PERFORMANCE CHARACTERISTICS OF SPERM DETECTION BY MICROSCOPY AND PURPLE COLOR TEST FOR ACID PHOSPHATASE IN VAGINAL SWABS OF SEXUAL CRIME SURVIVORS

MAMERTO S. BERNABE, JR.

Department of Biology, College of Science
Polytechnic University of the Philippines

Abstract: Acid phosphatase is an enzyme component of semen. Its detection is important in forensics as a presumptive evidence of seminal stain in sexual crimes. Although it is widely used in developed countries, the technology is not taken advantage by present institutions. To remedy the woes of concerned institutions, tests are carried out in the local setting to determine the sensitivity, specificity, positive and negative predictive values, and the rates of the false negative and false positive results of a purple color test for the presence of acid phosphatase. A field kit from the United States has been assessed and compared to the performance characteristics of sperm detection by conventional microscopy in the Philippine National Police – Crime Laboratory. Results from a paired observation has yielded slightly better performance in terms of sensitivity for the purple color test. This has been significant at more than 99.9% confidence.

Keywords: *sexual crime, test for semen, acid phosphatase, crime laboratory*

1. INTRODUCTION

In 1997, under President Fidel V. Ramos' administration, concerned citizens' groups coordinated with the United Nations Population Fund and the Office of the President, through the National Commission on the Role of Filipino Women (NCRFW), to lift the plight of Filipinos and to take steps in eliminating violence in the society. These groups included the Women's Crisis Center (WCC) and Women's Legal Bureau (WLB). They set themselves to tackle violence against women (VAW) and invited representatives from the Department of Health, East Avenue Medical Center, Department of Social Welfare and Development, Department of Interior and Local Government, Quezon City Local Government Units, Department of Justice, National Bureau of Investigation and the Philippine National Police.

Among the different manifestation of violence, sexual crimes became the focal point of most discussions. Unofficial figures put thousands of sexually-related complaints on women and child desks all over the country. Sexual assault continues to represent the most rapidly growing violent crime in America. Thus, rape was addressed with the deepest concern in most meetings, and measures were planned to lessen or eliminate other forms of violence on its victims.

They recognized that the inadequacy of contemporary systems in documenting the crime was an injustice and a form of violence. Evidence was ideally collected to find signs of physical injuries, to verify carnal knowledge, to date sexual intercourse to an extent, and to possibly identify the perpetrator. The lack or shortcoming in any of the purposes enumerated would be a crippling of justice.

Frequently in courts, evidence of ejaculate or semen within or around body orifice would represent incontrovertible proof of the sexual nature of the crime or offense. Often, the presence of semen would be an evidence of violence itself. The importance of the detection of semen could not be understated, as its identification was part of all sexual crime investigation protocols. Advanced nations utilize DNA techniques in analyzing sperms and zinc tests in spotting semen (Suzuki, 1983). More recently, immunologic studies, enzyme-linked immunoassays (ELISA) and fluorescence *in-situ* hybridization techniques (FISH) were employed to determine the presence of antigen (Keil, Bachus & Trojer, 1996; Scheithauer & Hoffman, 1991; Kamenev, 1990), antibody (Belec, 1994; Belec, 1995), semen, sperm, a male epithelial or inflammatory cell in a female (Collins, 1994), or a female cell on the penis (Collins, 2000). Many still kept antiquated methods of identifying sperms by microscopy and detecting semen with an acid phosphatase test. The latter two methods were gold standards in evaluating many new tests (Graves, 1985; Keil, Stubbings, 1985; Kamenev, 1990; Scheithauer, 1991; Collins, 1994; Bachus & Trojer, 1996).

Sperm microscopy was the state of the art in the Philippines. The finding of a sperm in a vaginal smear would be unequivocal proof of presence of an ejaculate. However, positivity from this microscopic examination would drop precipitously 24 hours after the act. Degeneration of sperm through time was the major disadvantage. Its limited value was further confounded by the habitual dilly-dallies of a Filipina sexual crime survivor. They would usually complain of the crime late, if ever they would report at all.

