Literature Review: Forensic Analysis or Identification of Semen

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Abstract

The main objective of this literature review on the articles is to find out one of the best techniques that can be used for the identification of semen in forensics.

Semen, also known as seminal fluid contains spermatozoa. This fluid is a mixture of fluids secreted by gonads. Many cases in forensics involve sexual offenses, making it necessary to examine the pieces of evidence such as clothes, area around the vagina, etc. for the presence of seminal stains. In the examination of the seminal fluid evidence, there are two types of tests performed in forensics. One is a presumptive test to find out whether the stain is semen or not and the other one is a confirmatory test for confirming that stain is seminal stain. In forensics, the most widely test used for the presumptive identification is Acid Phosphatase Test: it’s a spot test. Prostate gland secret enzyme Acid Phosphatase. When it reacts with alpha naphthyl acid phosphatase and brentamine fast blue, it gives a purple spot indicating the presence of semen. Confirmatory tests used in forensics are PSA and RSID, Prostate-Specific Antigen utilizes a test known as ABA Cards or P30 test to screen PSA. This test gives positive results even when no spermatozoa is present. RSID Semen Strip Test gives sensitivity and specificity to human semen. It identifies the presence of seminal vesicle specific antigen i.e. Semenogelin. This antigen is specific to semen so no cross-reactivity with other body fluids can occur.

Keywords: Immuno fluorescence, RSID, Prostate specific antigen. (PSA), Brentamine Fast Blue, Acid Phosphatase.

Introduction

On the 21st October of 2019, National Crime Records Bureau has released its report on the number of rape cases in the year 2018, which is 34,000. And the conviction rate is 27% i.e. 24,820 cases have remained unsolved. The main reason for the acquittal of the criminal could be the improper techniques used for the comparison of semen samples from the crime scenes and those of the criminals. There are a few tests that are time consuming and may delay the justice to the victim. [1]

Semen, also known as seminal fluid contains spermatozoa, fructose, citric acid, free amino acids, enzymes, and few minerals. There are two types of test performed for the identification of semen that are presumptive test and confirmatory test. Presumptive test is used for initial screening to identify whether the stain is a particular body fluid or not and Confirmatory test are used to identify the type of body fluid from which the stain originated and to determine whether it is human origin or animal origin.[2] Primarily including presumptive tests like microscopic identification, acid phosphatase test, etc, and confirmatory tests like PSA and RSID. These tests depend on the presence of sperm and other components of semen.

There are cases where sperm cannot be found like in the condition of oligospermia and azoospermia, and in such situations, identification of sperm could not be reliable. Acid phosphatase test, on the other hand, cannot be used for the identification of old samples due to the degradation of the enzyme. Though the PSA tests are highly sensitive, they give positive results for post ejaculate urine and adult male

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urine along with semen. Advancements in this field are to be done for rapid identification of sperm, more reliability, and high sensitivity.

This review article includes nearly all tests, both presumptive and confirmatory for the identification of semen along with the method used its limitations. This would help one to conclude the better test to be used for the identification of sperm.

**Presumptive and Confirmatory Tests**

1. **Microscopic Examination of Semen**
   Staining method is used for observing spermatozoa under microscope. The stains used were Eigrosin and Eosin stain.[3]

2. **Immuno Fluorescence Staining Kit**
   This method is alternative to microscopic examination for searching for spermatozoa in semen. This kit uses monoclonal antibody to detect the antigen present on the head of sperm. This kit will give green fluorescence when the antigen and antibody will combine with each other and indicating presence of spermatozoa.
   Limitation: This test is not used for dried semen as when the semen dries the spermatozoa becomes brittle so while washing and staining them there are chances that they can easily disintegrate. [3]

3. **Alternative Light Source (ALS)**
   Semen can be analysed by using ALS. When the forensic scientist reaches the crime scene the area is larger than the individual swab then they scan the area with the help of ALS.[4] The Wood Lamp is a specific device which emits wavelength in between 320 to 400nm and is small and easy to use. However, when wood lamp was tested it gives false positive result with ointments and cream. Another ALS was Blue maxx™ BM500 was tested and was 100 percent sensitive to semen.[5] Semen shows fluorescence due to presence of flavin and choline conjugated protein. The fluorescence color varies from blue to yellow.
   Limitation: Many molecules similar to choline and flavin shows fluorescence. When sample was exposed to factors like heat, humidity, oxidising agent and microorganisms it affects the fluorescence activity.[4]

