


REVIEW

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Recent advancements in identification and detection of saliva as forensic evidence: a review

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Abstract

Background Saliva is the most common biological evidence found at any crime scene next to blood. It is a clear liquid which makes it immune to any possible evidence of alteration by the perpetrator. In forensics, saliva is used as biological evidence and is very helpful in determining various aspects of an individual such as sex, individuality, ABO blood groups, microbial signature, biomarkers, or habits like smoking.

Main body Saliva shares a great resemblance with plasma as it encompasses similar organic or inorganic compound contents. In forensic casework, identifying any evidence is the primary goal to establish the groundwork for further investigation. Saliva may be found in the form of a pool or stained form, but its identification is challenging because of its transparency. It has been widely used as an informative tool in forensic situations like poisoning, hanging, or cases of drug abuse, etc. for more than two decades now. Over the years, many proposed ways or methods have been identified and described, which helped in the detection and identification of saliva as evidence.

Conclusion This review article represents the significance of saliva as important forensic evidence, along with the different forms it may be encountered at the crime scene. The use of diverse collection and detection methods, over the past few decades, has been discussed. An attempt has been made to collect the available data, highlighting the merit and demerits of different identification techniques. The relevant data has been collected from all the published and reported literature (1987–2021).

Keywords Saliva, Forensic evidence, Crime scene, Body fluid, Identification

Background

Identifying biological evidence obtained from any crime scene is crucial for forensic investigations. These evidences are reliable for linking crime to criminals and in the recreation of the crime scene. Establishing

the identity of body fluid or even identifying evidence as body fluid is enough to influence the case's outcome. The primary body fluids found at any crime scene are blood, semen, saliva, sweat, urine, and vaginal secretions. Each of them has their specific screening methods for identification. Blood is the most common biological fluid present at most crime scenes. In cases like homicide, murder, and sexual assault- blood, saliva, and semen is very common evidence obtained, as compared to others. In case of suicide, saliva and urine are commonly observed biological fluids. These fluids could be present either in a pool form or on any inanimate object that comes in contact with either the victim or assailant.

There is a common tendency of criminals to dispose of the weapon of offense or any other visible evidence like

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blood stains to avoid getting caught. However, saliva, a transparent liquid has an advantage over blood. Saliva is commonly found in cases such as homicide, sexual assault, burglary, and hanging. In homicide and sexual assault cases, saliva is usually deposited in bite marks. Also, proximity between victim and assailant(s) often leads to the deposition of saliva on each other or surroundings. In case of burglary, sometimes half-eaten fruits or edibles are the sources of salivary evidence, and in case of hanging, the unconscious dripping of saliva is very commonly observed. The evidence which is convenient to send to the laboratory is sent as it is, meanwhile the evidence which is not, is swabbed with sterile swabs and sealed and packed for screening analysis in the laboratory. These screening tests are presumptive, followed by a confirmatory test. The collection of these shreds of evidence is a challenge, too, as many factors influence the quality of evidence (Lee et al. 2001; Acosta et al. 2002; Magalhães et al. 2015).

Salivary gland secretions comprise a gingival crevicular fluid, exfoliated oral epithelial cells, and bacteria that are all combined to form the whole saliva. Saliva is a common fluid for forensic analysis due to the additional benefits of its noninvasive mode of collection, even by individuals with less experience, and the avoidance of intrusion into private functions when collected under direct observation. Saliva aids in many ways such as personal identification by DNA profiling, analysis of drug abuse, animal bite marks, or sex determination from bite marks. Saliva is the only biological fluid that shows similar characteristics to plasma (Saxena and Kumar 2015; John et al. 2018). Salivary fluid is an exocrine secretion that contains 99% water and a variety of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate, phosphate) as well as proteins that include enzymes, immunoglobulins and other antimicrobial agents, mucosal glycoproteins, traces of albumin, and some polypeptides and oligopeptides that are significant for oral health. Additionally, there is glucose, nitrogenous compounds like urea and ammonia, desquamated epithelial cells, leucocytes, and oral microbes (De Almeida et al. 2008).

Saliva has many perks and is considered advantageous over blood because unlike blood, its collection is non-invasive, easy, and safer, with a low risk of contamination, especially from transmitted diseases such as hepatitis (Mago et al. 2016; John et al. 2018). Since saliva exists as a clear liquid, it can be often ignored as evidence and is also less prone to any chances of alteration by criminals. It could be present in the pool or the stained form on any surface. Saliva gets deposited on human skin by various means like biting, licking, spitting, and sucking (Schenkels et al. 1995; Sweet

et al. 1997; Raj et al. 2018). It can also be assessed from prominent bite marks on food items (De Oliveira Musse et al. 2019) or other inanimate objects such as cigarette butts, straws, etc. (Pawar et al. 2018; Casey et al. 2013). An investigator should always assess the victim for any possible deposition of saliva samples. Since it is difficult to locate definite saliva sites on the body, the significant sites with high recovery chances of saliva are the face, neck, chest, shoulder, abdomen, forearm, and thighs (Jimsha et al. 2020). Apart from these significant body sites, the crime scene is also thoroughly investigated for any inanimate object with susceptibility to the presence of saliva on it. The inanimate objects received as evidence in various types of cases include cloth, personal belongings such as mobiles, cigarette butts, straws, cans, half-eaten edibles, and many more (Sweet and Hildebrand 1999; Pawar et al. 2018).