Frank Lundquist, working at the Legal Medicine Unit at the University of Copenhagen, developed the acid phosphatase test for semen in 1945. Acid phosphatase (ACP) is secreted by the prostate gland. A consummated sexual act necessary involves the ejaculation of seminal fluid into the vagina. Ejaculates contain prostatic secretions. Theoretically, even vasectomized suspects can consummate a sexual act although their ejaculate will not yield a sperm. Tamaki in 1989 demonstrated that ACP activity in seminal stains (23.8 $\pm$ 15.2 IU/mg protein) is significantly greater than vaginal fluid stains (0.088 $\pm$ 0.049 IU/mg protein), and recommended the test for forensic study (Tamaki, 1989). The test became the gold standard for new reagents or examination procedures being produced in the science to detect the same (Hooft & van de Hoorde, 1997; Hooft & van de Hoorde, 1992; Hooft & van de Hoorde, 1990).

Forensic laboratories used the test repeatedly to detect the presence of seminal stains in various surfaces, but the sensitivity of this test wanes at about 12 hours postcoitus (Spitz, 1993) and several substances or materials are known to interfere in controlled tests (Hooft & van de Hoorde, 1994).

Detection of ACP was done through various essays (Henry, 1996; Steinman, 1995; Kloosterman, 1984; Schiff, 1969; Kind, 1964). Test kits were available in the international market and were regularly evaluated (Simich, 1999). However, such kits were not widely available in the country. For the purposes of this study, the Federal Bureau of Investigation, through the United States embassy had supplied a test kit. The kit was designated here as the purple color test for phosphatase. Otherwise, it bore the commercial label of "SF298 Seminal Fluid Field Test Kit."

The study aims to determine the difference in the test performance characteristics of acid phosphatase determination and sperm detection by direct microscopy in vaginal swab specimen of survivors of alleged consummated sexual crimes.

In view of the impetus provided by concerned citizens groups, the paper aim to study and provide material knowledge for an additional test to ascertain the sexual nature of a crime. The ACP test was a widely-used adjunct in identifying seminal stains. The performance characteristics of this presumptive test for semen were determined and compared to the test performance characteristics of sperm detection by microscopy. That the latter procedure was a gold standard would not be contested in any way in the article. Sperm detection by microscopy as a definitive test for the presence of semen could hardly be replaced by any technology in the modern history of forensics.

The performance characteristics of the purple color test for ACP would be described in terms of its sensitivity, specificity, false negative and false positive rates, and its positive and negative predictive values. These characteristics will be measured having the sperm detection by microscopy as the gold standard, composite criteria as a gold standard. The composite criteria utilized the physical examination data, and the disclosure and dates of consummation.

2. METHODOLOGY

The composite criteria were used in order to compare the performances of the purple color and test microscopy. The differences between the two procedures would be subjected to statistical tests for paired observations for a rational interpretation. This research would fall under a cohort study.

Subjects

Sexual crime survivors, who were examined from June 1 to August 31, 2000 at the Medicolegal Office of Philippine National Police Crime Laboratory (PNPCL), were included in the study. There were 29 of them. All of them were women, aged 7 to 25 years (mean age of 16 years). Two were married. All of them gave consent for the examinations and reliable histories. Twenty-seven were submitted for purple color test of acid phosphatase, because the last one denied consummation of the act.

As results from both procedures for detecting semen were inversely related to elapsed time from coitus to examination, the time lapse in days were obtained from the difference of the examination date and the last incident rate.

Human resources

The research enlisted the services of the Medicolegal duty officers at the PNPCL. Six different officers examined the 28 survivors included in the study. One female officer saw 18 of them. The author examined the slides for microscopy and sperm detection at wet mount and with Papanicolau stain. An officer, who was trained by the U.S. embassy representative, prepared reagents prior to use. A medical technologist helped in the Papanicolau staining of the smears.

Reagents

The reagent sample of the purple color test for ACP was provided by the SPF298 Seminal Fluid Test Kit (Sirchie, North Carolina, USA) by Sirchie Fingerprint Laboratories, Inc. Reagent names were classified under the propriety product codes or catalog numbers, SF298S1 (Sirchie, North Carolina, USA), SF298S2 (Sirchie, North Carolina, USA) and SF298S3 (Sirchie, North Carolina, USA). An acid phosphatase reagent was labeled SF298AT (Sirchie, North Carolina, USA)**.** The test procedure claimed to utilize the property of a unique substance found in high concentrations in the semen. That property was the ability to hydrolyze phosphoric acid testers.