4. **Christmas Tree Stain**
   In this test the microscopic examination of spermatozoa is done for confirming the presence of semen. The main reagents which are used are Picroindigocarmine that will stain neck and tail portions of the sperm in green or blue color, and Nuclear Fast Blue (also known as Kernechtrot) which will stain sperm head in red color and tip of head i.e. acrosomal cap in pink color.
   Limitation: the sperm tails break easily while staining and washing. One should be trained enough to make differentiation between sperm head and other type of cells.[4]

5. **Seminal Acid Phosphatase (SAP) or Acid Phosphatase Test**
   Acid phosphatase is secreted from Prostate Gland in high amount. [4] SAP has the ability to catalyse the hydrolysis of phosphate and form a product that reacts with diazonium salt chromogen which causes color change. [5] The concentration of acid phosphatase decreases with time in vaginal secretion. The positive results are seen up to 36 hrs and disappears in 72 hrs if the body is refrigerated then it may change. [6]
Acidic solution of Alpha naphthyl acid phosphatase and Brentamine Fast Blue reacts with Acid Phosphatase and gives color change i.e. purple color. Shade of color varies with enzyme activities.

Limitations: The color can be negatively affected by the age and condition of stain. False positive results are obtained with human RBC, cauliflower juice etc. enzymes degrade when exposed to heat, mold or chemicals.

Other test similar to SAP are VAP (Vaginal Acid Phosphatase) which gives less false positive results as compared to SAP. Isoelectric focusing can be used to differentiate between SAP and VAP.

LAP (Leucine Aminopeptidase) it five less false positive result and becomes less sensitive on dilution.

GDA (Glycylproline Dipeptidyl Aminopeptidase) stains which are old as 24 years also shows positive results. This shows false positive results with vaginal fluid and strawberry etc. \[5\]

6. Florence Test

It is a test for presence of choline. Choline originates from seminal vesicles. In this test the solution of 10 percent HCl is used for extraction of stain and then its is treated with Potassium Iodide and Iodine solution. When dark brown crystals of choline iodide are formed it indicated presence of choline. The test also shows negative results with other bodily fluids such as vaginal fluid and semen from other species.

Limitation: There is high possibility of false positive result because of low sensitivity.

Another method for detection of choline is Isotachophoresis. It is complicated methods but has no false positive results, stains which are 10 years old also show positive result. \[5\][6]

7. Barberio’s Test

Different types of Polyamines are present in semen out of which Spermine is present in highest concentration. Previously used methods for detection of spermine was HPLC combined with simple extraction but it is not used now. Another test which is used is Barberio’s method. In this test when the solution of Picric Acid added to semen sample yellow crystals of spermine picrate was formed indication that stain is for semen.

Another crystal test used is Puanen’s test which uses reagent Naphthol Yellow S and gives orange color crystals in the presence of semen. \[5\][6]

8. Choline and Spermine Test

When the semen sample is in liquid or dry form then it is identified by doing TLC for analysis of presence of a unique combination of spermine and choline. Sample which is less in quantity up to 1 micro litre can be detected. The sensitivity of identification and detection is enhanced by using PCR. \[6\]

9. Rapid detection of human seminal plasma protein by membrane aspiration test

This test also called dot- immuno binding method .This method was developed for identification human seminal plasma (HSP) antigens biological materials such as vaginal swabs or extract from seminal stains in membrane based aspiration test sample was placed on dotted nitrocellulose membrane in disposable aspiration kit after drying and aspirations of quenching solutions on membrane, then aspiration of suspension solutions of gold particles (GP) which are coated with an immune serum to HSP on dotted nitrocellulose membrane take place. If HSP antigen is present Then red colouration obtains on dotted portion after several
repeated aspirations and within few minutes result can be obtained. 
Applicability: broad applicability due to its simplicity and rapidity. 
Limitations: GP size are too large, and decrease in reactivity of the GP reagent due to an increase in size.\[7\]

