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Detection of Spermatozoa - History to Till Now

Khanna J[1], Mehta R[2], Jangra A[*3] and Bhadaurja P[4]

[1]Student, M.Sc Forensic Science, GD Goenka University, Sohna, Gurugram, Haryana, India
[2]Assistant Director, Regional Forensic Science Laboratory, Bhondsi, Gurugram, Haryana, India
[3]Research Scholar, Amity Institute of Forensic Sciences, Noida,Uttar Pradesh, India
[4]Student, M.Sc Forensic Science, GD Goenka University, Sohna, Gurugram, Haryana, India

***Corresponding author:** Jangra A, Research Scholar, Amity Institute of Forensic Sciences, Noida,Uttar Pradesh, India, Tel: +91-7988457563, E-mail: anjali.jangra830@gmail.com

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Abstract

Semen is crucial evidence for some sex crimes, with its sole confirmation being sperm detection. The success of sperm detection is dependent on all levels of pre-analytic and analytic procedures. Mixed semen stains from multiple contributors are challenging samples in sexual assault casework, and it is crucial to obtain the DNA profiles of different donors to allow the evidence to play an important role in investigations and judicial proceedings. The spermatozoa are positive proof of sexual contact and their presence is strong evidence for the prosecution of any sexual assault case. The identification of seminal stains is frequently of great value in medicoloegal practise, particulary in case of alleged rape, sexual assault, sexual homicide or even adultery. One of the primary aims of the forensic laboratory sexual offence investigations is to simple and examine smears or other biological material taken from the assailant or the victim or stains found in cloths or linen or any other evidencce concerning the assault for the presenceof semen, with the potential to link them. Rape is usually an unwitnessed crime and evidence of semen can play a crucial role in corroborating the victim's allegations . The medical examination provides the opportunity to collect physical evidence and biological samples to confirm the presence of sperm and convict an assailant. This review article is related to all the techniques use for spermatozoa detection till now and their advantages and disadvantages.

Keywords: Crucial; Prosecution; Medicolegal; Evidence; Investigations

Introduction

Semen detection is often crucial evidence in male-perpetrated assault, which can lead to suspect identification. Presumptive detection can be grouped into 2 broad categories: seminal compounds or molecular biomarkers [1]. The spermatozoa are positive proof of sexual contact and their presence is strong evidence for the prosecution of any sexual assault case. Important events related to spermatozoa include the advent of assisted reproduction, male contraception and the effects of the interaction of sperm with microorganisms (viruses, bacteria, and fungi), among others. However, the story of the discovery of sperm has originated more than three centuries ago, in 1677 [2]. The identification of seminal stains is frequently of great value in medicoloegal practise, particulary in case of alleged rape, sexual assault, sexual homicide or even adultery [3]. The finding of spermatozoa during the forensic examination of sexual assault victims is of great importance. With modern day DNA-profiling techniques it constitutes a legally accepted proof,that a sexual act between the alleged perpetrator and the alleged victim has indeed taken place [4]. One of the primary aims of the forensic laboratory sexual offence investigations is to simple and examine smears or other biological material taken from the assailant or the victim or stains found in cloths or linen or any other evidencce concerning the assault for the presenceof semen, with the potential to link them. In cases of sexual assault, the forensic examination of evidence for seminal stains can actually be considered two step process. Firstly , the stain must be located. Secondly, the stain will be examined to prove its identity, possibly it may even be tested for the blood type of the individual from whom it originated [3]. We should also remember that a negative result for semen detection does not always mean that there was no male-perpetrated sexual assault. Several factors can lead to detection failures: prolonged delays before examination, vasectomized or orchiectomized perpetrators, nonejaculation, condom or spermicidal agent usage,contraceptive pill usage, menstruation, bleeding, immune system activation, and so on [1]. Currently, semen analysis is based on the recommendations of the fifth edition of the World Health Organization (WHO) Manual for the Examination and Processing of Human Semen that provides technologies and the reference values for evaluating semen parameters. They include standard procedures (macroscopic examination, initial microscopic examination, sperm count, motility, vitality, morphology,membrane integrity, assessment of leukocytes, immature germ cells, and testing for antibody coating of spermatozoa), optional tests (indices of multiple sperm defects, pan leukocyte immunocytochemical staining, interaction between spermatozoa and cervical mucus, computer-aided sperm analysis, and biochemical analysis), and research procedures (reactive oxygen species, human sperm–oocyte interaction tests, human zona pellucida binding tests, assessment of the acrosome reaction, zona-free hamster oocyte penetration test,and the assessment of sperm chromatin) [5]. Rape is usually an unwitnessed crime and evidence of semen can play a crucial role in corroborating the victim's allegations. The medical examination provides the opportunity to collect physical evidence and biological samples to confirm the presence of sperm and convict an assailant. Swabs should be routinely taken as semen may be recovered in the genital tracts even if the victim is so emotionally traumatized that penetration or ejaculation by the assailant was not disclosed during questioning [6]. ABO blood grouping method is an oldest method used in forensic biology for grouping purpose [7]. But now a days, DNA fingerprinting is an advanced technique used to solve crimes like gang rape [41].

