-mL tube was nested inside the 1.5-mL tube and the pair centrifuged at 15 000 *xg* for 1 min. This centrifugation step forced most of the liquid imbibed by the cutting into the tube containing the extract and served also to pellet any spermatozoa released from the cutting during extraction. The soluble extract

was tested for SAP activity by the semiquantitative STMP assay. Extracts that exhibited SAP activity were considered presumptively to contain semen. Confirmation of the presence of semen was sought first by microscopic search of the particulate matter concentrated at the bottom of the extraction tube. The observation of at least one intact spermatozoon was necessary for the diagnosis of semen presence. An extract which exhibited SAP activity, but was devoid of a discoverable spermatozoon, was tested for the presence of p30 by radial immunodiffusion [*8*]. Demonstration of p30 in the stain extract was considered confirmatory of semen presence.

Predictive Value Analysis of Test Results

Screening test results were evaluated by the predictive value method [*9*]. Within the context of this paper, the following definitions, which have been modified from Kolins [*10*], are useful for appreciating the implications of the predictive-value analysis.

(1) Sensitivity: The sensitivity of a screening test for semen presence has been expressed as the percent frequency that the test gave positive results when semen was present.

(2) Specificity: The specificity of a screening test for semen presence has been expressed as the percent frequency that the test gave negative results when semen was absent.

(3) Predictive value (PV) of a positive test response: The PV of a positive response defines the percentage of positive screening responses that were true responses (that is, semen was subsequently identified).

(4) PV of a negative test response: The PV of a negative response defines the percentage of negative screening responses that were true negatives (that is, semen absent).

(5) Efficiency of the test: The test efficiency is the percentage of the total responses that were true.

Results

Detection Sensitivities of SAP Tests Using BCIP and STMP as Substrates

A comparison was made of the minimum quantity of SAP detectable by the BCIP swab test and by the semiquantitative STMP test. Eight semen samples were diluted in serial doubling steps from 1:10 to 1:20 480 using HBS. Five-microlitre aliquots of each dilution of each semen sample were tested in the semiquantitative STMP test, and five-microlitre aliquots were applied directly to the tip of dry cotton applicator swabs for assay with BCIP. To establish a quantitative measure of the level of SAP present in these samples, separate aliquots were assayed by the quantitative STMP test.

For each semen sample, the limit of detectability of SAP activity occurred at the same dilution in both the semiquantitative STMP and BCIP procedures. Quantification of the level of SAP in each semen sample permitted calculation of the quantity of SAP present in the specimens at their limit of detectability. The IU SAP/mL diluted sample, at the qualitative detection limit, varied from 0.0145 to 0.0184 IU SAP/mL, with the mean being 0.0150 IU SAP/mL.

Hydrolysis of BCIP by Adventitious Phosphatases

Common adventitious body fluids and semen from animals other than man were tested for the presence of an acid phosphatase activity that would hydrolyze BCIP. Fresh blood or saliva, applied directly to the applicator swab, and tested with BCIP, failed to demonstrate acid phosphatase activity. In contrast, BCIP was hydrolyzed readily by the acid phosphatases present in human vaginal fluid and in semen from the dog, sheep, goat, pig, and orangutan.

Validation of BCIP Test Method with Case Evidence Stains

Table 1 shows the distribution of test results for the 4305 questioned stains that were presumptively screened for SAP activity. Semen was identified in a total of 777 stains. Within this population of stains, both the BCIP swab procedure and the semiquantitative STMP tests gave positive results in 732 instances. Of the stains which contained semen, 15 gave positive BCIP swab responses, but were negative by the STMP test. Conversely, 26 semen containing stains were negative for SAP activity by the BCIP swab test, but positive by the STMP test. Despite the presence of semen, 4 stains yielded negative results for SAP activity by both presumptive test procedures. Stains which lacked detectable quantities of semen totalled 3328. For 2868 of these semen-negative stains, both the BCIP and the STMP tests were negative. Positive BCIP and STMP test results were seen for 228 semen-negative stains. There were 319 stains that lacked semen and gave conflicting BCIP swab STMP test results.

The data in Table 1 were segregated so that the results for each of the two presumptive test procedures could be evaluated separately. Table 2 shows only the SAP test results obtained using the semiquantitative STMP procedure, and Table 3 shows only the results using the BCIP swab procedure. These tabular formats categorize the test results in terms of the numbers of true and false positive and negative test results for each screening procedure. An objective evaluation of the effects of false results upon screening test quality was derived by a predictive value analysis of the data [*9*].

Predictive value parameters for both screening tests are summarized in Table 4. These parameters reveal that both test procedures possess high sensitivity (STMP = 97.6% versus BCIP = 96.1%). However, the test procedure specificities were lower, with the semiquantitative STMP procedure showing 90.3% specificity compared to 84.4% specificity for the

TABLE 1—*Questioned semen stain results using the BCIP swab and the semiquantitative STMP test procedures.*

Test Results				
BCIP	STMP	Semen[a]	No. of Stains	% of Total
+	+	+	732	17
+	+	−	228	5.4
+	−	+	15	0.3
+	−	−	319	7.4
−	+	+	26	0.6
−	+	−	113	2.6
−	−	+	4	<0.1
−	−	−	2688	66.7
			4305	100

[a]Criteria for semen identification in questioned stain extracts was the observation of intact spermatozoa or presence of p30.

