



Saliva and Salivary Biomarkers: A Promising Frontier in Forensic and Clinical Diagnostics

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ABSTRACT:

With advancements in forensic and biomedical research, saliva has emerged as a crucial biological fluid due to its non-invasive collection method and rich source of diagnostic information across various investigative domains. This review explores the multifaceted applications of saliva in forensic science, particularly in personal identification, DNA profiling, drug detection, and biomarker analysis. In recent years, salivary microbiome profiling and the detection of salivary biomarkers have gained prominence. The presence of unique microbial species has enabled significant progress in disease diagnosis and forensic identification. Saliva is also instrumental in monitoring dental caries, periodontal diseases, oral cancer, and certain systemic diseases due to changes in its biochemical and microbial composition. This paper highlights the expanding role of saliva as a reliable, non-invasive substrate bridging forensic science and clinical diagnostics.

Introduction

Saliva has proven to be an invaluable biological fluid in forensic odontology, playing a crucial role in the identification and analysis of evidence, due to its easy, non-invasive collection method, rich diagnostic components, and applicability in diverse investigative contexts. Composed of secretions from salivary glands, epithelial cells, microorganisms, and crevicular fluid, saliva reflects both physiological and pathological states of the individual. In forensic investigations, it plays a pivotal role in personal identification, bite mark analysis, sex determination, and drug detection.^{1,2} Advancements in molecular biology have expanded its utility beyond DNA profiling to include salivary microbiome analysis, hormone detection, and epigenetic markers such as DNA methylation and RNA transcripts,^{3,4,5} thus highlighting the developments in saliva's growing relevance as a reliable and informative substrate in modern forensic science. In the present paper, we highlight the role of saliva in various aspects of forensic and clinical diagnosis.

Collection of Saliva Sample:

The collection of saliva is a delicate procedure typically performed using one of two swabbing methods, depending on the type of surface where the evidence is found. In the single swab technique, a sterile cotton swab moistened with distilled water or normal saline is gently rolled over the collection site without applying pressure to prevent picking up material from the surface itself. In contrast, the double swab technique involves two steps: initially swabbing the area with a moistened swab, followed by a second swab using a dry one.⁶ This method improves saliva recovery, particularly from absorbent surfaces such as skin, fabric, or partially consumed food. For non-absorbent surfaces, such as plastic or glass, the single swab method is typically used.⁷ During the collection process, care must be taken not to swab beyond the targeted area to avoid cross-contamination and the risk of obtaining mixed DNA profiles. The double swab method is generally favored over the single swab because it enhances the retrieval of biological evidence. Once collected, the saliva samples undergo various analytical techniques, which are categorized as either destructive or non-destructive. Destructive



techniques compromise the integrity of the evidence, making it unsuitable for further analysis or long-term storage. Whereas non-destructive techniques preserve the sample's integrity, allowing for additional testing or archiving after analysis.⁴

Saliva in Personal Identification:

Saliva is frequently found in bite marks in homicide cases, assaults, and other criminal incidents. However, bite mark analysis comes with many challenges, primarily due to the elastic and deformable nature of skin, as well as the lack of effective impression mediums. This has led to a significant focus on the saliva left behind during bites as an alternative method of investigation. Collecting saliva stains from skin, clothing, paper, or other surfaces can be difficult because these stains are usually invisible. Methods for detecting dried saliva stains include various techniques designed to make these stains more visible and analyzable.^{1,2,3}

Various chemicals and enzymes have been tested to detect dried saliva stains. One method for identifying saliva on stamps and envelopes utilizes a chemical that reacts with reducing sugars, resulting in the formation of a red insoluble precipitate. The enzymes most commonly used for this purpose include alkaline phosphatase, starch, and amylase. Additionally, salts such as nitrate and thiocyanate have been used for detection. However, these methods have limitations and show variable sensitivity, which can depend on the time of the saliva stain and the amount deposited. Techniques such as lasers and ultraviolet light, as well as quartz arch tubes and argon ion lasers, have also been employed in these investigations.^{3,4}

Fluorescent spectroscopy is another effective method for detection. The amino acid tryptophan that is found in α -salivary amylase produces a characteristic emission spectrum when subjected to fluorescent spectroscopy, produces good sensitivity for detecting dried saliva stains on the skin.^{3,4} This assay can also be utilized to determine the sex of the sample.^{3,4} Raman spectroscopy is a widely accepted technique for identifying body fluids due to its superior spatial resolution and its non-reactivity with water.^{8,9} Additionally, phenylalanine, which is present in amylase and lipase, produces distinctive peaks in saliva when analyzed using Raman spectroscopy. Thiocyanate, found in the saliva of smokers, also has identifiable spectral features.^{8,9} The spectral intensity of

saliva is significantly higher compared to other body fluids, allowing it to be distinguished even when mixed with blood or semen.^{10,11,12} Fourier transform infrared spectroscopy (FTIR) is a powerful technique that can detect various spectra of saliva based on the presence of alpha-amylase and lysozymes.^{13,14} However, one disadvantage of vibrational spectroscopy is that a single body fluid can produce multiple spectra. Because body fluids are complex mixtures, they exhibit inhomogeneous spatial distributions. Additionally, environmental degradation of samples can compromise results due to the risk of contamination.¹³