Factors Affecting Sperm Detection

Specimen collection

Legalities regarding the prioritization of collection site vary by jurisdiction. Preferably, specimens should be collected in relationship to the assault's account, but in cases where victims present memory lapses due to emotional and/or physical trauma, and so on, collection from at least from 3 orifices (oral, genital, and anal) as well as the surrounding areas is recommended. Using fluorescence with long-wave ultraviolet light can help collect specimens on the body's surface [1]. In rape cases, the condom plays an important role, since if it is found at the rape scene,it will be useful for the identification of the assiliant. Semen of the rapist may be found on the one side of the condom while blood found on the other side of the condom usually originate from the victim [3]. The medical examination provides the opportunity to collect physical evidence and biological samples to confirm the presence of sperm and convict an assailant.Swabs should be routinely taken as semen may be recovered in the genital tracts even if the victim is so emotionally traumatized that penetration or ejaculation by the assailant was not disclosed during questioning [6]. All physical and medical evidence obtained at the time of the examination must be complete and properly handled so that the legal case is not dismissed because of insufficient and mishandled evidence.Laboratory evidence in sexual assault cases includes vaginal, anorectal, or oral swabs; extragenital semen residue; foreign material such as hair and fibers; and clothing from the victim or bedding that could prove as evidence [7]. collect

carefully, avoiding contamination; collect specimens as early as possible; 72 hours after the assault the value of evidentiary material decreases dramatically; label all specimens accurately; dry all wet specimens; ensure specimens are secure and tamperproof; maintain continuity; document details of all collection and handling procedures [1]. Swabs taken from the vaginal, anorectal, or oral areas are transferred (smeared) onto separate glass slides. All smears should be spread as thinly as possible, especially anorectal smears. Simultaneous swabs should be taken and air-dried for prostatic acid phosphatase analysis and DNA fingerprinting if needed [7]. Contamination avoidance practice is essential for good results [1].

Ideal Collection Sites

Sperm has been found in the cervix at 179 hours, approximately 7½ days and in a child's vaginal vault at 5 days [1]. When Biological evidence are in non-transportable objects, the use of a dry or lightly moistened swab passed gently through and rotated in the same spot (swabbing method) is sufficient for recovery. In the case of wet evidence, care should be taken to dry them to avoid damage of Biological evidence, by the growth of microorganisms that cause degradation of DNA [8]. Specimens should be collected from dry areas of external genitalia using the double-swab technique; if the area is wet, dry swabs should be used. Both vaginal and cervical samples may be taken with dry swabs. To collect specimens from the oral cavity, oral rinses or using the double-swab technique with dry swabs is recommended for the cavity between gums and cheeks, under the tongue, around the teeth, gingival recesses, and back of the throat; this may also differ from jurisdiction to jurisdiction [1]. In oral intercourse cases, one or more mouth swabs should be taken by the police surgeon during the medical examination and sent to the laboratory to be tested for the presence of semen [9].

