Forensic Detection of Semen I. The Acid Phosphatase Test

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Introduction

Acid phosphatase is an enzyme secreted by the prostate gland that is present in large amounts in seminal fluid. It, like psa (prostatic specific antigen), is not unique to the prostate and can be found in other biological fluids including vaginal secretions. It is therefore considered a presumptive chemical test for the presence of semen and semen must be confirmed by other means (sperm detection or psa detection using membrane test systems).

Testing for the presence of acid phosphatase can be extremely helpful however, in locating semen stains on clothing and for testing swabs from sexual assault cases. A strong positive reaction generally indicates that semen is present and that further testing is warranted.

For an excellent review on the history of acid phosphatase detection, see Gaensslen [1]. A number of testing methods exist for the sampling of items for the presence of acid phosphatase. The enzymatic breakdown of sodium-α-naphthyl phosphate by acid phosphatase and the subsequent conversion of o-dianisidine to a colored compound by the free naphthyl is a recognized test procedure for the detection of semen [2]. The Serological Research Institute (SERI) produces a powder they call ap spot test. When the powder is reconstituted in water, it can be used to screen stains and swabs for the presence of semen.

The sensitivity and stability of the product are discussed.

Materials and Methods

Sensitivity

Acid phosphatase was obtained from Sigma Chemical Company. The product number was P-1146, Lot 051K7038 and was isolated from potato. 50 units were purchased, consisting of 7.5 mg of solid having an activity of 6.7 units/mg (50.25 units). The solid was dissolved in 200 µL of deionized water. 100 µL of this solution (25 units) was added to a cotton-tipped swab that was allowed to air dry. 50 µL deionized water was added to the remaining 100 µL, mixed and 100 µL of this solution (17 units) was added to a

cotton-tipped swab that was allowed to air dry. Subsequent dilutions were made in this manner resulting in dry cotton-tipped swabs having the following units of acid phosphatase: 25, 17, 5.6, 1.8, 0.6, 0.2, 0.05 and 0.02.

Testing of these dry swabs was conducted in the following manner. Deionized water was added to a small piece of Whatman filter paper #3. Each swab was pressed against the filter paper strongly between thumb and forefinger for ten seconds. A single drop of freshly prepared SERI ap spot test (Lot 1562) was added to each piece of filter paper and color changes were recorded after 10 minutes.

Stability

SERI ap spot test (Lot 1562) was prepared fresh daily and used for case analysis. The reagent was maintained in a small glass dropper bottle protected from light with tape at room temperature. At the end of the business day (approximately 8 hours), the reagent was placed in a plastic15 mL Falcon tube and refrigerated. The following morning, fresh ap spot test was prepared and kept on the lab bench along with the previous preparation. This procedure was followed for the three remaining days of the week.

Whatman #3 filter paper was moistened and a cotton-tipped swab containing 25 units of acid phosphatase was pressed to 5 areas of the paper (following the procedure described previously). The same procedure was followed with 17 units of acid phosphatase.

SERI ap spot test reagent (fresh, 1 day, 2 days, 3 days, 4 days and 5 days old) was added to the filter paper and color changes were recorded after 10 minutes.

The same methods were followed using SERI ap spot test reagents that were stored frozen for 1 to 5 days; however, the reagents were not removed from the freezer daily.

Results and Discussion

Sexual assault kits and clothing are routinely submitted to crime laboratories for examination for the presence of semen. Typically, forensic scientists conduct visual examinations for stains followed by examination with an alternate light source on clothing and bedding items. This is generally followed by testing of stains for the presence of acid phosphatase, an enzyme secreted by the prostate and found in high levels in semen. Swabs collected from sexual assault survivors are generally tested for the presence of acid phosphatase followed by tests for the presence of spermatozoa, and P30 if necessary.

It is customary to test stained areas and swabs collected from the survivor indirectly. In other words, a transfer method involving wet or dry cotton-tipped swabs or moistened filter paper applied as an overlay to a stained area or swabbing is employed. As recommended by Barnett, et.al. [3], presumptive test reagents should NEVER be applied directly to items of evidence.

Following this methodology, experiments were designed to determine the sensitivity of one acid phosphatase test. The Serological Research Institute (SERI) sells a product they call ap spot test. It contains sodium-α-naphthyl phosphate and o-dianisidine (Fast Blue B). If acid phosphatase is present in a sample and a drop of the ap spot test is added, the enzyme catalyzes the breakdown of sodium-α-naphthyl phosphate producing free naphthyl that reacts with o-dianisidine producing a purple colored compound.