The implication of saliva on the human body is very different from inanimate objects. The environmental and other external factors such as personal habits like showering or clothing could also hinder the amount of saliva recovered from the skin. Sweet and Shutler 1999, reported a case study, in which the saliva samples were effectively collected from a prominent bite mark present on a female body submerged in water for a period of four to five hours. They generated the DNA profile from bite marks that helped the investigation. Similarly, Chávez-Briones et al. 2015 reported a study on a homicide case where saliva was recovered from bite marks present on the breasts of the victim. They successfully generated a DNA profile from the saliva samples and matched it with that of the suspect.

The present review focused on the different detection and identification techniques or assays used to establish the identity of saliva as crucial forensic evidence during an investigation. It also summarizes the outlook of effective techniques used to identify saliva in the recent past. A comparative assessment in terms of the significance of various techniques has also been discussed. This paper is organized as follows-

- Collection of saliva samples by using different methods.
- Various preliminary tests were used in the detection of saliva. This includes polilight, Phadebas, SALIgAE, and immunochromatographic strip tests.
- Various advanced screening techniques for saliva. This portion includes a wide range of laboratory techniques used for the detection of saliva including DNA methylation, and microscopic methods, among others.
- Comparison of techniques based on their destructive and non-destructive type.

Main text

Collection of saliva samples

The collection of saliva is a very intricate process that is usually carried out by two swabbing techniques. The choice of technique depends on the type of surface on which evidence gets deposited. In a single swab approach, a wet sterile cotton swab which is moistened by distilled water/normal saline is rolled over the collection site. The swabbing is done without applying extra pressure to avoid the collection of the substrate material. Though, in double swabbing method, the sample site is swabbed twice. First, a wet cotton swab is rolled over the site, followed by a dry swab (Sweet et al. 1997). This method ensures maximum collection of saliva from the substrate. The Double swab technique is followed for absorbent surfaces such as skin, cloth, and half-eaten food items, etc. for non-absorbent surfaces, such as plastic evidence, and glasses, etc. the single swab technique is administered (Hedman et al. 2020). While swabbing, the investigator must be careful not to swab beyond the desired site as it could cause unnecessary contamination leading to improper or mixed DNA profiling. The double swabbing collection method has been preferred over single swabbing since the former ensures obtaining the maximum amount of evidence from the surface.

After collecting the saliva, the evidence is treated with a series of techniques. These techniques are broadly classified into two—destructive and non-destructive. The technique which destroys the integrity of evidence after the assessment is known as the destructive technique. In this the evidence treated, cannot be used for further processing or stored for later. Though, integrity of evidence is not affected after assessment in case of non-destructive techniques.

Preliminary tests

The preliminary tests state that saliva “might” be present in the submitted evidence, followed by confirmatory tests. Each test has its advantages and limitations. These tests consume a significant amount of time as well as evidence. The evidence loss in identification analysis cannot be retrieved to its original state unless the test is non-destructive. Due to this, there is very little evidence left to test for DNA profiling to establish the identity. The preliminary tests, a.k.a presumptive tests, for saliva, are based on the amylase enzyme activity. But this amylase is not solely present in saliva; it is also associated with other body fluid secretions (Wornes et al. 2018). There are two types of amylases, coded by AMY1 (α -amylase-1) and AMY2 (α amylase-2) locus present on chromosome 1. AMY1 type is present in saliva, breast milk, and sweat, while AMY2 is generally present in semen, pancreas, and vaginal fluid respectively (Sensabaugh 1982; Greenfield and Sloan 2002). α amylase-1 is found to be excessive in saliva as compared to other body fluids and can be easily distinguished from α amylase-2 by radial diffusion assay (Quarino et al. 1993).

