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<th>SUBJECT</th>
<th>FORENSIC SCIENCE</th>
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<td>Paper No. and Title</td>
<td>PAPER No. 12: Forensic Biology and Serology</td>
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<tr>
<td>Module No. and Title</td>
<td>MODULE No. 8: Analytical technique for identification of semen, presumptive &amp; confirmatory test</td>
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<th>Co-Principal Investigator</th>
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Anchor Institute: SGTB Khalsa College, University of Delhi
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1. Learning Outcomes

After studying this module, you shall be able to

- Know about various tests performed for semen analysis.
- Learn about techniques of semen examination.
- Identify unknown fluids found at the crime scene.
- Analyze various types of sexual assault cases.

2. Introduction: Semen & seminal stain

The ability to identify traces of body fluid discovered at crime scenes is a very important aspect of forensic investigations. Semen analysis is one of the most popular and informative forensic techniques, it is imperative that any potential body fluid sample is properly collected and not destroyed during the initial identification process. They can also help answer questions regarding the events of a crime. An analytical technique that could identify a particular body fluid rapidly, simply, and non-destructively at the scene of a crime would be a valuable tool for forensic investigators. In addition to blood, semen is one of the most prevalent body fluid found during criminal investigations, especially in cases involving sexual assault. There are currently several tests, both presumptive and confirmatory, that can be used to identify an unknown fluid found at a crime scene to be semen. An alternate light source (ALS) can be used as a finding aid to detect certain biological materials, including semen. Some commercial ALS instruments have been developed such as the Wood’s Lamp Bluemax. The most widely used methods for preservative and confirmatory identification of semen discuss below.

Seminal stain is an important biological fluid which requires identification and characterization in the investigation of sexual offences like rape, sodomy, bestiality, etc.

Normal semen reference pH range is 7.2 to 7.8. Variations in volume and low pH may be due to congenital abnormalities of the genital tract or to obstruction. A pH of 8.0 or greater can be associated with infection of the prostate, seminal vesicle, or epididymis.
3. Examination of semen & seminal stain

3.1 Visual & physical examination

**Color:** Thick, yellowish white, glairy, opalescent, secretion having a characteristic odor known as seminal odor.

**Texture:** On touch, seminal stains are starchy.

**Appearance:** Garments sent for forensic examination are usually dirty having variety of stains, in natural light some stains are reddish coloured, while others are brown, yellow or faint grey in colour.

These are often mixed with stains of blood vaginal discharge, urine and semen, so as to restrict the investigation to seminal stains only, preliminary examination is done under filtered UV light.

3.2 Microscopical examination

Microscopically, detection of spermatozoa is also considered as confirmatory evidence for the presence of semen in a suspected stain. In order to conduct microscopic identification, part of the stained garment should be dissolved in acidulated water in test tube. The test tube should be subjected to ultrasonic oscillation, which can separate the spermatozoa from the dried stain, followed by microscopic examination after fixing the same in dilute sulphuric acid. Since spermatozoa contain gram positive protein, the presence of spermatozoa will be confirmed by microscopic appearance of purple bodies.

3.3 Presumptive test

**Acid phosphate test:**

The Acid phosphate test is one of the best known and most widely employed techniques for the purpose of identification of semen. This test is adopted because of high acid phosphatase activity of semen. Thus by applying this test a suspected azoospermic or vasectomized person who is a suspect of sexual offence may be identified. Stability of acid phosphatase activity in seminal stain depends on temperature. If the stain is exposed to sunlight the acid phosphatase activity is slowly reduced and if the stain is heated at 60 °C for 5 min. this activity is completely destroy.
The enzyme acid phosphatase of semen hydrolyses the calcium alpha-napthyl phosphoric acid that liberates alpha naphthol. This couple with the age forming material to give a purple colour. **Reagent Preparations:**

**Buffer**

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<th>Component</th>
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<tbody>
<tr>
<td>Glacial Acetic acid</td>
<td>1ml</td>
</tr>
<tr>
<td>Sodium acetate anhydrous</td>
<td>2gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100ml</td>
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**Step 1 Reagent**

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<tr>
<td>Buffer</td>
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<tr>
<td>Sodium alpha-napthyl Phosphate, 0.25% (w/v)</td>
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**Step 2 Reagent**

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<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>50ml</td>
</tr>
<tr>
<td>Naphthanil diazo blue B, 0.5% (w/v)</td>
<td>250gm</td>
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Step 1 Reagent and Step 2 Reagent can be made up in bulk and aliquoted into test tubes and frozen.
When needed, one tube of each reagent can be thawed under warm running water for use.