Furthermore, it maintained that such substance was found at only very low levels in animal semen for it to interfere. Similar interference would be expected from vegetables juices of cauliflower, brussel sprouts and clover. Interference from bloodstains was disclaimed. The test would allegedly still be positive for three-year-old semen.

The chemical reagent for ACP may be *a*-naphthyl phosphate with *o*-dianisidine dye (Babson's method), or sodium thymolphthalein monophosphate (Roy's method), or *p*-nitrophenyl-phosphate substrate and tartrate inhibitor (Andersch's method). Seiden *et al.* (1983) preferred sodium thymolphthalein monophosphate over *a*-naphthyl phosphate due to the carcinogenicity of the associated *o*-dianisidine dye. Pragay *et al.* (1977) compared all reagents in terms of specificity and sensitivity, and came up with results where Roy's method fared best. The Andersch reagent is employed in a method similar to the detection of prostatic acid phosphatase in blood. A technique (Berg's method), which was used in most rape centers for the detection of acid phosphatase, was used by Schiff even on 25-year-old-specimen.

Protocol for the examination of sexual crime survivors

Upon consent of the survivor or her guardian, clinical data were recorded based on her accounts. Relevant clinical data obtained were the general data for subject identification, date of consummation of the alleged sexual acts, date of examination, and results of physical examination.

Swabs from the vaginal introitus of the survivors were immediately obtained for smears and purple color tests. The smears were examined microscopically (wet mount), and then preserved in ethyl alcohol and stained with the Papanicolau method. The researcher then identified the presence of sperm. The presence of at least one head and neck unit of the sperm constituted “positive” result for sperm. At the same time, the swab was tested for acid phosphatase.

Preparation of the purple color test for acid phosphatase

The SF298 Seminal Fluid Field Test Kit (Sirchie, North Carolina, USA) contained the dehydrated, gray white, powdery-fine reagent in several vials. Precalculated clear diluting fluid reagents, pieces of filter paper and disposable pipettes were included. A fresh reagent was created by mixing the powder with the fluids. The reagent turned black.

Administration of the purple color test

The fresh reagent was applied directly on the cotton swab of the suspected material. The blackened stain would turn purple if it was seminal in nature.

Papanicolau staining method

After examination of the fixed fresh smear under the microscope, wet mount for a quick look, the microscope slide was immediately redipped in ethanol 95% for at least 15 minutes prior to staining with the Papanicolau method. The wet mount examination of sperms under routine conditions was not ideal for the detection of sperms. A second slide for gram-staining was also made.

For the Papanicolau stain, fixing and rehydrating the smear, the microscope slide was treated with Harris hematoxylin, then rinse with tap water and appropriately decolorized with acid alcohol. It was rinsed again then dipped into ammonia water. Increasing concentration of alcohol dehydrated and prepared it for staining with the yellow dye orange G (OG6) and EA solutions, which contained eosin, light green and Bismarck brown. A cover slip was mounted over the slide prior to viewing.

Under the microscope, the small head of the sperm appeared dark blue, in contrast with larger epithelial cells that would have a pinkish cytoplasm.

Sperm detection by microscopy

The identification of at least one sperm head with a neck segment constitutes a positive result for microscopic detection of sperms. Sperms usually appeared as a small densely chromatic head capped with a clear acrosome at the tip, and neck connecting the long tail. A negative result meant no visualization of a spermatozoa or a definitive sperm head.

Purple color test for acid phosphatase

A purple color changed the black reagent after some contact with the questioned stain or material, meant positive for the presence of acid phosphatase. A negative reaction would keep the reagent color black.

Composite gold standard

The creation of the composite criteria was necessary to compare the performance of the two tests for semen. Because the two tests would corroborate disclosures of the consummation of the sexual crime, the composition would include and summate the data that would indicate the carnal knowledge. A positive corroboration of the composite criteria would mean the presence of physical genital injuries, disclosure, and disclosure dates within one week of examination. A negative value meant the absence of disclosure, or the absence of corroborative physical genital injuries if disclosure dates were within one week of examination.