Polilight detection

Polilight is a portable, high-intensity light source used to locate body fluids. This method is the only non-destructive type of presumptive technique for the detection of saliva (Table 1). It produces an intense narrow band of light with a wavelength between 310 and 650 nm (Vandenberg and Oorschot 2006). Forensic light source (FLS) is a common term adapted for any illuminating light source that aids in forensic investigation. It is also known as an Alternating light source (ALS) (Lennard and Stoilovic

Table 1 Various studies on polilight detection of saliva

Year of study	Substrate	Observation
Wawryk and Odell (2005)	Saliva stains on human skin	High-intensity LED, 370–480 nm, and Poliray, 450 nm—not potent enough to produce fluorescence
Carter-Snell and Soltys (2005)	Saliva stains on human skin	100% sensitive to UV light produced by mineral light, 254 nm, and evident products CE, 365 nm while only 14% towards Bluemaxx BM500
Vandenberg and van Oorschot 2006	Saliva from nylon cloth	Polilight PL 500, a 500W xenon arc lamp- detected 100% samples
Camilleri et al. (2006)	Various cloth material	Polilight PL 500—poor and non-specific detection
Seidl et al. (2008)	Saliva stain	compared two ALS—Laser and Mercury Arc lamp Lumatec Superlite-400. The laser gave slightly better results than the latter
Lee et al. (2012)	Saliva sample	portable LED-based multi-wavelength light source supplying supply near-UV, blue, and 415 nm
Karchewski et al. (2014)	Saliva on fabric [comparison study]	<ul style="list-style-type: none"> • Leeds Spectral Vision system (IR/455 nm) • Polilight-Flare Plus UV (365 nm) • Mini-CrimeScope 400 CSS (485 nm)- better than the other two
Tay et al. 2021	On fabric and diluted form	<ul style="list-style-type: none"> • Polilight PL400 (PL400) • Polilight PL500 (PL500) • LED-based Crime-lite 82S (blue) (CL82S)- better than the other two

2004). This method helps in visibility and enhances it for photography. This is rapid and less laborious, particularly for large surfaces.

Although, saliva is often difficult to locate under Polilight because of the lower fluorescence intensity (Camilleri et al. 2006); also, the absorbency of the surface acts as a hindrance to fluorescence (Vandenberg and Oorschot 2006). There are goggles having certain filters that allow only desirable wavelengths and give peculiar observations (Chuen and Eea 2010) (Table 2). The disadvantage of polilight is that the color of the material hampers the strength of the appearance of the stain (Vandenberg and Oorschot 2006) also, fluorescence patterns are similar to that of other body fluids, leading to non-specificity (Camilleri et al. 2006).

Phadebas test

The Phadebas test is a commonly used presumptive assay. Its active component is a blue dye-bound DSM-P, microsphere. The test relies on the alpha-amylase activity of saliva and is prone to multiple pseudo-positive results. A positive reaction is usually confirmed after the release of blue dye, which results from hydrolyses of starch in the presence of amylase (Hedman et al. 2008). It gives negative results with animal saliva, fruits, vegetables, and some cleaning solutions (Pang and Cheung 2008; Casey and Price 2010; Feia and Novroski 2013); other body fluids (Rao et al. 2020).

In a recent study, the Phadebas press test has also been reported to detect saliva stains on certain fabrics, and aged stained samples (up to 3 months) (Woodford et al. 2021). Some studies have shown its worth compared to other methods such as Polilight (Hedman et al. 2008), starch iodine, and SALIgAE (Myers and Adkins 2008). On the contrary, the inability of the Phadebas test was also reported to presume the identity of saliva (Olsén et al. 2011). It depends upon the type of evidence and the surface on which saliva is present.

Table 2 Excitation wavelength coupled with suitable goggles

Excitation light	Goggles – filters	Observation
Long UV	Only UV safety required	White-bluish stain
415 nm	Yellow – 555 nm interference filter	Saliva observed
450 nm	Orange – 555 nm interference filter	White color stain
470 nm	530 nm/555 nm interference filter	Saliva observed
490 nm	555 nm interference filter	Saliva observed
505 nm	555 nm interference filter	Saliva observed
532 nm	Specific goggles blocking 532 nm light	Orange color stain

SALIgAE test

It is a colorimetric approach to eliminate any possible false-positive reactions given by its counterparts methods- Phadebas and Polilight. Hence, it is a more sensitive approach for preliminary saliva detection (Pang and Cheung 2008; Liang and Roy 2014; Harbison and Fleming 2016). The potency and accuracy of SALIgAE have been studied many times with different approaches. It shows sensitivity, irrelevant to the surface on which the stain is present, like envelopes, soda bottles/cans, and mouth masks, and shows specificity towards human saliva (Miller and Hodges 2005). A positive reaction is the yellow color change of an otherwise colorless solution. However, the reaction time must be confined to 5 min to avoid false-positive by other body fluids, which generally takes more than 5 min (Lim et al. 2008). On comparative analysis of SALIgAE with other assays, it is found to be better and more sensitive than Phadebas.

Apart from these benefits, it possesses some disadvantages because of the colorimetric result (Myers and Adkins 2008). For analysis in samples mixed with blood, the significant disadvantage of this colorimetric assay is the generation of false positive reaction. It is also time-consuming, or the procedure involves maximum dilution of the sample beforehand, leading to disadvantages and abuse of evidence (Silenieks 2006; Myers and Adkins 2008; Park et al. 2015).