Results of Sensitivity Tests

Freshly prepared ap spot test gave positive results with acid phosphatase diluted to 0.18 to 0.6 units.* A photograph of the results appears in Figure 1.

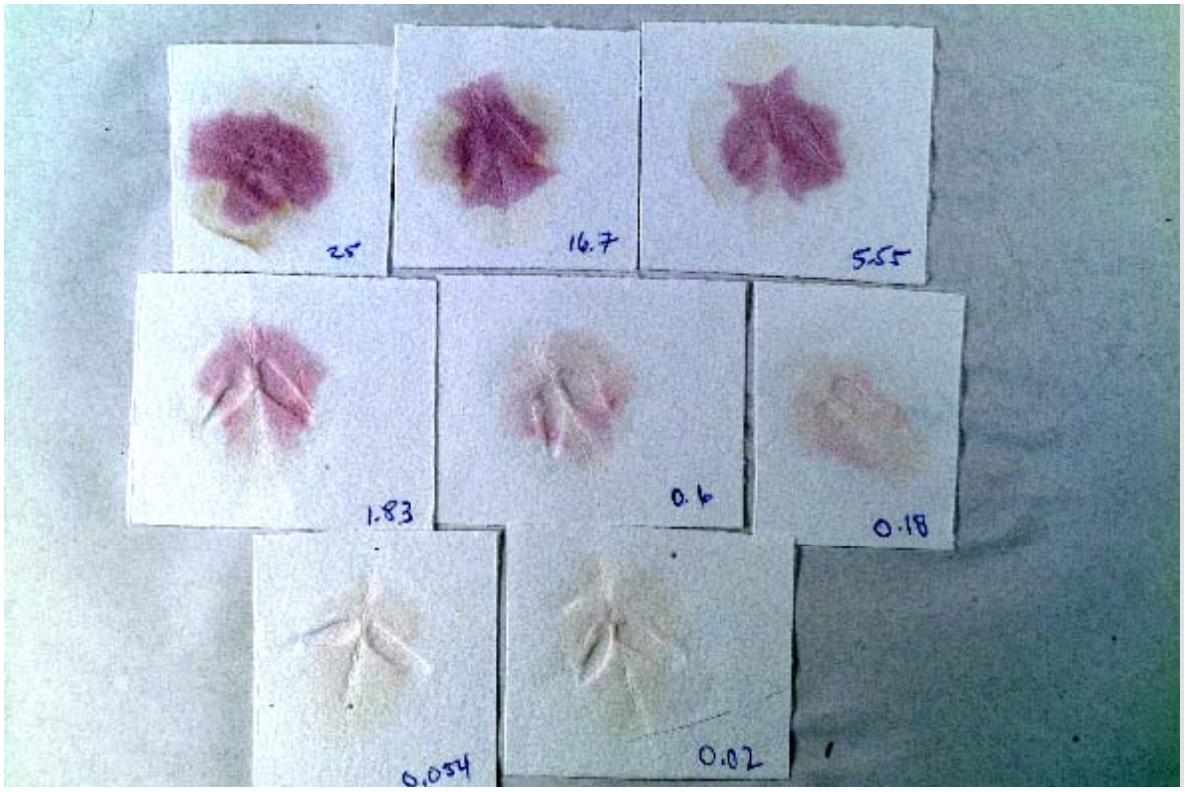

Figure 1. One drop of ap spot test added to moistened filter paper containing diluted acid phosphatase (25 to 0.02 units). Positive reaction (purple color change) obtained at 0.6 units.

Sensabaugh [4] published results of experiments designed to quantitate the levels of endogenous and postcoital vaginal acid phosphatase. He standardized data from several investigators including his data and obtained a range of endogenous vaginal acid phosphatase of 0.023 to 4.902 units. SERI's ap spot test would certainly react with these endogenous levels of acid phosphatase. Hence the presumptive nature of the ap test and the requirement that the presence of semen be confirmed in another manner.

*One unit will hydrolyze 1.0 μmole of p-nitrophenyl phosphate per minute at pH 4.8 at 37 °C

Results of Stability Tests

The directions supplied with SERI's ap spot test state to prepare the reagent daily. The Fast Blue B dye is light sensitive. At room temperature, on the lab bench and in the light, the ap spot test will begin to turn yellow and brown material will precipitate out.

The results of the stability experiments are shown in Figure 2.