10. Detection of prostate specific antigen by ELISA Method

Enzyme Linked Immunosorbent Assay (ELISA) used for the detection of prostate specific antigen (PSA OR P30) in sexual assaults cases. The sensitivity of ELISA is less than 1 ng/ml PSA of sample this method identified based on sensitivity and specificity of the test, this test did not give false - positive result against other body fluids and other contaminants but gives false - negative results when sample contaminated with detergents, due to this ELISA found that highly specific and sensitive and more efficient method for identification of semen sample.\[8\]

11. Rapid spot test for identifying suspected semen specimens

The concentration of acid phosphatase enzyme and zinc is higher in semen. The colour spot test for zinc and acid phosphatase reagents dried on cotton - tipped swabs which are impregnated with chromogenic reactants which at store at room temperature in closed containers prior to used. The substrate for this test is paranitrophenylphosphate and it is used in acidic buffer before the application on specimen it undergoes 3 min incubation period, under inert blue background Para nitrophenol visualised specimen in alkaline solution, semen would show green streaks due to concentration phosphatase in blue filed after 3 min incubation at room temperature. 
For zinc test swabs pyridylazonaphthaol used as colorimetric reactant and show dark yellow to bright pink colour when placed in contact with semen. 
Stability of test: for dried specimen zinc and acid phosphatase swabs show positive result for specimen stored at 230 days. Accurate result with small specimen. \[9\]

12. Semen – specific protein p30 by ABA card®PSA test

PSA or Prostate specific antigen is glycoprotein of prostatic origin. Semen contain PSA range from 200,000 to 5.5 million ng/ml; 4ng/ml is sensitivity of ABA card® there is no cross reactivity has been observed with various biological fluid when tested with PSA test. 
p30 is male specific protein and p30 antigen is strong evidence against forensic stain. The identification of semen can be done by using PSA test in this test strip is present which contains S,T,C well 200 micro litre semen sample placed in well S, if PSA is present in sample then it will bind with monoclonal anti human PSA antibody and mobile antigen- Antibody complex is formed and this complex move towards test area T through absorbent device, polyclonal Anti human PSA antibody get immobilised In test area T then sandwich formation take place by antibody- antigen- antibody complex . pink Colour band is formed in test area T, due to conjugated pink dye particles and shows positive test result. C control area contain an immobilized anti immunoglobulin antibody and bind with PSA antibody and pink dye conjugates and pink dye band formation take place in C area and indicate that test is working properly two band presents in T and C area indicate test gives positive result for semen identification. 
Limitation: -male urine gives positive result for PSA antigen and gives false negative result for semen sample having high concentration of PSA (high dose hook effect).\[10\]  
Sensitivity: ABACard® p30 show high sensitivity for both fresh and frozen diluted sample.\[22\]
13. Evaluation of Prostate-Specific Antigen (PSA) Membrane Test Assay for the Forensic Identification of seminal fluid

In PSA membrane based test assay have three test PSA check - 1, Seratec® PSA Semi quant and One step ABA Card PSA all these method used mobile monoclonal anti-human antibodies which are conjugated with dye particle and bind with human PSA and antigen-antibody complex is formed and migrate on membrane to reaction zone on test device where Polyclonal antihuman PSA antibodies reside which are in immobilized form Sandwich formation occur due to antigen-antibody-antigen complex concentrates the dye particles, and line formation indicate that there is presence of human PSA and mobile monoclonal anti-human PSA antibodies migrate towards control zone which are unbound where Ig antibodies reside which are immobilized. Complex is formed concentrating dye particles one line formation take place at control zone that means test is valid. And the test results give in 10 minutes. Provide result in more rapid fashion than other PSA detection method and have same sensitivity as ELISA.[11]


PSA membrane test kits have three PSA kits which utilized immunochromatographic assay for forensic examination of semen after the evaluation three PSA test kits depending on sensitivity and specificity SMITEST PSA card have highest sensitivity other than two PSA test kits, PSA test is semi-quantitative assay for determination PSA up to 4 nanogram/ml SMITEST PSA card test are get affected by heat and other contaminants such as body fluids reagent.[12]

15. BioSign PSA membrane test for identification of semen stains in forensic

BioSign prostate specific antigen test is a membrane test device used in forensic science laboratories for detection of seminal stains. BioSign PSA was used for its specificity, sensitivity and cost. Mainly used for to replace ELISA (enzyme Linked Immunosorbent Assay) method for PSA detection and also gives negative results for animal semen and other body fluids.[13]