Immunochromatographic strip test

A lateral flow immunochromatographic strip works on engaging two monoclonal antibodies present in saliva, making them more sensitive (Casey and Price 2010). A common example of this strip is Rapid Stain Identification (RSID) kit. This test is different and much more sensitive than Phadebas or SALIgAE because of its serological approach instead of colorimetric (Fig. 1). Many studies have compared these three approaches, and RSID emerged to be much more sensitive and time-effective than its counterparts. This qualitative assay generates positive or negative results depending on the presence or absence of a red or blue line on the “Test zone” after 10–15 min of sample addition (Sinelnikov et al. 2013). The accuracy, reproducibility, and sensitivity of RSID to detect saliva on different surfaces (cigarette butts, cans, bottles, etc.) are as low as 1µL (Old et al. 2009). This technique is efficient, portable, and potent to remove any ambiguity if it occurs. Since it detects the presence of alpha-amylase, which is also present in other body fluids such as urine, and vaginal secretion, the sensitivity of RSID with saliva is much greater (Sari et al. 2020).

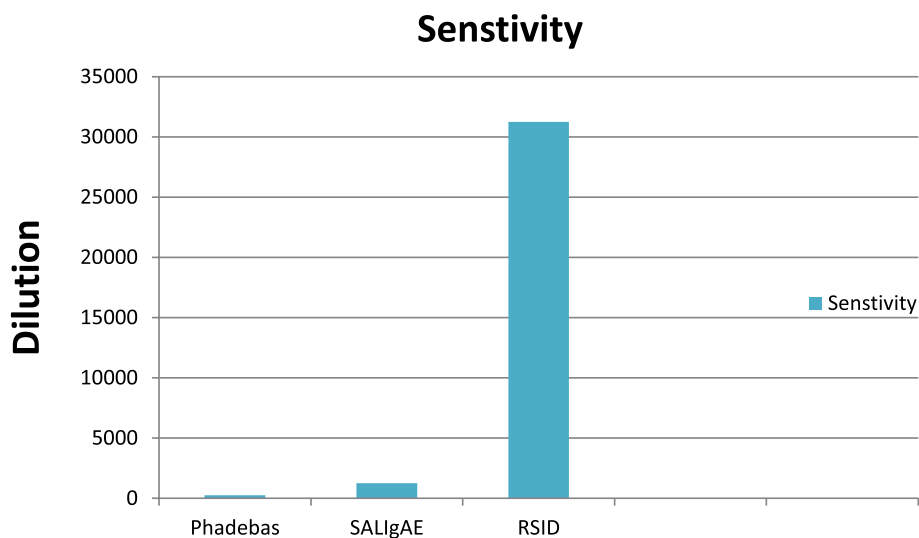


Fig. 1 Sensitivity comparison of Phadebas, SALIgAE, and RSID test

The efficacy of an immunochromatographic strip also depends upon the type of substrate on which the sample has been placed. Saliva has a tendency to get absorbed into porous material or substrates thereby making it more difficult to examine as compared to non-absorbent, non-porous surfaces. Also, environmental conditions are responsible for the degradation of saliva, like- light, heat, air, and humidity (Castelló et al. 2017). Though RSID can detect saliva with up to 10,000-fold dilution and amylase with up to 20,000-fold dilution (Old et al. 2009), these conditions may generate different results. The RSID technique has also been reported on samples degraded by humid soil and showed positive results which otherwise showed negative results by Phadebas (Ohta and Sakurada et al. 2019). This assay also showed efficacy in detecting saliva from expired blood spatters or blow artifacts. The other assays either failed or showed faint or pseudo-positive, but RSID gave positive results (Silenieks 2006; Park et al. 2015; Thompson et al. 2022).

Advanced screening/detection techniques

Various detection techniques are considered confirmatory to establish the identity of saliva. Some of these tests have been followed for decades, and some are still emerging.

Immunological techniques

Immunology assays are antigen–antibody-based reactions. These assays not only aid in detection but in species determination as well. Enzyme-linked immunosorbent assay (ELISA) was performed using horseradish peroxidase conjugate combined with monoclonal antibodies to detect the alpha-amylase activity in saliva (Komuro

et al. 1995). This assay has shown no cross-reactivity with either pancreatic or bacterial amylase, but there were still some false positives- 13% of other body fluid (Quarino et al. 2005). To overcome this, Statherin (STATH), a low molecular weight phosphoprotein secreted by the Parotid gland, was used for detection since it is only present in saliva. The findings gave positive for saliva with no cross-reactivity in ELISA. Also, old-age and mixed saliva samples were readily detected (Akutsu et al. 2010).

Other proteins, known as proline-rich proteins (PRPs), are present in two forms- acidic salivary PRP HaeIII sub-family 1/2 (PRH1/2) and basic salivary PRP 2 (PRB2). They are specific for saliva, especially the PRH1/2 expressed explicitly in salivary glands (Fábián et al. 2012). These PRPs were used in ELISA to detect saliva and to compare the sensitivity, efficiency, and specificity with STATH (Igoh 2015). Similarities in the detection rate and sensitivity of STATH and PRH1/2 followed by PRB2 were observed. However, the specificity towards saliva of PRH1/2 was higher than STATH, stating the former to be of forensic importance.