Figure 2. Photograph of filter paper with 25 and 12 units of acid phosphatase and drops of fresh, 1 day, 2 day, 3 day and 4 day old ap spot test reagent.

A decrease in activity was observed in 1 day old ap spot test reagent, however it still reacted fairly well. By two days, the activity of the reagent dropped significantly and by four days, the reagent has lost the ability to detect 25 units of acid phosphatase.

Tests were conducted to determine whether freezing the reagent could enhance stability. The results of this experiment are shown in Figure 3. As can be seen in Figure 3, freezing did enhance the stability of the reagent. However, the frozen reagents were not

removed from the freezer and thawed on the laboratory bench daily, but remained in the freezer.

Figure 3. Varying units of acid phosphatase (25 u, 17 u, 6u and 2 u) deposited on filter paper to which ap spot test, frozen for various days (fresh, 1 day, 2 days, 5 days), was added.

The ap spot test stored frozen for 5 days worked as well as the freshly prepared reagent. However, once thawed and left on the lab bench, this reagent would degrade just as fresh or refrigerated reagent.

Interpretation of the color change indicating a positive result can be subjective. As seen in Figure 1, a deep, dark purple color change, especially if it occurs rapidly, strongly indicates the presence of semen and would demand further testing. Light results such as 0.6 to 0.18 units (Figure 1) may be the result of very weak semen stains or endogenous acid phosphatase levels.

Occasionally, color changes having a tannish hue are found on swabs taken from survivors, especially rectal swabs. Figure 4 shows one such result. This is a typical result from rectal and anal swabs and should not be confused with a positive AP reaction.

Subsequent testing for P30 and spermatozoa was negative. Certainly, these results can't be ignored, but the experienced analyst will recognize these as negative results, and not a true purple color change indicating the presence of semen.

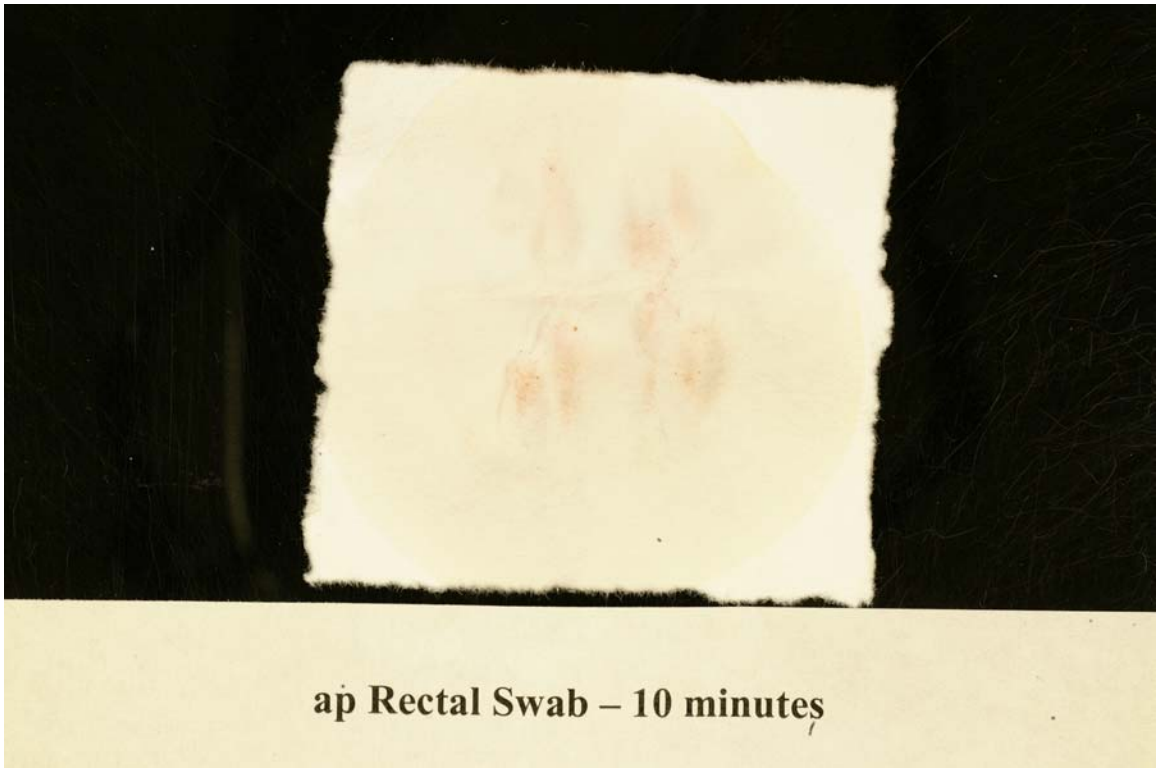

Figure 4. Typical results obtained from ap spot test added to filter paper transfer from a rectal swab. No semen was present on the swab.