Packeging & Transportation of Samples to Laboratory

The ideal swab can effectively collect semen and sperm Drying processes to prevent growth of mold and bacteria destructive to an evidentiary sample are very important; they also can minimize chances of these contaminations.Paper bags are preferable to plastic because of air ventilation and moisture prevention. If it is impossible to dry the specimen before delivery such as with the collection of large wet specimens at the crime scene, for example bedsheets, jurisdictions must have guidelines on shipment duration guarantees,packaging (drying cabinets), labeling, and refrigeration or freezing.All specimens must always be considered to be under the chain of custody [1]. Items suspected of containing of seminal stains must be handled carefully and must be submitted for laboratory processing [3]. The specimen should be kept at room or body temperature during transportation [10].

Materials and Methods

Examination and Detection of Spermatozoa

Presumptive Test

Acid phosphatase test is one of the most common tests used by forensic laboratories for the detection of semen. Sometimes in sexual offences the seminal fluid got mix with various other body fluids. Therefore, its detection after mixing with other body fluids is one of the most challengeable tasks for forensic laboratories by using acid phosphatase test [12]. Although presumptive tests such as AP test, prostate-specific antigen (PSA), and semenogelin (Sg or SEMG) are not confirmation of semen, they are still valuable evidence. Positive results from these tests, may, for example, lead to DNA evaluation in which identity could be established or be the only clue a legal team has [1]. The acid phosphatase reaction is a typical test in which the suspected seminal stain or an extract of it on a filter paper is reacted with a solution of the substrate, a monophenolic phosphoric acid or its ester, in acetate buffer of pH 5. The enzyme acid phosphatase hydrolyses the substrate to the corresponding phenol and phosphate ion. The phenol formed is simultaneously coupled with a suitable diazonium salt as a chromogen to give a characteristic colored dye stuff, which is a positive test for the presence of a seminal stain [11].

Preparation of acid phosphatase solution-

0.2 gm of sodium acetate and 0.1 ml of acetic acid was taken into a conical flask and stirred with glass rod until dissolved. Then 0.025 gm brentamine fast blue B salt was added and covered with aluminum foil and heated in water bath for two minutes to dissolve. Then 0.0125 gm sodium α naphthyl phosphate was added and stirred to dissolve. Then 10 ml distilled water was added into the solution. Then solution was placed in amber colour bottle for further analysis [12].

Prostate Specific Antigen(PSA/P30)

PSA has become the hallmark test for evidence of ejacu-

lation by men who have been vasectomized or who are azoospermic (where there is no production of sperm). It is a prostate-specific glycoprotein produced by the prostate. It has also been found in normal breast tissue and a percentage of breast tumors. Besides semen from both vasectomized and normal men, positive findings were also found in post-ejaculate urine and urine from adult men. Semen stains, stored at room temperature for up to 30 years, have yielded a positive PSA result [30]. A significant indicator of recent sexual activity is the protein p30. Under the analytical conditions employed in forensic laboratories, p30 is wnique to seminal plasma and it is measured with a crossed electrophoresis technique. This protein is a useful semen marker particularly in cases of azoospermia. At appropriate concentration levels, this proten usually not found in the vaginal cavity beyond 8 hours following intercourse. Occassionally p30 is positive in the face of a negative acid phosphatase. Prostate Specific Antigen(PSA) has been found to be present in high concentration in semen. Simple senistive and reproducible methods have been developed for analysis of the presence of PSA, including the tandem-E PSA Immunoenzymetric Assay. This method can be used to identify the presence of PSA that is of seminal origin in a biological stain in forensically significant specimens [3].