Other immunological techniques such as immunoelectrophoresis have also been attempted for saliva detection but showed multiple cross-reactivities, making them unreliable for the detection of saliva (Virkler and Lednev 2009).

Microscopy techniques

Microscopic techniques like SEM (Scanning Electron Microscope) coupled with EDX (Energy Dispersive X-Ray) can identify a specific metal concentration for detection. Some of the trace elements like sodium, phosphorus, sulfur, chlorine, potassium, and calcium are also present in saliva at different concentrations. However, potassium is shown to have the most prominent peak in

saliva samples and thus can be used for detection purposes (Seta 1977; Dube et al. 2020).

RNA profiling

RNA is unstable and prone to degradation, but many studies have shown its stability in samples and its massive use for forensics (Juusola and Ballantyne 2005; Chong et al. 2015; Park et al. 2006). The availability of RNAseq by Massively parallel sequencing (MPS) has made the rapid discovery and characterization of novel transcripts and other RNA regions such as non-coding, micro, and small RNA, possible (Trapnell et al. 2010; Roewer 2013). MicroRNA is a class of small non-coding RNA (ncRNA) molecules with 18–24 nucleotides that act as essential regulators for multiple cellular processes (Courts and Madea 2011) and are less susceptible to environmental decay (Sirker et al. 2017). The degradation rate of RNA in various biological samples over 6 months shows a similar decay pattern that can be analyzed to estimate the sample's age (Simard et al. 2012).

The first two saliva-specific RNA genes to be identified were statherine (STATH) and histatin3 (HTN3) (Ballantyne and Juusola, 2009; Haas et al. 2008; Haas et al. 2009; Chong et al. 2015; Watanabe et al. 2017). Later, Mucin7 (MUC7) gene was also reported along with STATH and HTN3 genes in degraded saliva samples of about two to 6 weeks (Sharma and Vogel 2009). The technique used to analyze these genes are reverse transcription endpoint PCR and RT-PCR (Real-time Polymerase Chain Reaction) (Haas et al. 2009). However, Liu et al. (2020) further used capillary electrophoresis (CE)-based method for the examination of these saliva-specific genes. Other than these, five other stable RNA genes—SPRR3, SPRR1A, KRT4, KRT6A, and KRT13—also provided good results for up to 180 days old saliva samples (Zubakov et al. 20082008).

Reverse transcription-loop-mediated isothermal amplification (RT-LAMP) is a new technique used for detection, a one-step amplification of a specific RNA sequence. This method is sensitive and provides high specificity (Tsai et al. 2018; Jackson et al. 2020; Layne et al. 2021).

DNA methylation

DNA, compared to RNA, is a more stable molecule in biological fluids (Marguet and Forterre 1994; Park et al. 2014). Methylation is a genetically programmed type of DNA modification in mammals, occurring at the 5' position of the cytosine in the CpG dinucleotide sequence (Miranda and Jones 2007). It plays an essential role in the development and differentiation of cells by controlling gene expression through changes in the chromatin structure and tissue-specific patterns (Hashimshony et al. 2003; Straussman et al. 2009;

Schilling and Rehli 2007). DNA methylation is an epigenetic modification that can provide important information if explored vividly (Frumkin et al. 2011; Varriale 2014). The DNA methylation markers show similarity in cellular and extracellular DNA, which suggests their positive examination in the absence of cells (Fu et al. 2015). So far, multiple DNA methylation markers for saliva have been reported for forensic purposes (Table 3).

DNA methylation can be done by various techniques, the most common is a chemical modification of cytosine residues by sodium bisulfate (Gomaa et al. 2017). This modification can speed up by using high-concentration bisulfate at high temperatures (Shiraishi and Hayatsu 2004). However, bisulfite conversion can lead to the undesirable side effect of DNA degradation, which is not ideal if the sample concentration is low or poor quality (Lin et al. 2016). Methylation-sensitive restriction enzyme PCR (MSRE-PCR), an alternate strategy, was introduced to overcome the issue, but it worked only for semen identification (Lin et al. 2016; Wasserstrom et al. 2013). Methylation SnaPshot is the second most used technique; it has the advantage of simultaneous analysis by constructing multiplex methylation SnaPshot. Another introduction to this is multiplex Snapshot microarray which combines different markers. Bisulfite genomic sequencing is a fundamental gold-standard methylation technique because it provides a qualitative, quantitative, and efficient approach to identifying 5-methylcytosine at single base-pair resolution (Gomaa et al. 2017). Next Gen Sequencing has been suggested as an aid to overcome any possible false positives (Gauthier et al. 2019).

