July - December 2021 Volume 30 Issue 2
PRINT ISSN: 2277-1867
ONLINE ISSN: 2277-8853

JOURNAL OF FORENSIC MEDICINE SCIENCE AND LAW

Official Publication of Medicolegal Association of Maharashtra

MULTISPECIALITY, MULTIDISCIPLINARY, NATIONAL
PEER REVIEWED, OPEN ACCESS, MLAM (SOCIETY) JOURNAL
Indexed with Scopus (Elsevier) & Index Copernicus (Poland)

Editorial Office Address
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JOURNAL OF FORENSIC MEDICINE SCIENCE AND LAW
(Official Publication of Medicolegal Association of Maharashtra)
Email.id: mlameditor@gmail.com

PRINT ISSN: 2277-1867
ONLINE ISSN: 2277-8853

Original Research Article

Role of Acid Phosphatase Test over Detection of Spermatozoa in Vaginal Swabs – A Cross-Sectional Study

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Article Info

Received on: 15.09.2021
Accepted on: 30.11.2021

Key words
Acid Phosphatase Spot Test,
Hematoxylin and Eosin Stain,
Spermatozoa,
Sexual Assault.

Abstract

Background: Detection of intact spermatozoa is considered the gold standard in being corroborative evidence for recent sexual intercourse, however in many cases; the survivor is brought late for medical examination. Hence, the acid phosphatase test may be helpful. **Aims and objective:** i)To find out specificity and sensitivity of acid phosphatase test with hymenal tear. ii)To compare results of acid phosphatase test to that of detection of spermatozoa. **Materials and Methods:** This was a cross-sectional study, conducted at three tertiary hospitals for three years. The study group included sexual assault survivors brought for medical examination. Acid phosphatase was detected in vaginal swabs by the use of alpha-naphthyl phosphate as substrate and brentamine fast blue b. Microscopic examination was done for spermatozoa following hematoxylin and eosin stain. **Results:** Out of 67 cases, one tested positive for spermatozoa. 12 tested positive for the acid phosphatase test. Acid phosphatase positivity was 100% cases with a fresh hymenal tear, 16.7% with an old tear, and 7.4% with no tear, sensitivity was 25% and specificity was 92.59%. The sensitivity of the detection of spermatozoa was 2.5%. **Conclusion:** Acid phosphatase test has better sensitivity and specificity to that of detection of spermatozoa and should be routinely used in sexual assault cases.

1. Introduction

Investigations play a pivotal role in confirming cases of sexual assault and act as corroborative evidence that has varied and evolved. Earlier, matching of blood groups of the suspect with that in the crime scene was confirmatory evidence but now forensic technology has evolved to the arena of DNA analysis where the DNA of the accused is matched with the biological sample at the crime scene.

However, the finding of single intact spermatozoa from vaginal swabs remains the surest marker of recent sexual intercourse. The persistence of spermatozoa, seminal blood group antigens, choline and seminal acid phosphatase in the human vagina after sexual intercourse had been studied earlier. Anne Davies and Elizabeth Wilson collected many studies and summarised and found that after

How to cite this article: Nath A, Das RK, Sarmah H. Role of Acid Phosphatase Test over Detection of Spermatozoa in Vaginal Swabs – A Cross-Sectional Study. J For Med Sci Law 2021;30(2):16-21.

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sexual intercourse, spermatozoa were usually found within three days, seminal acid phosphatase remained detectable up to three days, choline was detected only on swabs taken within one day.[1]

The possibility of getting positive results reduced after fourteen hours. Those swabs taken within forty-eight hours of intercourse was seen useful in detecting obvious level of seminal blood-group antigens. The chances of obtaining a positive result decreased with an increasing time interval after intercourse.

2. Aims and objectives:

i) To find out the specificity and sensitivity of acid phosphatase test with hymeneal tear.
ii) To compare the results of the acid phosphatase test to that of detection of spermatozoa.