Semen Detection Techniques:

1. Microscopic Examination- A phase-contrast microscope is recommended for all examinations of unstained preparations of fresh semen.An initial microscopic examination of the sample involves scanning the preparation at a total magnification of ×100 [13]. When spermatozoa are located through microscopic examination the stain is identified as having been derived from semen. Seminal stains are detected definitely by finding spermatozoa under the microscope,without or with staining [3].

2. Ultraviolet light techique- It is routine procedure to search a crime scene for semen and other fluids using this simple and non-destructive method [14]. The Wood's lamp (WL) is a source of ultraviolet radiation emitting wavelengths of approximately 320 to 400 nm. This portion of the electromagnetic spectrum is called long-wave ultraviolet or black light [15]. The Wood's lamp emits ultraviolet light and has been established as a useful evaluation in rape cases because it is purported to cause semen to fluoresce [3]. However when the WL was put to the test against other fluids, it was not very specific and sometimes did not even detect semen stains, and it gave false positive results for ointments and creams [14]

3. Other Alternate Light Source- Another commercial ALS, the BluemaxxTM BM500, was tested in a similar way and was 100% sensitive to semen stains. Also, physicians using the ALS were able to distinguish semen from other products 83% of the time after receiving training on how to use the device [14]

4. Fluorescence Microscopy- Fluorescence microscopy and confocal microscopy are used to assess various sperm characteristics such as the membrane, acrosome and chromatin. One advantage of fluorescence microscopy is the ability to identify fluorescent signals in individual cells [43]

5. High powered UV-VIS Light Source- portable forensic light source, which emits wavelength from 430 nm to 700 nm (UV to VIS) and filters were used. The very high intensity light source detected semen in darkness and daylight using wavelength between 415 nm to 490 nm. The semen responds to illumination by longer wavelength frequencies of UV light (~350 nm) which is invisible to the human eye. When the substance is illuminated, it absorbs the energy and exhibits luminescence at a lower energy (longer wavelength) frequency of visible blue light. The advantage of this is that you can make invisible semen stains appear visible to the human eye. In addition, excitation and emission spectra of untreated dry semen indicate clearly that there is an alternative to using UV radiation when searching for semen stains. Due to its high intensity the portable forensic light source detects biological stains in darkness and daylight and can therefore not only be used in laboratory but also at the crime scene that can't be darkened. Visualization is optimum when filters are chosen that blocks the bright excitation and ambient light. Careful detection of semen stains on undergarments of gang rape victim and their grouping by simplest and oldest ABO grouping method besides using DNA fingerprinting solved the case [41].

6. Laser Capture Microdissesction- The laser capture microdissection (LCM) technology represents a significant improvement in cell separation methods. It combines existing light microscopic instrumentation with laser beam technology and allows targeting of specific cells or tissue regions that need to be separated from others [16] laser capture microdissection (LCM)/ LMD slide detection techniques are often used with varying levels of success in sperm DNA and RNA, especially in regard to detection after the use of common staining methods [1]. The main advantages of LMD are the ability to physically separate mixture components,usually sperm cells from other cells, while retaining the former and discarding the latter. With the LMD

technology,fewer cells are needed to obtain a genetic profile from any given sample [17]. There are two main classes of LCM systems: ultraviolet (UV) cutting systems and infrared (IR) capture systems.After visualization via microscopy, the cells of interest are isolated by focused laser energy (UV systems) or transferred to a thermoplastic polymer with formation of a polymer-cell composite (IR systems) [16].

The MMI Cell Tools software was used to command visualization, microdissection, capturing, and reporting cells of interest. The procedure included a full slide scan for each slide with a 49 objective. Cutting was performed with the 209 objective for Sperm Hy-Liter stained samples and the 409 objective for samples stained with H&E. Microdissected sperm cells were automatically captured (glued by the mounting membrane) directly onto MMI Isolation Caps of 0.5-mL microtubes without any kind of manipulation, thus preventing sample contamination by the operator. Approximately 50 sperm cells were selected to be captured per slide. Captured sperm cells were microscopically inspected whenever necessary during the procedure to confirm integrity and relative position of the samples on the MMI Isolation Cap [17].