Experience cannot be overemphasized and senior forensic biologists should make a habit of passing on their knowledge to new analysts. Schiff [5] stated "after 14 year's (sp) experience with the AP test, the author has found it to have great merit as a test for the identification of seminal fluid in the absence of spermatozoa". He continues, "because he has used the test qualitatively rather than quantitatively, he has established no arbitrary, numerical cutoff as to when the test is to be declared positive and when negative". He states that the test "is only as dependable as the physician, chemist, or pathologist who performs it".

Schiff lists three guidelines that he maintains should be followed in conducting the AP test:

1. The reagents must be freshly prepared.

   He found that the diazo-coupling agent that originally was clear and lightly tinted began to precipitate after 12 hours. This author has experienced the same result and recommends that it be prepared fresh daily.

2. The examiner must follow the same protocol in every case.

   In other words, press a swab to a piece of filter paper for the same time period applying the same pressure each and every time. Add the same number of drops of AP spot test and wait the same length of time each and every time. Developing consistency in the conducting the test will make the analyst more comfortable in interpreting the results.

3. The examiner must not deviate from his/her method of reading the test.

   After the analyst gains confidence in conducting the test, and performs a sufficient number of confirmatory tests on various test results, the analyst will come to realize what is a true positive reaction.

Conclusion

Testing for acid phosphatase remains a valuable presumptive test for the screening of swabs collected from sexual assault survivors and for the testing of stains found on clothing and bedding. The experienced forensic biologist knows that all stains that fluoresce are not necessarily semen and all semen stains do not fluoresce. In addition, semen is a heterogeneous fluid and portions of a deposited stain will contain various levels of acid phosphatase, P30 and spermatozoa. Examination of a pair of panties with an alternate light source and extraction of all the stains that fluoresce followed by psa analysis may yield semen, however, it may not, and it does not appear to this author to be the best use of time and expenses. Acid phosphatase mapping is an inexpensive and quick method for screening such stains.

Years ago, forensic biologists (serologists) were taught what was termed "a systematic approach to the analysis of semen evidence" developed by Blake, Sensabaugh and Bashinski [6]. The three major steps consisted of locating the stain, estimating the amount of semen found and genetic analysis of the stain. With the advent of DNA, it seems possible that one could just cut a stain from a pair of underwear, extract it and generate a DNA profile. Obtaining the subject's DNA profile on the underwear, where it shouldn't be, should be conclusive proof of guilt. And perhaps it is. However, this analyst, trained in the "old school" feels that a more thorough analysis is warranted. Acid phosphatase mapping in locating stains and sperm quantitation of positive stains are important steps that can only aid the DNA analyst in interpreting the results.

It behooves the forensic biologist to utilize all of the methods available for optimum semen detection.

References

1. Gaensslen RE. **Sourcebook in Forensic Serology, Immunology, and Biochemistry**, Research Foundation of the City University of New York, 1983.
2. Babson AL, Read P and Phillips G. The importance of the substrate in assays of acid phosphatase in serum, American Journal of Clinical Pathology, 32 (1), July 1959, pp. 1-5.
3. Barnett PD, Blake ET, Super-Mihalovich J, Harmor G, Rawlinson L and Wraxall B. Discussion of "Effects of presumptive test reagents on the ability to obtain restriction fragment length polymorphism (RFLP) patterns from human blood and semen stains", Journal of Forensic Sciences, 37 (2), March 1992, p.369.
4. Sensabaugh GF. The quantitative acid phosphatase test. A statistical analysis of endogenous and postcoital acid phosphatase levels in the vagina, Journal of Forensic Sciences, 24 (2), April 1979, pp. 346-365
5. Schiff AF. Reliability of the acid phosphatase test for the identification of seminal fluid, Journal of Forensic Sciences, 23 (4), October 1978, pp. 833-843.
6. Blake ET, Sensabaugh GF and Bashinski J. A systematic approach to the analysis of semen evidence, Fall Semiannual Seminar of the California Association of Criminalists, November 1980.