Polilight is a portable, high-intensity light source used to locate body fluids that emits a focused narrow band of light with wavelengths ranging from 310 to 650 nm.¹⁵ This method enhances visibility for photography, making it rapid and less labor-intensive, especially for large surfaces. However, locating saliva with Polilight can be challenging due to its lower fluorescence intensity,¹⁶ and the absorbency of the surface may hinder fluorescence.¹⁵ There are specialized goggles equipped with filters that allow only certain wavelengths, providing distinct observations.¹⁷ One disadvantage of Polilight is that the color of the material can affect the visibility of the stain.¹⁵ Additionally, the fluorescence patterns of different body fluids can be similar, leading to a lack of specificity.¹⁶

Saliva Testing for Drugs of Abuse:

Oral fluid testing for drugs of abuse offers significant advantages over urine testing. One major benefit is that oral fluid samples can be collected under direct observation, which reduces the risk of sample adulteration or substitution. Drugs typically enter oral fluid through passive diffusion from the bloodstream, but they can also be introduced into the oral cavity through oral consumption, smoking, or intranasal methods. Additionally, drug metabolites can be detected in oral fluid. The drugs that can be detected in saliva include amphetamines, barbiturates, benzodiazepines, phencyclidine, cocaine, and opioids. Furthermore, saliva can be used to detect recent marijuana use through radioimmunoassay. A major psychoactive component of marijuana can be detected in saliva for at least four hours after it has been smoked.¹⁸ Saliva is a promising matrix for detecting on-site recent drug use, especially for cocaine and amphetamines, using Drugwipe immunoassay.¹⁹



DNA Profiling in Forensics:

DNA profiling is useful in identifying victims in mass disasters, associating dismembered body parts, determining familial relationships and paternity, and providing evidence in criminal investigations. The specificity of DNA profiles ensures a high degree of accuracy in identification, and the acceptance of DNA evidence in legal proceedings underscores its forensic significance. Teeth are an excellent source of DNA since they remain largely unaffected by environmental factors which allow proper isolation and quantification of DNA which is essential for successful analysis. Most commonly DNA in fingerprint analysis is extracted by organic method (phenol) or by Chelex 100 (Bio Rad Laboratories, Inc), FTA paper (Whatman Inc, Clifton, NJ) or isopropyl alcohol.²⁰

DNA samples are often amplified through a process called polymerase chain reaction (PCR) for the purpose of DNA typing, specifically using short tandem repeats (STRs). The variations found in STRs, particularly in small fragments, enable the analysis of DNA from samples that are significantly degraded. In addition to genomic DNA, cells derived from saliva also contain mitochondrial DNA (mtDNA). The sequence of building blocks in mtDNA can be analyzed to aid in identification. One of the primary advantages of mtDNA is that each cell contains a high copy number due to the abundance of mitochondria and can be successfully used even when nuclear DNA testing fails or when genomic DNA cannot be analyzed due to degradation.³

Salivary Biomarkers in Forensic Applications:

Biomarkers exist in various forms, including antibodies, microbes, DNA, RNA, lipids, metabolites, and proteins. Changes in their concentration, structure, function, or activity can be associated with the onset, progression, or even regression of a particular disorder, or they can result from how the body responds to it.²¹ A set of reliable and reproducible biomarkers unique to specific diseases is often referred to as a biomarker or molecular signature. Understanding and evaluating the significance of an individual's biomarker signature can be useful in determining the presence, location, and even likelihood of disease. Therefore, biomarkers serve as a valuable and effective tool in the detection, risk assessment, diagnosis, prognosis, and monitoring of the disease.²²

Saliva harbors a diverse microbial flora despite its antimicrobial components, with an average microbial

load of around 750 million oral bacteria per milliliter. Among these, *Streptococcus salivarius* is the most prominent and frequently used as a microbial marker due to its exclusive presence in saliva. Other key microbes include *Streptococcus mutans*, associated with dental caries; *Streptococcus sanguinis*, a dominant species in oral biofilms; and *Neisseria subflava*, part of the normal flora of the oral and respiratory tracts. Additionally, gram-negative bacteria such as *Veillonella atypica* and *Prevotella maculosa* are noted, although they are less stable under environmental degradation compared to gram-positive counterparts (Table 1).^{4,22}

Table 1: Key Oral Microbiomes Identified in Saliva^{4,22}

Microorganism	Role
<i>Streptococcus salivarius</i>	Most abundant; considered a hallmark of saliva. Used frequently in microbial detection assays (PCR, LAMP).
<i>Streptococcus mutans</i>	Associated with dental caries; found in saliva.
<i>Streptococcus sanguinis</i>	Abundant in oral Biofilms.
<i>Neisseria subflava</i>	Part of the normal flora of the oral cavity and respiratory tract.
<i>Veillonella atypica</i>	Gram-negative oral bacterium; used in degraded saliva sample detection.
<i>Prevotella maculosa</i>	Gram-negative oral bacterium; shows lesser stability in degraded conditions compared to gram-positive.