Spectroscopic technique

The advancement and recent developments in body fluid analysis using vibrational spectroscopy have shown excellent potential for an alternative approach (Elkins 2011; Virkler and Lednev 2008). This technique is universal, non-destructive, and label-free (Vyas et al. 2020).

Table 3 DNA methylation markers for saliva

Salivary markers	Reference studies
BCAS4	Madi et al. (2012), Silva et al. (2016), Gauthier et al. (2019)
cg26107890	Park et al. (2014)
cg09652652-2d	Lee et al. (2015)
cg09652652 and cg09107912	Lin et al. (2016)
SOX11	Forat et al. (2016)
cg09652652	Holtkötter et al. (2017)
HPCAL1	Ghai et al. (2020)

UV-Visible spectroscopy UV-Visible spectroscopy has been improvised over the years, with many advancements and changes from a short range to a broader range (Fiedler et al. 2008; Zapata et al. 2015a). Multiple studies have been done to show the specific wavelength range of different body fluids. However, the similar response of one body fluid to another fluid's characteristic spectra, questions the technique's specificity. This technique is prone to multiple false positives as other substances may hinder results (Zapata et al. 2015b).

Fluorescent spectroscopy Fluorescent spectroscopy uses tryptophan, an endogenous fluorophore in alpha-amylase, as a prevalent probe in dried saliva stains on human skin. It shows a characteristic emission spectrum at 345–355 nm (Soukos et al. 2000; Nanda et al. 2011; Sikirzhyski et al. 2011).

Raman spectroscopy Raman is a widely used and accepted technique in body fluid identification because it provides better spatial resolution and doesn't react with water (Sikirzhyski et al. 2012; Virkler and Lednev 2010). Apart from that, Phenylalanine, present in amylase and lipase, gives peculiar peaks of saliva in Raman and Thiocyanate, which is found in the saliva of a smoker (Virkler and Lednev 2008). The saliva spectra are high compared to other body fluids and can be discriminated against when mixed with blood or semen (Quarino et al. 2005; Hardy et al. 2022). Compared to other amino acids, the spectra of tryptophan are single maximum. This assay can also be used to determine the sex of the sample (Muro et al. 2016).

Fourier transform infrared spectroscopy Another robust technique is Fourier transform infrared spectroscopy (FTIR), which can detect multiple spectra of saliva based on alpha-amylase and lysozymes (Orphanou et al. 2015; Takamura et al. 2018). However, a disadvantage of vibrational spectroscopy is the multiple spectra of a single body fluid. Since a body fluid is a complex mixture, it gives inhomogeneous complex spatial distributions. Moreover, environmental degradation of samples hinders results due to multiple contaminations (Orphanou et al. 2015).

Microbial detection for saliva identification

Healthy saliva is a mixture of proteins, carbohydrates, antibacterial proteins, white blood cells, and many other components. Though it has antibacterial immunoglobulins and WBCs, forming the first line of defense, it does nurture an array of microbial flora (Dash and Das 2018). On average, the total microscopic count is approximately 750 million oral bacteria cells

per milliliter of saliva, and of these, streptococci are the most abundant. The presence of these bacteria solely in the saliva has been studied over the years by various techniques such as PCR (Nakanishi et al. 2009; Ali et al. 2013; Choi et al. 2014) and loop-mediated isothermal amplification (Nakanishi et al. 2011). Over the years, the interest in microbiome identification of body fluids has increased drastically among forensic scientists. There have been multiple studies that show definite positive detection but are performed differently (Fig. 2).

Streptococcus salivarius is one of the most common bacteria, followed by *Streptococcus mutans*, responsible for dental caries (Ali et al. 2013). *Streptococcus salivarius* (a prominent member of oral microbiota), *Streptococcus sanguinis* (most abundant species in oral biofilms), and *Neisseria subflava* (normal flora of the oral cavity and respiratory tract) are the most common microbes used to identify saliva by different methods combined with RT-PCR such as OB-mRT-PCR (Jung et al. 2018) and colorimetric detection of bacterial DNA amplicons on an immunochromatographic strip (ICS) (Lee et al. 2018).

These techniques are novel, more convenient, user-friendly, and cost-effective. The cross-examination of samples by conventional methods generates a result that favors microbial detection and is specific for body fluid and species. The sensitivity of this method is high in mixed samples as well (Li et al. 2019).

In degraded samples, the identification of saliva using microbial profiling has shown high sensitivity compared to other techniques. Saliva in samples degraded up to 30 days has been analyzed by examining the 16S rRNA V4-V5 region and successfully amplifying only 92% of total saliva samples (Fig. 3) (Dobay et al. 2019).

Another study reported the efficacy of oral gram-positive and gram-negative bacteria for DNA-based identification of highly degraded salivary samples. It was observed that oral gram-positive bacteria served as a better source for DNA-based identification as the same could be detected successfully in environmentally exposed and UV-irradiated samples (Table 4) (Ohta and Sakurada et al. 2019).

