TECHNICAL NOTE

Kevin Noppinger,[1] *B.S.; Rodger Morrison,*[2] *B.S.;*
Nancie H. Jones,[2] *B.S.; and Harry Hopkins, II,*[3] *B.S.*

An Evaluation of an Enzymatic Choline Determination for the Identification of Semen in Casework Samples

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ABSTRACT: This study compares the detection of choline in seminal stains by both an enzymatic method and by the standard Florence crystal test. The tests were conducted on 293 actual casework samples. In those samples identified as containing semen, choline was detected twice as often by the enzymatic method compared to the Florence method (84.6 versus 40.3%). The choline results were correlated with spermatozoa and acid phosphatase tests. The enzymatic detection of choline in seminal stains was found to be a fast, easy, sensitive, and reliable test.

KEYWORDS: forensic science, choline, semen, semen stains

The examination of alleged sexual assault cases commonly includes the identification of human seminal stains. Seminal stains are unequivocally identified by the presence of spermatozoa. With some 350 million sperm in the average male ejaculate [*1*], it might seem to the inexperienced analyst that the identification of one or two spermatozoa should not be very difficult. The experienced forensic serologist knows this is not always the case. There are a number of factors that lead to the destruction of spermatozoa making their available numbers in a stain very low or absent altogether. Also, the number of oligospermic or aspermic males, whether for clinical or surgical reasons, continues to increase [*2*].

In the absence of identifiable spermatozoa, the forensic serologist must rely on other tests to identify seminal stains. These tests are directed toward components of semen which are either unique to semen or are found in significantly higher levels in semen than in other body fluids or in natural sources. Some of the tests reported include the detection or quantitation

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[1]Crime laboratory analyst, Florida Department of Law Enforcement, Pensacola Regional Crime Laboratory, Pensacola, FL.

[2]Criminalist and forensic laboratory analyst, respectively, Alabama Department of Forensic Sciences, Huntsville, AL.

[3]Crime laboratory analyst, Florida Department of Law Enforcement, Orlando Regional Crime Laboratory, Orlando, FL.

or both of: prostatic acid phosphatase [3–5], choline [6], spermine [7], leucine aminopeptidase,[4] lactate dehydrogenase-C4 (LDH-C4) [8], gamma-glutamyltranspeptidase (GGT) [9], and the protein E1 or p30 [10–12]. Each of these tests have their own advantages, and more importantly, disadvantages.

The detection of choline is one of the oldest tests for semen. Although choline occurs in other substances such as tomatoes, it is not known to occur together with significant amounts of prostatic acid phosphatase in anything other than semen. Therefore, strong positive tests for both choline and prostatic acid phosphatase is considered by some as confirmation of the presence of semen [13].

Choline occurs in seminal plasma as a result of the enzymatic hydrolysis of phosphorylcholine. Phosphorylcholine, secreted into the seminal plasma mostly from the seminal vesicles, breaks down rapidly into free choline and inorganic phosphate after ejaculation, by the action of prostatic acid phosphatase [1]. Human semen contains approximately 2.5 to 3.8 mg phosphorylcholine per millilitre of semen [14]. The concentration of choline in normal semen was found by Takatori et al. [24] to be 0.9 to 1.4 mg/mL. Mann [1] reported free choline in semen to be as high as 20 mg/mL.

The classical test for choline is the microchemical test described by Florence [6] which has become known as the Florence test. In this test an extract of the stain is placed on a microscope slide and is treated with a solution of iodine and potassium iodide. If choline is present, brown needle shaped crystals of choline periodide will appear. The major limitation of this test is the rather high number of false negative results obtained, primarily as a result of its lack of sensitivity. More recently, a number of papers on thin-layer chromatography procedures have been written [15–23]. These papers represent a number of different solvent systems for separating the seminal fluid components and a number of different development reagents for visualizing and identifying the choline. These procedures show varying degrees of sensitivity, but all are time-consuming and require not less than 1 h to determine if choline is present in a stain. In 1981, two papers were published on the enzymatic detection of choline in seminal stains [24,25]. These tests are based on the reaction of choline oxidase with choline. The key reaction is the production of hydrogen peroxide by the oxidase action on choline, whereby hydrogen peroxide reacts with either *N*-ethyl-*N*-(3-methyl phenyl)-*N'*-acetylethylenediamine (EMAE) and 4-aminoantipyrine in the presence of peroxidase to produce a purple color. These tests were, in fact, easily performed and showed a high degree of specificity and sensitivity.

The present study was designed to evaluate the enzymatic detection of choline in actual case samples, and correlate the results with the Florence test, acid phosphatase test, and the presence or absence of spermatozoa.

Materials and Methods

Samples

All samples for testing were from actual casework materials. At the time of examination, the stains were from approximately three days to several months old. Dried stains on clothing were maintained at room temperature until examined. Vaginal, oral, and anal swabs were stored at 4°C until examined.

During this study, 142 sexual assault cases with 433 stains were examined. Of those, 102 cases with 293 stains contained sufficient stain material to conduct all 4 tests (spermatozoa, acid phosphatase, enzymatic choline, and Florence choline) on each stain. All four tests were conducted regardless of the outcome of any one, that is, a negative acid phosphatase test did

[4]M. E. Lawton and J. G. Sutton, "A Study of Leucine Aminopeptidase as a Possible Means of Identifying Semen," HOCRE Report, Home Office Central Research Establishment, Aldermaston, England, 1981.

not preclude the other 3 tests. If vaginal, anal, and oral swabs were present in a case, all were examined regardless of the victim's statement. The substrates for the 293 potential seminal stains included: vaginal, oral, and anal swabs; genital swabs; cervical swabs; dried secretions on the subject's body; stains on synthetic fiber material; natural fiber material; paper products and tampons; semen stains mixed with saliva, blood, and vomitus; and stains on condoms. The known seminal stain used as a positive control on the choline and acid phosphatase tests was a dried seminal stain on tissue paper maintained at room temperature for 6 years.