**Procedure:**

**Place a small piece (2 x 2 mm) of suspected seminal stain**

1. Stain material on Whatman filter paper or other suitable test paper. Use proper standards and controls including positive, negative and unstained controls.
2. Add 1-2 drops of Step 1 Reagent and allow to react for 30 seconds. (No colour should develop at this stage).
3. Add 1 drop of Step 2 Reagent. Record the result after 10 seconds.
4. A positive reaction is recorded upon rapid development of a purple colour, which is indicative of semen.

This is not a confirmatory test for semen.
3.4 Thin layer chromatography:

This method is used for the detection of choline and spermine. In TLC, it is possible to detect choline and spermine simultaneously in seminal stain. It has been possible to detect choline and spermine in old seminal stains events after the lapse of 5 years by this method. There are 2 types of reagents used in TLC method they are-

- Dragendorff’s reagent
- Potassium iodoplatinato reagent

4. Examination through UV light

Eleven commercially available pieces of clothing of different fabric types were tested:
- 3 - 100% cotton; 3 - 100% polyamide; 1 - 100% polyester; 1 - 95% cotton / 5% spandex; 1 - 73% cotton / 24% polyamide, 1 - 3% spandex; 1 - 80% polyamide / 20% Spandex. This resulted in different colors and color combinations of fabric, including white and black.

The fabric was classified into three groups:
- Bright (white, bright blue and pink),
- Medium (blue, green, yellow, orange, red) and
- Dark (black, brown).

Light

To test the excitation of the samples, we used a newly developed, very high intensity light source manufactured by Lumatec GmbH, Germany. The portable system is suitable for heavy use at crime scenes because of its low weight (approximately 6 kg), an unusually robust full metal shell, compactness and a battery that allows 60 minutes of operation away from an electrical outlet. A mercury lamp inside of the unit produces a high intensity of UV (320-400nm) and visible light (400-700 nm) which was developed to detect biological stains even in daylight. And at the end of the flexible light tube results in a beam applicator focused on a spot or spread to a fan-like triangle.

Wavelengths can be chosen by eleven adjustable filter positions (400-700,350-500,400-500,320,400,415,440,460,490,550,570). In combination with the eleven excitation settings, white (>400 nm), Orange (>500 nm) and red >590nm) goggles were used to block the excitation light and to visualize fluorescence more precisely.
5. Confirmatory test (chemical test)

5.1 CROSS-OVER ELECTROPHORESIS

Seminal Material can be identified by demonstrating the presence of p30, a semen specific protein. One method of doing this is Cross-Over Electrophoresis. An extract of the suspected stain is placed in the cathode well of a gel plate and anti p30 is placed in the anode well. Electrophoresis is commenced for 20 minutes at 200 volts, forcing two components together. When the antigen (p30) meets the antibody, a precipitin band is formed. The presence of a precipitin band within an extract of an unknown stain proves that the stain contains seminal material, anti-p30.

Plate Preparation:

Clean 1” x 3” or 2” x 3” microscope slides with detergent. Rinse and polish dry. With a diamond tipped scribe, number the slides in the upper right-hand corner. Slides should be consecutively numbered.

Pre-coating Plates:

Note: Omit this step if gel bond is used instead of glass slides. (In order for final 1% agarose to adhere to the glass slide, it is necessary to pre-coat the slide with a thin layer of 0.2% agarose).
Add 1 gm of 0.2% stock agarose to 10 ml distilled water and boil until dissolved. Paint a thin coating of this solution onto the pre-numbered glass slides with a soft brush. Wipe the edges of the slides with tissue paper or Kim wipe to remove any overflow. Allow to air dry. Store pre-coated slides in a numbered slide box for later use. They can be kept indefinitely.

Coating Plate with Gel:

On a level surface, place a pre-coated slide onto a small inverted beaker, allowing the slide edges to be suspended. Place one or two tubes containing 3.5 ml each of 1% agarose gel in a water bath to liquefy.

1 – 3.5 ml tube for a 1” x 3” slide
2 – 3.5 ml tube for a 2” x 3” slide

Pour the liquefied 1% agarose onto the slide. Surface tension and pre-coating of slide will prevent the liquid from overflowing the edges. A smooth level surface is formed. Allow it to cool and harden.