The latest advancement in this technique is developing a novel smartphone-based bacteria sensor that assesses two oral bacteria—*S. salivarius* and *S. sanguinis* (Li 2020). The test strip gives dose-sensitive color (blue-emitting silicon carbide quantum dots and red-emitting gold nanoclusters) when introduced under a 365-nm UV lamp. The color intensity is directly proportional to the concentration of bacteria in the sample. This method can identify two bacteria simultaneously in 20 min, unlike other oral bacteria detection methods, and is the latest advancement in the forensic microbiome.

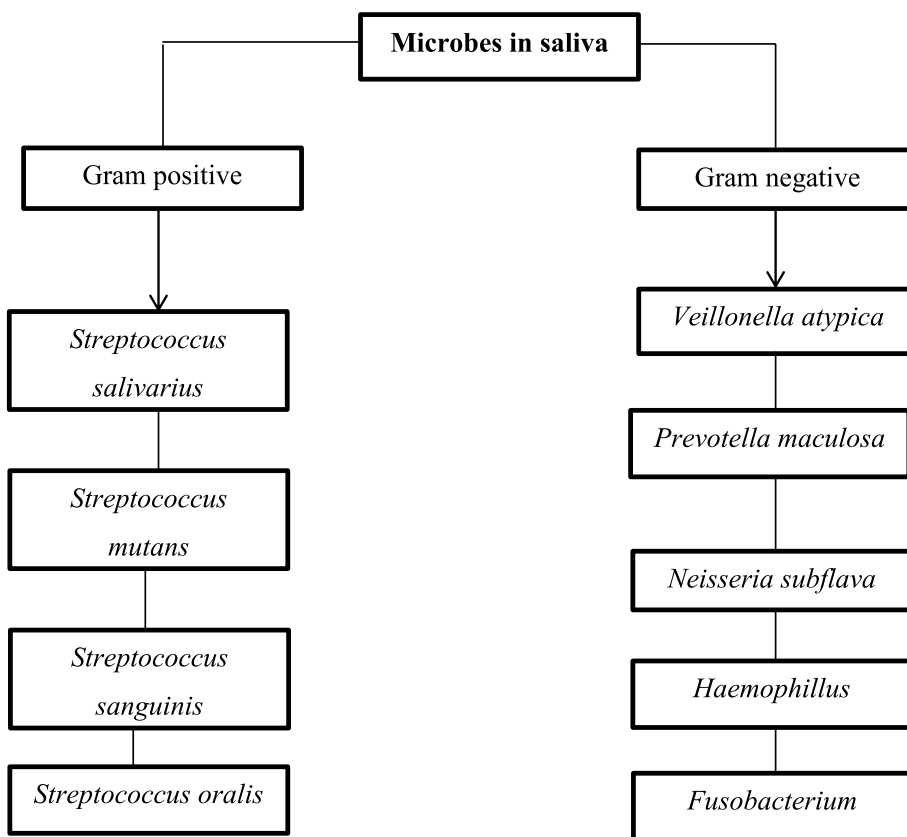


Fig. 2 Flowchart showing the different types of Microbes in saliva

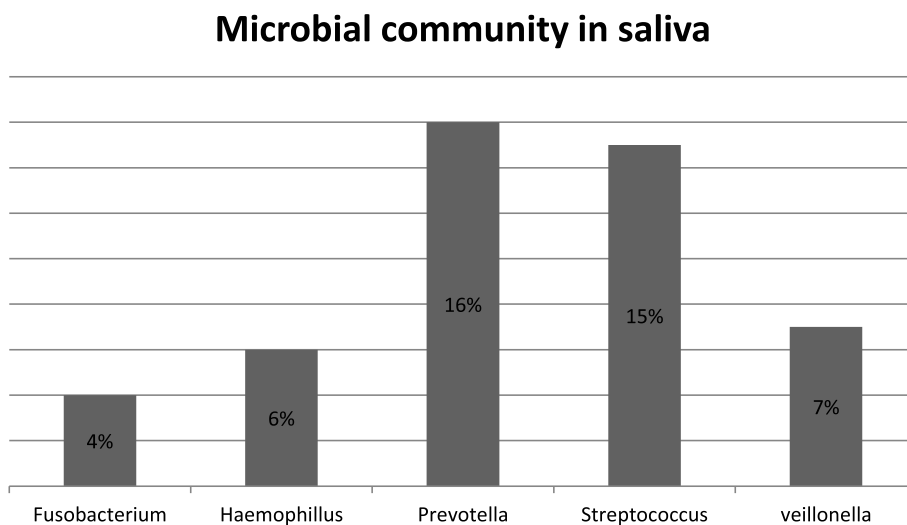


Fig. 3 Microbial community in saliva

Destructive and non-destructive assessment of saliva samples

The above-mentioned assessment techniques for saliva

can be further classified into destructive and non-destructive categories. Destructive methods often result in the consumption of samples in a way that the samples cannot be used for further processing for DNA analysis.