7. Fluorescent in situ hybridisation- Fluorescent in situ hybridization (FISH) facilitated the detection of sperm aneuploidy.Sperm FISH is most commonly used to determine the proportion of aneuploidy present in sex chromosomes and autosomes of infertile men. sperm FISH analysis is increasingly included in infertility diagnostic protocols, providing useful information to enable the affected couple to make difficult, but informed reproductive choices [18]. Since not all cases of sexual assault result in the deposit of semen, recent research with Fluorescence In situ Hybridisation(FISH) has been found to be a very sensitive and specific method for detection of the Y- chromosome from male cells in the absence of semen. This method demonstrates the presence of epithelial cells of male origin in the postcoital vaginal tract for extended amounts of time after sexual assault, using a commercially available probe [3].

Slides were first treated with a FISH Pre-treatment Reagent Kit,which included a 12-min incubation step in protease solution at 37 8C, followed by room temperature solutions of PBS for 5 min and 70% ethanol for 1 min.After being allowed to air dry, FISH was performed using a CEP1X SpectrumOrangeTM Y SpectrumGreenTM DNA Probe Kit [19].

The limitations of FISH include the inability to study structural anomalies as well as the limited evaluation as only the segments of chromosome identified by the probes are detected [20].

8. Computer Aided Sperm Analysis- CASA technology does allow the objective analysis of sperm motility kinematics, as well as sperm concentration and percent motile in washed preparations, provided that speci fi c expert recommendations are followed. CASA analysis of sperm subpopulations with particular patterns of motility has proven invaluable in research , and can be used in human infertility diagnosis and pre-assisted conception workup to identify sperm subpopulations with either the appropriate kinematics to penetrate into cervical mucus or that show hyperactivated motility under capacitating conditions. It must be stressed that population-averaged values of sperm kinematic measures are meaningless; biologically important subpopulations must be identified on a cell-by-cell basis using multi-parametric definitions based on several kinematic measures [21]. To improve the methodology of assessing sperm quality and to make it objective and quantitative,actual sperm velocity can be measured eithermanually or automatically. At its simplest, this involves videotaping sperm movement via a microscope and then replaying the tape frame-byframe while marking the position of each sperm on an acetate film fixed to the video monitor.From the measurement of the length of the tracks over the time and a knowledge of the frame rate of the video recorder, it is possible to calculate a value for sperm velocity. Such methods are not only extremely time-consuming, but there is also an element of subjectivity in choosing which individual sperm to track [22]. Future validation of methods for the accurate identification of spermatozoa (and their accurate differentiation from non-sperm objects) might allow the extension of CASA technology to the automated analysis of human semen, but this will require additional imaging capabilities such as fluorescence because phase contrast optics cannot achieve such object discrimination in human semen, with its high levels of debris and other cellular elements [21].

9. Sperm Chromatin Structure Assay- The SCSA ® is the pioneering assay for the detection of damaged sperm DNA and altered proteins in sperm nuclei via flow cytometry of acridine orange (AO) stained sperm. The SCSA ® is considered to be the most precise and repeatable test providing very unique,The SCSA ® is considered to be the most technician friendly, time- and cost-efficient, precise and repeatable DNA fragmentation assay, with the most data and the only fragmentation assay with an accepted clinical threshold for placing a man at risk for in-

fertility. SCSA ® data are more predictive of male factor infertility than classical semen analyses The SCSA ® values have a very high level of repeatability provided that no significant event has happened between collection times, e.g., hot tubs, medications, fever, illness, etc. The SCSA ® is the easiest and most rapid DNA fragmentation test in the laboratory. The SCSA is also the most precise and repeatable with CV of ~1–3%. Due to the low cost of supplies, numerous measurements can be made for both research and clinical applications [23]. The major use of the SCSA test has been to determine thepercent of sperm with fragmented DNA. The original termfor describing the % of sperm in a semen sample with frag-mented DNA was Cells Outside the Main Population [24].