5.2 Florence test:-

This test was discovered by Dr. Florence in the year 1886. When Florence reagent (Potassium Iodide + Iodine + Water) is applied to the slide it produces rhomboidal shape dark crystals of choline periodide. Similarly, any tissue or biological material containing sufficient high choline concentration would give positive Florence Test. According to Davis and Wilson, if swab is collected within one day of sexual act, choline can be detected. At the same time if swab is taken after 14 hours of intercourse, there is a possibility of negative result.

5.3 Barberio’s test:-

Barberio’s test was invented by Barberio in the year 1905. When the questioned stain is allowed to react with picric acid it leads to the formation of yellow needle shaped spermine picrate crystals, including the presence of seminal stain.
6. Detection of Seminal Stain

Sensabaugh in 1977 reported the event of isolation of p30 in human seminal fluid. It is a glycoprotein of prostate origin. Human seminal stain of up to one year old gave positive result for p30, vaginal washing collected from the victim of sexual assault will give negative result for p30. Detection of seminal stain is examined by crossover-electrophoresis. Suspected stain extract is placed on the cathode and p30 is placed on anode and electrophoresis is conducted for 20 minute at 200V leading to the formation of precipitin bands confirming the presence of p30.

7. Morphology of human Spermatozoa

The maturation of a cell in the seminiferous tubule to mature oval-shaped spermatozoa (with an acrosomal cap) in the ejaculate involves many differentiating steps. Some cells will prematurely terminate the process or result in other shapes; tapering heads; pinheads, round heads. It is important to note that abnormally shaped sperm usually do not participate in fertilization, but if fertilization occurred, there is no evidence to support an increased risk for malformation of the developing fetus. A semen sample usually contains 50% or more normal spermatozoa.

Items that have been tested presumptively positive for seminal fluid using the AP test can be confirmed either by microscopic detection of spermatozoa or chemical detection of a semen-specific protein. Positive swabs or a small cutting from a positive stain can be smeared onto a microscope slide and then stained for visualization.

Two common stains used for visualization of spermatozoa are nuclear fast red (red stain) and picroindigocarmine (green stain) and are sometimes referred to as the Christmas tree stain because of the red–green color combination. Once stained, epithelial cells (a group of cells such as skin cells and cells that line body orifices) and spermatozoa have a specific appearance.

Microscopically identifying spermatozoa is an absolute indicator that semen is present on an item. It is also useful because the relative quantity of spermatozoa and epithelial cells can be assessed. This determination becomes important during subsequent DNA analysis because spermatozoa contain male DNA while most epithelial cells in a male–female sexual assault will contain female DNA from the complainant.
8. Protein confirmation of semen:

Traditional p30 detection tests utilize electrophoresis or diffusion methods such as crossover electrophoresis and Ouchterlony's double diffusion, or ELISA. Commercial test kits for p30 are also available and have become prevalent in forensic laboratories because of their sensitivity and ease of use. All of these methods require a small cutting of an AP-positive stain to be incubated in water or saline until rehydrated. Afterwards, the liquid is separated from the cutting by centrifugation so that the stain will be retained in a liquid form instead of dried to the cutting. At this point, a portion of the extracted stain can be used to test for the presence of p30.

All of the methods that detect p30 rely on the formation of an antibody–antigen complex. If semen is present in a stain, the binding of p30 to the test antibody produces a visual result. The lack of a result in any of the tests would indicate that the stain does not contain semen or that not enough is present to facilitate a visible reaction. This test can be used alone to confirm semen, or it can be used in conjunction with the microscopic method described in the previous section.
9. Application of Forensic in Semen Examination

1- Semen analysis is an important laboratory test and should be thought of in the same way as any other diagnostic assay is used in determining treatment plans for infertility.

2- Semen analysis is very important in assault cases as it is very useful for detection of semen in cloths.

3- Isolation and identification of seminal stain found on various suspected area such as cloths, bed sheet, pillow, blanket, etc.

4- Analysis of various protein present in semen or seminal stain.

5- Seminal stains are examined generally in rape cases, Sodomy, Bestiality, Buccal coitus and in cases of Sexual perversions.

6- Detection of sperm cell in Vagina and other body parts.

7- Feacal matter examination.
10. Summary

1-Through the various techniques used in semen analysis, it can be examined that the stain found at the suspected area is semen, by analyzing the spermatozoa presence.

2-Spermatozoa found in case of human is generally spindle-shaped having a motile tail and a head in which components such as p30, choline, spermine, amino-acids and some proteins are found.

3-A very fine and clear structure of spermatozoa can be seen by the help of compound microscope.

4-The presence of choline can be detected by using enzymes such as cholinesterase in semen or seminal stain.