TABLE 2—*Results of semen stain screening using semiquantitative STMP method.*

	Number of Stains		
	Positive	Negative	Total
Semen present	758	19	777
Semen absent	341	3187	3528
Total	1099	3206	4305

TABLE 3—*Results of semen stain screening using BCIP-swab method.*

	Number of Stains		
	Positive	Negative	Total
Semen present	747	30	777
Semen absent	547	2981	3528
Total	1294	3011	4305

TABLE 4—*Comparison of semen stain screening test parameters.*

	Percentage	
	STMP-Conventional	BCIP-Swab
Sensitivity[a]	98	96
Specificity[b]	90	84
Predictive value of positive responses[c]	69	58
Predictive value of negative responses[d]	99.4	99
Efficiency[e]	91	87

[a]$\text{Sensitivity} = \dfrac{\text{True positives (TP)}}{\text{TP} + \text{False negatives (FN)}} \times 100$

[b]$\text{Specificity} = \dfrac{\text{True negatives (TN)}}{\text{TN} + \text{False positives (FP)}} \times 100$

[c]$\text{Predictive value of positive responses} = \dfrac{\text{TP}}{\text{Total number of positives}} \times 100$

[d]$\text{Predictive value of negative responses} = \dfrac{\text{TN}}{\text{Total number of negatives}} \times 100$

[e]$\text{Efficiency} = \dfrac{\text{TP} + \text{TN}}{\text{Total number of test results}} \times 100$

BCIP swab test. The PV of a positive test result for the STMP test was seen to be 68.9% and for the BCIP test, 57.7%. Thus, in the case of the STMP test, 31 positive responses out of 100 positive responses would be false. Similarly, 42 positive BCIP swab responses out of every 100 positives would be false. In contrast, both tests were superior at predicting the absence of semen from suspected stains. The PV of a negative result for the STMP test was 99.4%, and 99.0% for the BCIP test. Thus, for every 1000 stains which yield negative results by the STMP procedure, 6 would be false. For the BCIP procedure, 10 out of 1000 negatives would be false. A comparison of the overall test efficiencies reveals that both were quite efficient, but the semiquantitative STMP test was slightly superior because of its increased positive predictive value.

Discussion

The goal of this study was to evaluate the BCIP swab method for presumptively identifying semen in sexual assault evidence stains submitted to the FBI Laboratory for examination. The BCIP swab test procedure requires that the suspected stain be stroked with a moistened cotton tipped applicator swab. SAP catalytic activity transferred to the swab was detected by assay with the histochemical phosphatase substrate BCIP. Compared with the conventional procedure employed by the FBI Laboratory, in which suspected semen stains were cut, ex-

tracted, and assayed with the substrate STMP, the BCIP test procedure appeared to offer a considerable potential savings in time and effort.

Despite its immediate attractiveness as a replacement for the semiquantitative STMP test, a number of issues had to be addressed before this procedure could be considered by the Laboratory for routine use. The detection sensitivities of the BCIP swab and semiquantitative STMP tests appeared equal. Thus, no sacrifice in sensitivity would be incurred by the use of the BCIP swab procedure. An important feature of the use of BCIP as a substrate for SAP is its failure to serve as a substrate for erythrocyte acid phosphatase. This property is shared with STMP [*3*] and enables the presumptive detection of semen when mixed with blood in stains.

The validity of the BCIP swab procedure for the presumptive identification of semen became apparent as the results were examined following its application to more than 4000 case stains. The BCIP swab procedure, like the semiquantitative STMP procedure, is an extremely reliable method for identifying and screening those stains which do not contain semen. The sensitivity and specificity of any test procedure are related inversely [*9*]. As the sensitivity of a test procedure increases, at the expense of specificity, the number of false negatives (FN) decreases. In the case of a presumptive screening test for semen presence, the most desirable test is one in which sensitivity is at its greatest, so as to minimize the occurrence of false negatives. The trade-off is reduced specificity and an increase in the number of false positives (FP). Because these tests for semen are presumptive and not confirmatory, the occurrence of false positive test results is not a pitfall that would lead to incorrect examination conclusions. Note that of the 19 FN seen with the semiquantitative STMP test and the 30 FN seen with the BCIP swab procedure, only 4 were negative for both test procedures. This suggests that some factor other than the absence of SAP catalytic activity in the stain itself was responsible for the FN results with the BCIP swab procedure. One obvious cause for FN with the BCIP swab method is inadequate transfer of SAP activity to the swab from the stain during the swabbing step.

This study has shown that the presumptive screening of questioned semen stains can be effected reliably by the BCIP-swab technique. The major advantage conferred by the use of this technique in case stain examinations lies in its ability to target those stains which lack semen without having to invest cutting and extracting time before the assay of SAP. In terms of the present study, all 4305 stains had to be extracted before SAP tests were run by the conventional STMP test. In contrast, had stains for confirmatory semen testing been selected on the basis of the BCIP-swab test results, it would have been necessary to extract only 1294 questioned stains. Use of the BCIP-swab test would have reduced the number of cuttings and extractions by 70%.

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Address requests for reprints or additional information to
F. Samuel Baechtel, Ph.D.
Forensic Science Research and Training Center
FBI Academy, Bldg. 12
Quantico, VA 22135