10. Direct Selection of Immotile sperm- It is important to note that samples in which spermatozoa are immotile, or where the percentage of motile spermatozoa is low, are not suitable for SU and DGC methodologies.This is the case of sperm samples obtained by testicular sperm extraction (TESE) in azoospermic patients (AO). Nowadays, in the routine practice of fertility clinics only vague and subjective morphological criteria are followed to select the immotile spermatozoa prior to ICSI, like the identification of spermatozoa with normal head and tail. However, in the last decades a number of efforts have been made in order to find a selection method for this type of samples, aiming to discriminate viable spermatozoa directly under the micromanipulator irrespectively of their motility. Another sperm selection method that has been investigated is the hipo-osmotic swelling test (HOS). This technique is based on the fundament that the tails of viable spermatozoa swell or curl under hypo-osmotic conditions due normal membrane function,thus allowing their identification and recovery under the microscope, potentially for their use for ICSI [25].

11. DNA Analysis- The first use of DNA testing in a forensic setting came in 1986; two girls were sexually assaulted and then brutally murdered in 1983 and 1986, in Leicestershire, England. This case showed an innocent being accused and 1 year later the guilty responsible one being found and processed. The advantage of the organic extraction method is that it can obtain genetic material from difficult samples (degraded and/or low amount of DNA) and can successfully remove the presence of inhibitors for the PCR. While this method remains one of the most reliable and efficient, it is also very time-consuming, uses hazardous chemicals, and, because of the greater hands-on effort and multiple tube transfers involved, introduces increased risks for contamination and sample mishandling. The recovery of evidence in cases of SA is a great challenge for the DNA forensic analysts, because it requires the separation of DNA from epithelial (the victim) and sperm (perpetrator) cells [8].

DNA was isolated from the samples using both a standard organic extraction and a differential organic extraction Additionally, various extraction kits were evaluated throughout the work and included the DNA Investigator kit (QIAGEN, QIACube protocol), the Erase Sperm Isolation kit (Paternity Testing Corporation), the PrepFilerTM Forensic DNA Extraction kit (Applied Biosystems (AB) by Life Technologies, and the DifferexTM System (Promega). All extractions were initially performed in accordance with the manufacturer's recommended conditions [26].

The removal of epithelial cell DNA from the sperm fraction of the sample has been a time consuming step that has met with limited success. Another aspect of the problem is that traditionally this process was very difficult or impossible to successfully automate. The method used in a crime laboratory to obtain the sperm cell and epithelial cell DNA profiles from the mixed stain is referred to as differential extraction. The method of differential extraction in use for over two decades has relied on preferential lysis of the epithelial cells using a solution that will lyse epithelial cells but not sperm cells. This allows the scientist to separate the two types of cells [27]. Genetic profiling in forensic science often requires the isolation of a population of cells from complex biological mixtures. Sexual assault cases are the most frequent type of case submitted to crime labs, and often contain mixed samples of male and female DNA When ample sperm is present in a sample, spermatozoa are isolated by preferential lysis of epithelial cells present in the sample. Sperm can then be separated from the cell debris by centrifugation [28].

12. Bio-mimetic Sperm Selection- Bio-mimetic is the use of technologies and/or processes that mimic a naturally occurring event. Several mechanisms have been suggested that mimic the selection taking place in the female reproductive tract [29]. These methods either separate the spermatozoa from SP or, in addition, permit selection of the better quality spermatozoa from the rest of the ejaculate. Basically, the methods can be divided into those that result in removal of seminal plasma only (sperm washing), and methods that select spermatozoa on the basis of certain characteristics, such as sperm migration (based on sperm motility), filtration (based on membrane integrity), and colloid centrifugation (based on sperm motility, morphology, viability and chromatin integrity). Colloid centrifugation can be subdi-

vided into density gradient centrifugation (DGC) and Single Layer Centrifugation [29].