3. Materials and Methods:

The present study design was cross-sectional. The study samples were collected from 03 tertiary care medical institutes. The study period was for three years (August 2014 to August 2017). Alleged sexual assault cases seeking medical aid or examination in these institutes either by themselves or were brought by police were included in the study group after their written consent. In the case of children, consent was taken from parents. Their identifying data were kept confidential with the authors. Format of the Ministry of Health and Family Welfare for examination of Survivor of Sexual Violence was used to record the data.[2]

The following data were noted – History of Penetration of penis/Insertion of other parts of body or objects, Body part assaulted, Duration between incident and reporting, presence or absence of genital or extra-genital injuries. Depending on the history of the incident, the following samples were selected and processed: A) Vaginal swabs. B) Anal/Rectal swabs. C) Oral swabs. D) Skin swabs E) Piece of undergarments and/or other clothing with suspected seminal stain.

Sterile cotton swabs were made from pieces of dried lateral veins of coconut leaves (each measuring 10 cm long and sterilized). Sterile cotton balls were wrapped at one end of the stick using aseptic measures. Each sample was subjected to two tests- i) microscopic examination for detection of spermatozoa after smearing on a glass slide and staining with hematoxylin and eosin stain and ii) acid phosphatase test by subjecting the swab dipped in normal saline to alpha-naphthyl phosphate and the diazo dye brentamine fast blue B. Each smear was air-dried and stained with hematoxylin and eosin stain. Following the collection of vaginal swabs, the smear was made on a clean glass slide (75mmX25mm) by horizontally smearing the swab at one go to avoid clumping of vaginal epithelial cells. The glass slide is then numbered at one end by a marker and kept on the tabletop for drying under normal air and room temperature. After drying, the glass slides were stained with hematoxylin and eosin dyes.

The staining procedure included firstly staining with Haematoxylin solution for 20 minutes, washing under tap water for 1 minute, staining with Eosin solution for 10 minutes, and finally washing for 1 minute under tap water. Slides were air-dried. The slide was then mounted on a microscope and visualized for spermatozoa along with vaginal epithelial cells under 4X, 10X, and 100X lenses. In case the findings were positive for the presence of spermatozoa, photographs of the slide under a microscope were clicked. For the acid-phosphatase test, first of all, preparation of glacial acetic acid solution and preparation of acid phosphate buffer was done. For the preparation of the glacial acetic acid solution, to 1 part of distilled water, we added 1 part of glacial acetic acid. For the preparation of acid phosphate buffer, 10 grams of anhydrous sodium acetate is added to 5ml glacial acetic acid in a measuring cylinder and the volume is adjusted to 500 ml by adding water.

After preparing the buffer, for the main test 0.025grams of sodium α naphthyl phosphate was measured and kept in a beaker. 10 ml of acid phosphate buffer was added to it and gently stirred with an iron stirrer to form solution "A" 0.050grams of fast blue b salt was measured and kept in a beaker. 10 ml of acid phosphate buffer is added to it and gently stirred with an iron stirrer to form solution "B". In a porcelain tray piece of samples (swab/cloth) was kept in each slot and a piece of filter paper as control is kept at another slot. To each slot, a drop of solution A and a drop of solution B was added.

Colour change was observed. The purple colour indicated the presence of acid phosphatase and was noted and recorded accordingly. All the data were entered into SPSS software version 21 and were expressed as numbers and percentages Tear of the hymen was cross-tabulated with acid-phosphatase test using Fisher's exact test and the p-value of less than 0.05 was considered to be significant. The sensitivity and specificity of both the microscopical examination of spermatozoa and acid phosphatase spot test were evaluated.

4. Results:

Out of 67 cases analyzed, in 40 cases (59.70%), there was a history of penetration by the penis. In 22 cases (32.83%), no history of penetration/insertion was present but the only history of touching or fondling private parts was present. In 4 cases (5.97%) there was a history of insertion by fingers and in 1(1.49%) case there was a history of insertion by a pen. (**Table No. 1**).