3. RESULTS AND DISCUSSION

Twenty-eight female survivors were in the study. Table 1 shows 28 case numbers and corresponding results or comments. Under the column of "Acid Phosphatase Test," "NR" stood for *no record* where the officers-on-case did not report an equivocal result. *Positive* was represented by the symbol, "+", while "-"corresponded to *negative*. The same symbols meant the same under the columns, "Presence of Sperm" and "Recent Physical Injuries". A positive recent physical injury signified the presence of a genital injury consistent with the disclosed dates of the crimes. Inconsistency in the injury and disclosure would be tantamount to a "value." "NA" meant *not assessed* "Time Lapse in Days" was derived from the difference of disclosure dates and examination dates.

One of the survivors denied consummation of the rape attempt. Physical examination revealed no genital or extra-genital injuries. The officer on her case did not performed the test for ACP nor took vaginal swabs and looked for sperms. The survivor was 15 years old, single and nulliparous.

Two cases were unfit for the study due to poor preservation of specimen submitted for microscopy and unclear slide labels. Of the 25 remaining, 11 more had equivocal color changes of the ACP reagent. When the black reagent did not turn purple, but it turned dark blue or gray, the color change would be meaningless or at best equivocal.

Table 1. Summary of the case report and clinical results used in the study.

Case	ACP Test	Presence of Sperm	Recent Physical Injuries	Time Lapse in Days
1956	+	+	+	1.00
1960	NR	-	-	9.00
1996	NR	-	-	0.00
2079	-	-	-	4.00
2083	NR	NA	-	0.00
2097	NR	-	+	2.00
2099	NR	-	-	17.00
2100	NR	-	-	34.00
2150	-	-	-	2.00
2151	NR	-	-	88.00
2152	+	+	+	1.00
2188	+	+	+	5.00
2189	-	-	-	17.00
2190	NR	-	-	1.00
2329	+	-	+	1.00
2330	-	-	-	7.00
2334	-	-	+	1.00
2347	NR	-	-	3.00
2355	NR	-	-	33.00
2356	-	-	-	1.00
2359	-	-	-	9.00
2362	-	-	-	2.00
2403	-	-	+	0.00
2413	+	-	-	1.00
2423	+	NA	-	0.00
2430	NR	-	-	6.00
2433	NR	-	-	10
2477	NR	NR	-	NA

Legend: (+) *= positive;* (-) *= negative; NR = no record; NA = not assessed*

Performance characteristics of ACP with sperm detection as the gold standard

Of the 14 survivors, five were positive for ACP purple color test (Table 2). With the presence of sperm as the gold standard, the sensitivity of the color test was 1.0 and the specificity was 0.81, with the false positive rate of 0.18. The positive predictive value is 0.60. The index of sensitivity and specificity (1.8) was high. The zero false negative result could be attributed to the small number of samples.

Table 2. Two by two table for ACP test with sperm detection as gold standard, and performance values for the various characteristics of the ACP tests.

ACP	S+	S-	Total
A+	3	2	5
A-	0	9	9
Total	3	11	14

Sensitivity (true positive rate)	1.0
Specificity (true negative rate)	0.8
Positive predictive value	0.6
Negative predictive value	1.0
False negative	0.0
False positive	0.2

Legend: A+, positive for ACP; A-, negative for ACP; S+, positive for sperms; S-, negative for sperms

Performance characteristics of the two methods against a composite gold standard

The composite gold standard of the other parameters to corroborate the consummation of a sexual crime was created from Table 1. The 14 sets of observations were illustrated on Table 3. Tables 4 and 5 were the 2x2 table of each test. Table 6 compared the performance characteristics of both tests. The values in this table were computed from the data of Tables 4 and 5.

Table 3 shows data for the 14 survivors. All of them disclosed consummation of the sexual act but only 11 had an examination within one week from disclosure dates. Seven of 11 had genital injuries (Table 1). Put in another way, 11 out of 14 had sexual intercourse within one week of examination. Five of them were positive for acid phosphatase test (Table 4), while only three had detectable spermatozoa in their Papanicolau smears (Table 5).

Table 3. List of cases for the comparison of purple color test for ACP and sperm detection by microscopy. Their performance characteristics were measured against a composite gold standard.