13. Cryopreservation of Human spermatozoa-The procedure that makes it possible to stabilize the cells at cryogenic temperatures is called cryopreservation, also known as an applied aspect of cryobiology or the study of life at low temperatures [31]. Cryopreservation technologies are integral for managing male-factor infertility [32]. Cryobiology is a multidisciplinary science, studying the physical and biological behaviors of living materials (e.g., cells and tissues) at low temperatures. Cryobiology contains many disciplines such as, cellular biology, theriogenology and molecular biology, engineering and mathematics, veterinary and human medicine, intensive and extensive farming on land and in watery environments .Optimization of the cryopreservation procedure of spermatozoa needs all the above-mentioned disciplines because of the complex cellular structure, activationand capacitation mechanisms of spermatozoon [33]. Cryopreservation of human spermatozoa—introduced in the 1960's —has overcome many space and time limitations and now forms integral part of assisted reproduction technologies (ARTs). This technique becomes particularly important in cases of preservation of male fertility before radiotherapy or chemotherapy [31]. Cryopreservation is a process that maintains biological samples in a state of suspended animation at cryogenic temperature for any considerable period and is used to preserve the fine structure of cells. The major steps in cryopreservation are the 1) mixing of CPAs with cells or tissues before cooling, 2) cooling of the cells or tissues to a low temperature and its storage, 3) warming of the cells or tissues and 4) removal of CPAs from the cells or tissues after thawing [34].

14. Open- CASA- which allows users to study three classical sperm quality parameters: motility, morphodmetry and membrane integrity (viability) and offers the possibility of analyzing the guided movement response of spermatozoa to different stimuli (useful for chemotaxis, thermotaxis or rheotaxis studies) or different motile cells such as bacteria, using a single software. This software has been released in a Version Control System at Github. This platform will allow researchers not only to download the software but also to be involved in and contribute to further developments. The Open CASA software has been implemented in Java language. The starting point was the source code previously developed by Wilson-Leedy & Ingermann, which uses internally Image libraries for image processing and analysis. The software architecture was designed to facilitate the subsequent development of new features, so the code was separated in different packages depending on its functionality. This categorization helps to reutilize the previously implemented code, e.g., functions related to the identification of cells in an image, or to extract kinetic parameters from a set of trajectories, and it makes the source code files shorter and less complex [36].

15. Detection of Spermatozoa in mouth- Semen is usually identified by finding spermatozoa. During the course of the laboratory examination, there have been cases in which the saliva sample taken for the purpose of identifying the individual's blood group has been found to contain spermatozoa. Spermatozoa can persist for several hours in the mouth, the longest time recorded being 13 hours. It is therefore appropriate to take oral samples even if the offence occurred sometime before the medical examination [42].

Analysis of Aged Seminal Stains by Current Forensic DNA Approach

Few studies have estimated the age of semen stains, which can play an important role in forensic investigations. If the age of a semen stain was known, investigators could potentially verify alibis, identify suspects, determine the time of crimes and indirectly estimate the PMI [39].

1. **STR Analysis**-Aged seminal stains may be successfully analysed for identification purpose bycurrentforensic methodsincluding miniSTRs, particularly suitable on degraded DNA,but the selection of markers not only on DNA degradation index, but also on DNA amount is mandatory to obtain a reproducible and eventually composite profile [37].

Presently, real-time PCR assays based on the mRNA levels of semen-specific genes and immuno-chromatography assays of semen-specific proteins are considered novel semen identification methods in addition to traditional methods, such as the acid phosphatase test and microscopic test. Moreover, DNA typing methods, such as analysis of short tandem repeats (STR), have replaced ABO blood typing in personal identification.Thus, recently, the semen examination methods used have changed significantly. The results of these newly developed techniques might differ from those of traditional methods. STR analysis could be used for extremely aged seminal stain samples and may be useful in forensic practice and criminal trials. In future, older unsolved sexual crimes may be solved using current methods [38].

2. Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR)- semen stain age analysis via FTIR can provide key information in determining the time of a

crime and for estimating PMI. ATR-FTIR, combined with chemometric methods, shows great potential as a convenient and efficient tool for age estimation of semen stains.Moreover, the method could be applied to routine forensic investigations in the future. FTIR, combined with chemometrics, provides an efficient method for estimating the age of human semen stains based on time-dependent changes in the spectra [39].

Wet Mount Examination for the Presence of Sperm in Sexual Assault Cases

Many protocols for the examination of sexual assault victims include the preparation of vaginal wet mount slides in the hospital emergency room or other site of victim examination. These are assessed microscopically to determine whether sperm are present and if so, whether the sperm are motile. The presence of motile sperm is believed to be an indicator of relatively recent sexual contact, usually within 8–12 h postcoitus. The wet mount findings are recorded in the report on the sexual assault victim examination, and the wet mount slides themselves are often included in the sexual assault evidence kit submitted to the crime laboratory. The finding from a wet mount examination might possibly assist the sexual assault investigation in two respects: (i) it may provide guidance to the crime laboratory in the subsequent analysis of the evidence in the sexual assault kit and (ii) it may provide information useful in the interpretation of other evidentiary findings in the case. The observation of motile sperm on the wet mount is commonly interpreted as indicating recent sexual activity, and this would be a useful corroborative information regarding the time of the assault and other aspects of the investigation [40].

Conclusion

Sperm presentation is the confirmation of semen, which may be crucial in some sexual assault cases. The success of sperm detection largely depends on examiners, including forensic physicians and nurses who perform specimen collection, whoever preserves the sample for transfer to laboratory, as well as laboratory scientists or officers who do the testing. All of them must have up-to-date knowledge, competence, and careful skills, and perform their duties with prudence and completeness. Unfortunately, tests in a general laboratory are limited because the specimen will be separated for preliminary testing, staining tests, and DE. Preservation should be paramount, as evidence may be reopened for yet unknown future confirmatory tests. Seminal fluid has been known to mix with other body fluids in sexual offences. As a result, using the acid phosphatase test to detect it after mixing with other body fluids is one of the most difficult tasks for forensic laboratories [12]. While presumptive tests like the AP test, PSA, and semenogelin (Sg or SEMG) do not confirm the presence of sperm, they are still useful proof. One major factor influencing spermatozoa detection was the interval between ejaculation and swab collection. The ABO blood grouping system is one of the oldest grouping methods in forensic biology. However, DNA fingerprinting is now a sophisticated method used to solve crimes such as gang rape. Finding spermatozoa under a microscope, without or with staining, is a surefire way to identify seminal stains. Wood's Lamp was less specific, failing to detect semen stains in some cases and giving false positive results for ointments and creams. The inability to research structural abnormalities, as well as the restricted evaluation since only the fragments of chromosome found by the probes are detected, are two of FISH's drawbacks. Computer-assisted sperm analysis (CASA) systems have proven their usefulness and potential for evaluating sperm quality and enhancing the prediction of a seminal dose's fertility potential in the field of assisted reproductive techniques. Direct selection of immotile sperm is based on the idea that under hypo-osmotic conditions, the tails of viable spermatozoa swell or curl due to normal membrane function, allowing for their detection and recovery under the microscope, and possibly for ICSI. Spermatozoa can survive in the mouth for many hours, with the longest period being 13 hours. As a result, oral samples should be taken even though the offence happened before the medical test. . STR analysis can be useful in forensic practise and criminal trials for extremely aged seminal stain samples. Older unsolved sexual assaults can be solved in the future using existing approaches. Based on time-dependent shifts in the spectra, FTIR in combination with chemometrics provides a reliable tool for estimating the age of human semen stains. Wet mount examination will provide valuable corroborative evidence about the time of the attack and other aspects of the investigation.

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