Table no. 1: History of penetration/insertion.

History of penetration/Insertion	Number	Percentage%
No penetration/Insertion	22	32.83
Penetration by penis	40	59.7
Insertion by fingers	4	5.97
Insertion by object	1	1.49
Total	67	100

From history, most common body part assaulted was vagina (n= 42, 62.68%), followed by labia majora and minora (n=15, 22.38%), mouth (n=2, 2.98%), anus (n=1, 1.49%). There was no assault on the urethra. Other body parts like breasts, thighs, buttocks, etc were assaulted in 7 cases (10.44%). (**Figure no. 1**).

Figure no. 1: Frequency of various body parts assaulted.

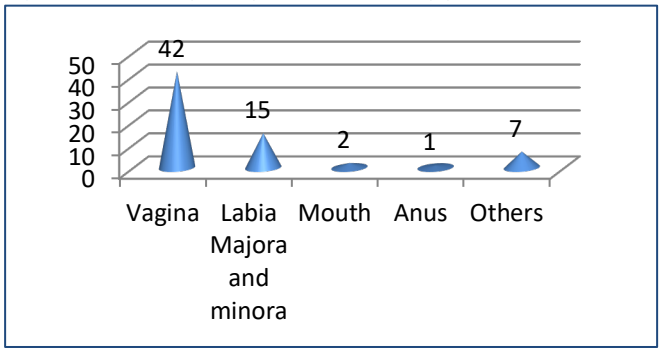


Table no. 2: Duration between incident and reporting.

Duration between incident and reporting	Number	Percentage
< 24 hrs	1	1.49
2-4 days	2	2.98
5-7 days	2	2.98
1-2 weeks	11	16.41
2-4 weeks	8	11.94
1-2 months	11	16.41
2-6 months	12	17.91
>6 months	20	29.85
Total	67	100

In no case was a condom used. In all of the cases, survivors washed, urinated, defecated, and wore new clothes before coming for medical examination. Although 67 cases were taken into account during the study period only one case was reported within the same day of occurrence of the incident (n=1, 1.49%), 2 cases (4.98%) were reported in between 2-4 days, 2 cases (4.98%) reported in between 5-7 days, 11 cases (16.41%) reported between 1-2 weeks, 8 cases (11.94%) reported between 2-4 weeks, 11 cases (16.41%) reported between 1-2 months, 12 cases (17.91%) reported between 2-6 months, 20 cases (29.85%) reported beyond 6 months (**Table No. 2**).

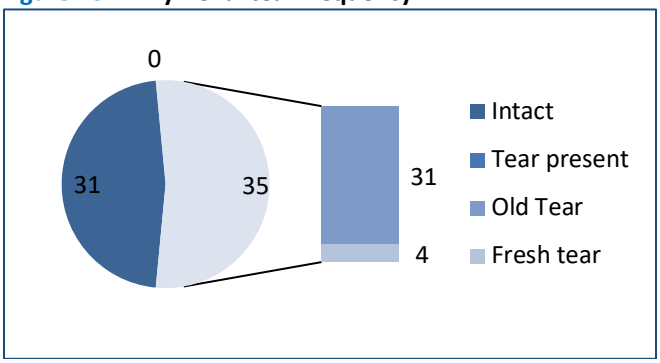
Examination of the survivors revealed the following injuries- In 2 cases injury marks were found over the breasts. No injury marks in the anus or oral cavity were found. In 57.57% of cases, the genital injury was present. In 35 cases injury was found in the form of tear of the hymen. 4 cases revealed fresh tears over hymen while 31 cases showed an old healed tear. In 29 cases, the number of a tear over hymen was more than 1 in number (**Table No. 3**).

Table no. 3: Position of genital and extra-genital injuries.