Spermatozoa

Swabs and dried stains (approximately 0.5 cm^2) were actively extracted in 1/2 mL of physiological saline. A portion of the extract was placed on a glass slide, dried, stained with Christmas tree stain [*26*], and examined microscopically.

Acid Phosphatase

Acid phosphatase tests were conducted as described in the *Biology Methods Manual* [*13*]. The reactions were graded 0 through 14 at 30 s.

Florence Choline

The Florence tests were performed as described in the *Biology Methods Manual* [*13*] and were scored as either positive or negative.

Enzymatic Choline

The enzymatic choline tests were conducted as reported by Suzuki et al. [*25*], and were graded 0 through +4 at 5 to 10 min.

Chemicals

All reagents were purchased from Sigma Chemical Company, St. Louis, MO, with the exception of *N*-ethyl-*N*-(3-methyl phenyl)-*N'*-acetylethylenediamine (EMAE) which was kindly provided by Kyowa-Hakko-kogyo Co., Tokyo, Japan.

Results and Discussion

Table 1 illustrates the comparison of the 4 tests on the 293 casework samples. Of the 293 potential seminal stains, spermatozoa were identified in 170 (58%) of the stains. Of the 170 sperm positive stains, 161 (94.7%) were acid phosphatase positive, 139 (81.8%) were positive for choline by the enzymatic method, and 77 (45.3%) were positive for choline by the Florence test.

Two (1%) samples were positive for choline by the enzymatic method and contained sperm but were negative for choline by the Florence test and were negative for acid phosphatase. Both of these samples were from vaginal swabs.

Two samples gave positive enzymatic tests for choline while all other tests were negative. Both of these samples were oral swabs and are considered as false positives. No false positive results were recorded for the Florence choline test.

Forty-one samples gave positive acid phosphatase results but were negative for both spermatozoa and choline.

Of the 293 samples, 201 (68.6%) were identified as seminal stains by the presence of

TABLE 1—*Comparison of spermatozoa, acid phosphatase, enzymatic choline, and Florence choline results from 293 casework samples.*[a]

Sp	AP	Suz.	Fl	No.	%
+	+	+	+	72	24.6
+	+	+	−	65	22.2
+	+	−	+	4	1.4
+	−	+	+	0	0.0
+	+	−	−	20	6.8
+	−	+	−	2	0.7
+	−	−	+	1	0.3
+	−	−	−	6	2.0
−	+	+	+	4	1.4
−	+	+	−	27	9.2
−	+	−	+	0	0.0
−	−	+	+	1	0.3
−	+	−	−	41	14.0
−	−	+	−	2	0.7
−	−	−	+	0	0.0
−	−	−	−	48	16.4
			Total	293	100

[a]Sp = sperm
AP = acid phosphatase
Suz = enzymatic choline
Fl = Florence choline

sperm or a combination of significant levels of acid phosphatase and choline. Table 2 illustrates the comparison of the results on the 201 samples identified as seminal stains.

Of the 201 samples, 5 (2.5%) were positive for choline by the Florence test but were negative for choline by the enzyme test. All 5 samples were confirmed as seminal stains by the presence of spermatozoa. Of the stains, 4 were on clothing, and the fifth stain, which was also negative for acid phosphatase, was a dried secretion stain from the pubic area.

Of the 92 samples which were not identified as seminal stains by the previously stated criteria, 1 sample gave positive results for choline by both the enzymatic and Florence methods but was negative for sperm and acid phosphatase. This sample was from a condom and had remained in a liquid state for several days, unrefrigerated.

TABLE 2—*Correlation of spermatozoa, acid phosphatase, enzymatic choline, and Florence choline results from 201 casework seminal stains.*

201 Casework Seminal Stains		
170	Contain spermatozoa	84.6%
31	No identifiable sperm	15.4%
192	AP positive	95.5%
9	AP negative	4.5%
175	Choline positive	87.1%
26	Choline negative	12.9%
76	Suz. positive, Fl positive	37.8%
94	Suz. positive, Fl negative	46.8%
5	Suz. negative, Fl positive	2.5%

Conclusion

The detection of choline in seminal stains represented by actual casework samples becomes a fast, easily performed procedure, showing a relatively high degree of specificity and sensitivity when the enzymatic procedure described by Suzuki et al. [*25*] is used. In this study of 201 samples identified as containing seminal stains, 170 (84.6%) gave positive choline tests by the enzymatic method, while only 81 (40.3%) gave positive choline tests by the Florence method.

The two false positive results obtained by the enzymatic method in oral swabs are relatively weak and should not pose a serious problem to the experienced examiner.

Overall, the authors found the enzymatic test for choline to be superior to the Florence test and encourage its use in the routine examination of sexual assault cases.

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Address requests for reprints or additional information to
Kevin Noppinger
Florida Department of Law Enforcement
Pensacola Regional Crime Laboratory
160 Governmental Center, Rm. 308
Pensacola, FL 32501