16. Detection of semenogelin by one-step immunochromatographic assay

Method and Limitation: The method is based on the immunochromatographic identification of semenogelin produced in seminal vesicles using pAb Sg-II (rabbit immunoglobulins [Ig] G) and mAb Sg-II (mouse hybridoma: Sg F11. The two antibodies bind with the seminal plasma motility inhibitor (SPMI; 14 kDa) as a final fragment peptide of Sg. The test can be used as a confirmatory test and can give rapid results than the ELISA test and dot-blot immunoassay. Degradation up to 5 years, pH and heat does not affect its identification. It does not give any positive reaction to even male urine.[14]

17. Immunohistochemical staining of human sperm cells in smears from sexual assaults cases

Human sperm heads should be staining with fluorescently labelled mouse antibody and can be visualised by fluoroscein or Alexa 488 filter using SPERM HY- LITER TM KIT and using DAPI filter nucleic acid stain used to visualised both sperm cell nuclei and nuclei from epithelial cells. Sperm sample smears and vaginal smears slides are stained with SPERM HY - LITER TM KIT and after staining unmounted slides are controlled by fluorescence microscopy and sperm cells are counted.
En Vision+
Slides are stained with En Vision+ and mainly target primary mouse antibody. Show same result in both En vision+ and SPERM HY- LITER TM KIT but En Vision show browner colour than SPERM HY- LITER TM KIT to stained sperm cells. Combining both staining is a fast process give result in 30 min. [15]

18. Rapid stain identification of Human Semen
RSID semen is lateral flow immunochromatographic assay that uses monoclonal antibodies of mouse which specific for semenogelin of Human. Conjugation between antibodies and gold nanoparticle taken place and placed beneath the pad of sample window. On test line T second antibody is placed and attach to membrane of conjugate pad. Control line consist of IgG antibody of anti-mouse. Transportation of sample, antibodies on membrane take place by bulk fluid flow on membrane. Red line present on test line show semenogelin antigen-antibody-gold colloidal complex present and red line present on control line C show test working properly.
Specificity: No cross reactivity with other biological fluid and animal semen.[22]
Limitation: using RSID undiluted semen sample are not used due to viscosity of sample. High dose hook effect gives week positive or false negative result.
Sensitivity: moderately high sensitivity for fresh diluted semen sample. Very low sensitivity for frozen diluted sample. [16]

19. DNA Methylation-based Forensic Semen Identification Assay
Method and Limitation: The method follows the principle that DNA extracted from different tissues are differentially methylated at specific genomic loci. The method is based on methylation-sensitive restriction endonuclease digestion of DNA and then fluorescent PCR, after which these amplified products are differentiated on capillary electrophoresis. The test was highly specific for semen samples and did not give any false-positive results for any forensically relevant body fluid or tissue. [17]

20. Light scattering study on semen analysis methods/techniques.
To study the characteristics and parameters of semen and its density and the morphology of the spermatozoa using computer assisted semen analyser (CASA). CASA plays unique role for assessment of sperm concentration (counts) and mobility (movement) and understand the sperm function. CASA technology has (SQA-V) Sperm Quality Analyzer it combines video microscopy with computer algorithm and find result of semen in 75 seconds (ISAS) Integrated semen analysis system based on image analysis. Sperm class Analyzer (SCA) used for semen analysis fast and accurate method. IVOS sperm Analyzer it is integrated visual optical system also have CEROS sperm Analyzer uses phase contrast microscope.
Limitation: CASA have subjective settings. Used different algorithms of mathematics. High and low sperm concentration can be counted by CASA. [18]

21. Confirmatory detection of sperm and semen via Proximity ligation real-time PCR
Proximity Ligation Real Time PCR (PLiRT-PCR) used for sexual assaults cases for identification of sperm and semen. PLiRT PCR two protein have chosen, cysteine - rich secretor proteins 2 (CRISP-2) and prostate - specific antigen (PSA) and CRISP-2 is found inside acrosome of spermatozoa PLiRT - PCR detect and quantitate the expression of Protein markers through an antibody- protein binding followed by qPCR. PSA have higher sensitivity
than CRISP- 2 protein and PSA have higher concentrations than sperm cell. [19]