Position of genital and extra-genital injuries		Number of cases
Hymen	Old tear	31
	New tear	4
Vagina	Congestion	7
Labia minora	Congestion	3
Posterior labial commisure	Tear	4

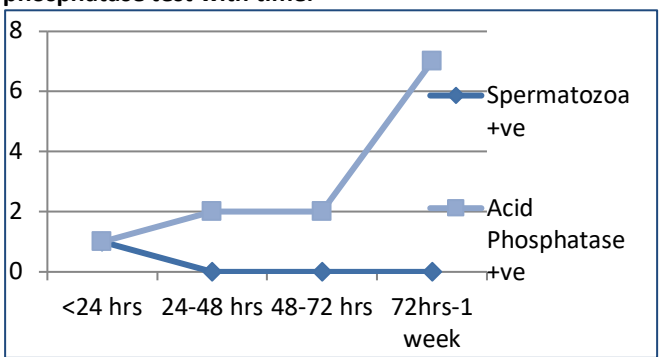
The most common position of tear was 5 o'clock, followed by 3 o'clock, 7 o'clock, 9 o'clock, and 11 o'clock. In 7 cases congestion was present over the vagina. In 3 cases congestion was present over the labia minora. In 4 cases, it was revealed tear on the posterior labial commissure. (**Figure no. 2**).

Figure no. 2: Hymenal tear frequency.



One sample showed positive for intact spermatozoa as shown and 12 samples showed positive for acid phosphatase enzyme. The case reported within 24 hours only showed positive for spermatozoa. The acid phosphatase test was positive for one week (**Figure no. 3**).

Figure no. 3: Positivity of spermatozoa and acid phosphatase test with time.



The sensitivity of the acid phosphatase test to tear of the hymen was found to be 25% and specificity was found to be 92.5%. The sensitivity of detection of spermatozoa to tear of the hymen was found to be 100% and specificity was found to be 2.5%. Relation between tear of hymen and acid phosphatase test has been tested by applying Fisher's exact test and the result was found to be significant. (**Table No. 4**)

Table no. 4: Coss tabulation between Hymenal tear and acid phosphatase test.

Type of Tear of hymen		Acid phosphatase test		Fishers exact test
		Positive	Negative	
Old tear	Count	6	30	P= 0.001
	% within Tear of hymen(old/ new)	16.7 %	83.3 %	
New tear	Count	4	0	
	% within Tear of hymen(old/ new)	100. 0%	0.0%	
No tear	Count	2	25	
	% within Tear of hymen(old/ new)	7.4%	92.6 %	

5. Discussion:

Florence in 1896 gave a fascinating audit of the techniques utilized in examining alleged survivors of rape preceding the nineteenth century. Even though spermatozoa were found in 1677, acknowledgment of the realities that the cells were one of a kind to seminal fluid and that the sperm cell was the main factor in reproduction was not recognized for some time until the 19th century. In sexual assault cases, sperm cells were not considered as evidence in seminal fluid. That no suitable methods were available for the purpose undoubtedly accounts for that situation in part, but perhaps more importantly, examinations of alleged survivors were carried out by members of high repute instead of doctors. Bayard in 1839, elaborated utilization of microscope for examining spermatozoa from seminal stain.[3] He standardized the procedure and thereby this methodology became accepted globally. The earlier chemical methods were gradually abandoned. However, efforts were still in motion for standardizing non-microscopical methods for examining and classifying different body fluid stains. Brouardel emphasized microscopy as the principal technique for identifying seminal stains in 1879. The oldest literature about the persistence of spermatozoa in the vaginal canal was given by Pollack in 1943. In his survey, he mentioned widely varying estimates of the time that spermatozoa survive in the vagina and gave various estimates from 30 minutes to 17 days.[4] Pollack also noted that seminal stain stiffened fine fabric. Sharpe in 1963 in his research paper stated that motile forms are found in the vagina for six hours after coitus, the average time being three hours.[5] During menstruation, the survival period for spermatozoa in the vagina averages four hours. The time varies with the state of the fluid in the vagina. Such extensive researches arise from and just remind us of the need for proof of the heinous crime of rape. It could be surmised that given the volume of such harsh realities of the present, researchers had made excellent discoveries in past to assist prompt criminal proceedings of the future. Morrison in 1972 thought that sperm cells survived longer following coitus in the first 14 days after menstruation.[6] The ability to find sperm dropped markedly after 48 hours post-coitus. If intercourse had occurred on the fifth postmenstrual day, there was less chances of getting sperm in the vagina up to 9 days after intercourse. In one exceptional case, even after the 12th day of sexual intercourse, spermatozoa were detected on a cervical smear as no other cervical smears were positive after 10 days following intercourse. In pregnant women, it was found that till one-week spermatozoa were detectable in the vagina. Morrison noted that, in rare instances, sperm could be found on cervical smears taken after the end of a menstrual period where coitus had occurred during menstruation. Twenty hours post intercourse; spermatozoa were positively detected in rectal smear by Enos and Beyer in 1977.[7] They also noted that in cases where oral sex has been reported