Case	ACP Test	Presence of Sperm	Composite Gold Standard
1956	+	+	+
2079	-	-	+
2150	-	-	+
2152	+	+	+
2188	+	+	+
2189	-	-	-
2329	+	-	+
2330	-	-	-
2334	-	-	+
2356	-	-	+
2359	-	-	-
2362	-	-	+
2403	-	-	+
2413	+	-	+

Legend: (+) = *positive;* (-) = *negative*

Table 4. Two by two table for ACP test with composite gold standard.

SP	Gold Standard		Total
	GS+	GS-	
S+	3	0	3
S-	8	3	11
Total	11	3	14

Legend: (A+) = *positive for ACP;* (A-) = *negative for ACP;* (GS+) = *positive recent intercourse;* (GS-) = *negative recent intercourse*

Table 5. Two by two table for sperm detection by microscopy with composite gold standard.

ACP	Gold Standard		Total
	GS+	GS-	
A+	5	0	5
A-	6	3	9
Total	11	3	14

Legend: (S+) = *positive for sperms*; (S-) = *negative for sperms*; (GS+) = *positive recent intercourse;* (GS-) = *negative recent intercourse*

Table 6 demonstrated the differences in the performance characteristics of the purple color test for ACP and sperm detection by microscopy. The performance of ACP test was slightly better than the performance of sperm microscopy. It has a higher sensitivity (0.45 vs. 0.27) and sensitivity-specificity index (1.45 vs. 1.27), and a lower false negative rate (0.43 vs. 0.55).

The data was tested for significant difference using statistical formulae for paired observations. The McNemar's test was done as well as the Fisher's exact test. Under the first test, as *p* values of the two tests approach zero, there was no significant difference between the performances of purple color test and sperm microscopy, but larger samples would be needed to assess the probability. However, with the Fisher test, the probability of having such difference of results chance was about 0.00097. In simpler terms, the better performance of acid phosphatase test in terms of higher sensitivity and lower false negative results over sperm microscopy was about 9.99% significant. As a corroborative test, the purple color test could be reliable to be used in the absence of facilities for sperm microscopy.

The data showed a higher sensitivity of the purple color test for ACP than the microscopic examination for the sperms of the smears, using the gold standard composite of physical examination data and time lapse based on disclosure dates (Table 6). Other characteristics were equal in both tests. This finding supported the high sensitivity-specificity index value (1.8) of the purple color test even with the sperm detection as the gold standard. As the index value approached 2.0, the test would become an ideal indicator of its purpose.

Sperm microscopy, however, would be irreplaceable. Its lower but still high performance could be attributed to factors that affect the collection, preservation and processing of the materials. Even the time of collection would be paramount. The acid phosphatase test would also be subjected to interference from materials of nonhuman origin that could also hydrolyze the phosphoric acid esters.

Table 6. Test performance characteristics of the ACP test and microscopy for sperms.

Test Performance Characteristics	For Acid Phosphatase	For Sperm Detection by Microscopy
Sensitivity (True positive rate)	0.45	0.27
Specificity (True negative rate)	1.00	1.00
Positive predictive value	1.00	1.00
Negative predictive value	0.33	0.33
False negative	0.43	0.55
False positive	0	0

Nevertheless, this research affirmed the value of an acid phosphatase test in forensic investigation. Its importance would be especially tangible in field works and scene of crime operations, where facilities for microscopy would not be readily available.

4. CONCLUSIONS

The performance characteristics of the purple color test for acid phosphatase had comparable, or even better outcomes to sperm detection by microscopy in corroborating the allegations of the sexual crime survivor. In this paper, it turned out to be a better corroborator (higher-sensitivity-specificity index of 1.45), a better indicator (higher sensitivity of 0.45 and lower false negative rate of 0.43), and a reliable test that displayed 100% specificity and positive predictive values, and zero false positive rate.

Its significantly higher sensitivity and lower false negative rates than sperm detection by microscopy warrants as another very helpful procedure in assessing sexual crimes.

5. RECOMMENDATIONS

It is highly recommended that the protocols for the examination of sexual crime survivors be revised to accommodate routinely the test for acid phosphatase. This is very useful adjunct to objectively corroborate sexual crime survivor statements. All complainants would greatly benefit: the meek youngster, the housewife, the old, and the indecisive.

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