22. **Confirmatory test for semen detection by using Combination of Prostate - Specific antigen detection and micro - Raman spectroscopy for.**
In this test semen sample is diluted with double distilled water and placed on aluminium foil-wrapped microscope slides and left to dry the semen sample overnight at room temperature, before measurement, then used monochromatic light of Raman spectroscopy is a molecular spectroscopy based on inelastic scattering of monochromatic light by semen sample, during the process a photon either transfer energy to the sample stroke or receive energy from it. Advantage: - combination of PSA test with Raman spectroscopy provide confirmatory semen detection even no sperm cells are detected.
-Non destructive
-Required trace amount of sample
-Simultaneously identity variety of body fluid
Limitation: -Complex analysis of Raman spectroscopy measurement is main limitation its routine used in forensic biology
-Required dedicated expert for mathematical function and statistical computation. [20]

23. **Multiplex mRNA Profiling Identification of Fluids**
It is uses multiplex Reverse Transcription PCR detection. There are two genes which are specific to semen these are Protamine 1 (PRM1) and Protamine 2 (PRM2). The methodology is based upon analysing and profiling of expression of these genes in the semen. The genes are identified by detecting the presence of mRNA which is coding for the genes. The methods used for detection of mRNA is Capillary Electrophoresis or Laser Induced Fluorescence. [21]

24. **Dot blot assay for semen identification**
Seminal vesicles produced semenogelin (sg), and it help for coagulation Sg consists of two proteins Sg-1 and Sg-2 proteins released during liquefaction Sg-1 mostly help for coagulation Sg contains Seminal vesicle - specific antigen (SVSA) seminal plasma motility inhibitor (SPMI) the stability and specificity of the sg protein are examined by dot-blot-immunoassay using Recombinant Sg-1, and Sg- 2 and their anti-sg-1 and sg-2 polyclonal antibodies to test the feasibility of sg protein for identification of semen. Aliquot of sg antigen transferred to polyvinylidene difluoride (PVDF) membrane the membrane was incubated with phosphate-buffered saline(PBS) which is blocking solutions then add NaCl contain 3% bovine serum albumin(PSA) and add anti sg-1 or sg-2 antibodies in PBS at room temperature then wash the membrane three times then incubate with HRP- conjugated goat anti - rabbit IgG serum and HRP product show blue colour using Tris-HCL and detection of Sg antigen using sodium dodecylsulfate polyacrylamide gel electrophoresis SDS - PAGE by immunoblotting. [23]

25. **Nano trap Sg as a semen detection kit**
One step detection test based on immunochromatographic assay for the semenogelin protein using Nano trap Sg. Mainly used monoclonal and polyclonal antibodies against recombinant sg-2 then sample diluted with extraction buffer saline then red-purple colours vertical line found in membrane test which shows concentration of Sg - 2 Nano trap Sg is an immunochromatography membrane test. [24]
Discussion

The study was regarding the presumptive tests and confirmatory tests that can be used for the purpose of semen identification. Every test has a drawback, it may be the time taking nature, its accuracy, sensitivity, specificity, cross-reactivity, or the conditions in which it cannot be used. So, there are more than 20 tests (both presumptive and confirmatory) that have been reviewed under this study. The study demonstrates that most of the tests have a few limitations which are discussed along with the review.

Even the most commonly used presumptive tests like the microscopic test and acid phosphatase test have their drawbacks being the microscopic test cannot be used when there is no sperm or less sperm count as the sperm structure is identified under the microscope, while acid phosphatase gives false-positive results when reacted with human RBC and its color can be affected by age and condition of the stain.

Though the most renowned confirmatory tests for semen identification are Rapid Stain Identification Series and Prostate-specific antigen test, they are mostly used by Forensic laboratories in sexual assault cases. These tests mostly used are based on their specificity, reliability, and sensitivity and cannot be trusted due to their limitations. So further studies should be done based on objectivity, reliability, less time consuming, cost effective, faster and gives accurate results.

Conclusion

There is no valid and totally reliable test for semen detection for sexual assaults cases. Mainly, acid phosphatase test is used as presumptive test and prostate specific antigen and Rapid stain identification of human semen are used as confirmatory test in Forensic Science Laboratories, for more accurate and reliable identification of semen, combination of test is used.

References


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