Table 4 Efficacy of oral gram-positive and gram-negative bacteria for DNA-based identification

Type of bacteria	Heat (40–80 °C) denaturation	Microbial decomposition (1–5 days)	UV irradiation (0.01–1 J/cm ²)
Gram-positive bacteria (<i>Streptococcus salivarius</i> and <i>Streptococcus oralis</i>)	Detected	Detected (5-day sample)	Detected (> 50%)
Gram-negative bacteria (<i>Veillonella atypica</i> and <i>Prevotella maculosa</i>)	Detected	Detected (1-day sample)	Not detected

An overview of various destructive and non-destructive techniques used in the examination of saliva samples has been presented in Table 5.

Conclusions

Saliva holds much relevance in forensics as any other body fluid. Its non-invasive collection, wide benefit, and colorless appearance make it good evidence. The detection process of saliva offers a destructive and non-destructive assessment. The detection process provides information on the quality and quantity of saliva present on the substrate. After establishing the identity of saliva, it becomes a major source of DNA, which is further processed for DNA profiling.

The preliminary detection is based on the presence of α -amylase, saliva-specific antibodies, and the fluorescence activity of saliva. The polilight-based detection is portable and low cost but shows specific colored light that is impossible to read on bright backgrounds. The light emitted by saliva mimics the fluorescence of other body fluids, which could lead to either false identification or more time consumption until other techniques verify the identity. The Phadebas and SALigAE tests rely on the presence of α -amylase present on the test saliva samples. These tests are simple to use but have a disadvantage of

cross-reactivity, time efficacy, and wastage of samples. Immunochromatographic kit-based detection of monoclonal antibodies provides better sensitivity than the former three, but tends to give false negative results with other body fluids.

In advanced screening, the detection of saliva must be established for further downstream processing of evidence. Each technique is unique but possesses some disadvantages such as the RNA being highly unstable, and its profiling for identification can be costly and time-consuming. The DNA methylation technique can identify body fluid and individuals with single processing. Among all these, the NextGen sequencing technique, so far, is a much better approach but NGS is costly and cannot be available everywhere.

On the other hand, the spectroscopy techniques are robust, non-destructive, and possess considerable merits. The only demerit is its multiple spectra of a single body fluid, which could worsen if the sample is contaminated or mixed. These techniques maintain the integrity of the sample and produce more satisfactory results. Microbial profiling is the new advancement and interest for detection, falling under forensic microbiology. However, this technique requires skills and is expensive. The survival rate of microbes in the environment is much more

Table 5 List of destructive techniques used in saliva analysis

Techniques	Type of technique	Success rate
Phadebas test	Destructive	It has many false positives since amylase is present in body fluids other than saliva as well
SALigAE test	Destructive	false-positive values have been traced in breast milk, rat saliva, clean urine, fecal/feces, and semen samples
RSID	Destructive	Highly sensitive and rapid test
Immunological test	Destructive	Multiple cross-reactivities are seen in this analysis
RNA profiling	Destructive	The highly unstable nature of RNA causes hindrance
DNA methylation	Destructive	The success rate of this technique is very high
UV-Vis Spectroscopy	Destructive	Other body fluids often show similar ranges
Microbial profiling	Destructive	The very high success rate on normal and degraded samples
Polilight	Non-destructive	It has a low success rate as semen gives the same fluorescence as saliva, resulting in false positives
Microscopic analysis	Non-destructive	Mixed samples cause variation in the concentration of metal
Fluorescent spectroscopy	Non-destructive	Shows characteristic fluorescence for tryptophan
Raman spectroscopy	Non-destructive	It has a good success rate
FTIR spectroscopy	Non-destructive	Mixed and degraded samples do hinder the result

than any nucleic acid or protein. It can be concluded that despite having an ample amount of techniques for identification and detection, there is still a lack of a definitive technique for saliva examination. The technique which is robust, feasible, cost-effective, and does not compromise the integrity of the exhibit still needs to be explored.

Abbreviations

DNA	Deoxyribonucleic acid
FLS	Forensic light source
ALS	Alternate light source
AMY1/AMY2	α -Amylase 1/ α -amylase 2
RSID	Rapid stain identification series
ELISA	Enzyme-linked immunosorbent assay
STATH	Statherin
PRPs	Proline-rich proteins
SEM	Scanning electron microscope
EDX	Energy dispersive X-ray
RNA	Ribonucleic acid
MPS	Massive parallel sequencing
HTN	Histatine
qPCR	Quantitative polymerase chain reaction
RT-LAMP	Reverse transcription–loop-mediated isothermal amplification
MSRE-PCR	Methylation-sensitive restriction enzymes–polymerase chain reaction
UV-Vis	Ultraviolet-visible
FTIR	Fourier transform infrared
WBC	White blood cells
RT-PCR	Real-time polymerase chain reaction

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