by the survivor, oral swabs must be examined. Possibility of Sperm getting identified by Papanicolaou staining in oral swabs was up to 6 hrs after the incident and could survive oral hygiene practices. In 1978, Enos and Beyer stated that the spermatozoa heads presence in anal area or the rectum need to be interpreted cautiously. In dead women's vagina, spermatozoa appeared to survive longer. Wilson in 1974 reported a case in which sperm were recovered from a rape-murder survivor 16 days after the incident where the body was found in a mountainous area with low temperature zone.[8] The survival of spermatozoa depends upon the natural environment to which the body was subjected. As eosin stains the cytoplasmic organelles in varying shades of pink, red, or orange, hematoxylin is generally used in combination with eosin. Eosin is acidic, negatively charged dye and binds to positively charged proteins in the cytoplasm. The acid phosphatase test is most widely employed techniques for semen identification, apart from sperm cell identification itself. The detection of the enzyme acid phosphatase has been comprehensively discussed by Kind in 1964 as a searching tool, for locating seminal stain on garments and surfaces and the application of the phosphatase assay using p-nitrophenyl phosphate as substrate.[9] In 1947, Kaye recommended the acid phosphatase test for seminal stain identification.[10] He employed phenyl phosphate as a substrate. Seminal fluid stains that have a minimum of 30 King Armstrong units of AP activity were found and any stain containing that level of activity or higher was considered to be of seminal origin. In 1949, Kaye reported that a wide variety of substances contained less than 5 King Armstrong units of activity per ml, or per cm^2 of stained cloth. These substances were viz. vaginal secretions, urine, serum, blood, menstrual blood, saliva, perspiration, pus, nasal mucus, gastric juice, faeces, and many foods and beverages. Values above 25 K-A units/cm^2 stain were considered positive for semen. Even when seminal stains were as old as 6 months, they still gave positive reactions with the acid-phosphatase test. In the present study, only one survivor reported within 24 hours which is in accordance with the study conducted by Sukul et al and Tamuli et al.[11,12] Our study was alike the studies conducted by Tamuli and Bandopadhyay where old hymenal tears were found to be more than fresh hymenal tears.[13] In only one case, spermatozoa were detectable in smear resembling studies of Sarkar and Sharma who also found less number of spermatozoa positivity.[14,15] Alike Schumann, we also found more acid phosphatase positivity than spermatozoa positivity.[16] In our study, we found positivity of acid phosphatase till one week after alleged sexual assault. Collins and Kim A. M.D found positivity till 2.5 months after sexual intercourse and Standefer JC and Street EW found positivity till 1 month after sexual intercourse.[17,18]

Conclusion:

As acid phosphatase shows positivity longer than detection of spermatozoa in the smear in cases of alleged sexual assault, therefore this should be routinely done as a standard test while dealing with such cases because there is an invariable delay in reporting to law enforcement agencies or medical establishments.

Conflict of interest: None.
Ethical Clearance: Yes.
Source of Funding: None.

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