Microbial profiling has emerged as a novel and reliable method for saliva identification, especially useful in forensic cases involving degraded or mixed biological samples. Techniques like Polymerase Chain Reaction (PCR), loop-mediated isothermal amplification (LAMP), OB multiplex real-time PCR (mRT-PCR), and colorimetric immunochromatographic strips (ICS) are employed to detect these microbes with high specificity. Remarkably, saliva samples degraded up to 30 days have been successfully identified using 16S rRNA sequencing, with a detection success rate as high as 92%.⁴ Moreover, recent advancements include the development of a smartphone-based bacterial sensor capable of detecting *S. salivarius* and *S. sanguinis* using quantum dots and gold nanoclusters, providing a rapid and portable tool for forensic applications. These microbiome-based methods offer high sensitivity, specificity, and practicality, making them a promising addition to conventional saliva detection techniques.⁴

Saliva and Disease Correlation:

Saliva plays a crucial role in the diagnosis and monitoring of various oral diseases due to its rich composition of biomarkers, including enzymes, immunoglobulins, cytokines, hormones, and oxidative stress markers. Saliva not only reflects the oral health conditions but also affects systemic health, owing to its close interaction with blood serum and oral tissues. In the context of dental caries, salivary pH, buffering capacity, flow rate, and microbial content (like *Streptococcus mutans* and *Lactobacillus*) are key indicators. Elevated levels of these cariogenic bacteria in saliva are directly associated with increased caries risk. Additionally, enzymes such as salivary amylase also play a role in carbohydrate metabolism, influencing caries development.²³

In periodontal diseases, saliva contains elevated levels of inflammatory biomarkers such as interleukin-1 β (IL-1 β), tumor necrosis factor-alpha (TNF- α), matrix metalloproteinases (MMPs), and C-reactive protein (CRP). These markers reflect tissue inflammation, collagen degradation, and immune responses occurring in periodontal tissues.^{24,25} Regarding oral cancer, especially oral squamous cell carcinoma (OSCC), saliva shows significant changes in cytokines (e.g., IL-6, IL-8), growth factors, mRNA, microRNA, and protein markers

(Table 2). These salivary components have shown potential in the early detection and prognosis of oral malignancies.²⁶ Further, studies have shown the presence of abnormal oral bacterial growth increases the risk of pancreatic cancer.^{27,28,29} Thus, saliva serves as a non-invasive, cost-effective diagnostic medium with strong potential in detecting and monitoring a wide spectrum of oral diseases through the evaluation of specific biomarkers.

Oral Disease	Salivary Biomarker Involved	Significance
Dental Caries ²³	- <i>Streptococcus mutans</i> , <i>Lactobacillus</i>	Reflects cariogenic potential;
	Salivary pH Buffering capacity Salivary amylase	- changes in pH and microbial load increase - caries susceptibility
Periodontal Disease ^{24,25}	- IL-1 β - TNF- α - MMPs (e.g., MMP-8, MMP-9) - C-reactive protein (CRP)	Indicators of inflammation, tissue destruction, and immune response
Oral Squamous Cell Carcinoma ²⁶	- IL-6, IL-8 Tumor markers (e.g., Cyfra 21-1) mRNA, microRNA Growth factors	- Useful in early detection, diagnosis, and prognosis - of oral cancer
Oral Infections ⁴	- Antibodies (IgA, IgG) Microbial DNA/RNA (e.g., HSV, Candida)	Reflects pathogen presence; viral/fungal infections.

Saliva is increasingly recognized as a valuable source of biomarkers through various -omics technologies:

1. **Transcriptomics:** Saliva contains mRNA species and microRNAs (miRNA), offering potential for disease-specific biomarker discovery. It has shown promise in diagnosing conditions like Sjogren's



syndrome and several cancers, indicating its utility in non-invasive transcriptomic profiling.^{30,31,32}

2. **Proteomics:** Though saliva has only about 30% of the protein content of blood, it contains a wide range of functional proteins. Studies have identified specific salivary protein profiles associated with diseases like oral cancer, diabetes, AIDS, periodontal disease, and breast cancer, positioning saliva as a powerful diagnostic medium.^{33,34,35,36,37,38,39}

3. **Methylomics:** Saliva-based DNA methylation analysis has proven useful in detecting epigenetic changes associated with cancers such as OSCC and HNSCC. It also shows potential for age-related disease prediction and forensic applications. Emerging studies have successfully differentiated bodily fluids using tissue-specific differentially methylated regions (tDMRs), highlighting the future role of salivary methylomics in forensic identification.^{40,41,42}

Conclusion:

Saliva serves as a powerful and versatile biological fluid with significant relevance in both forensic science and oral disease diagnostics. From a forensic perspective, saliva is increasingly recognized as a vital form of trace evidence owing to its non-invasive collection, resistance to tampering, and its utility in establishing personal identity through DNA profiling and microbial or biomarker signatures. The broad spectrum of traditional and advanced methods for saliva detection, including biochemical assays, RNA profiling, DNA methylation markers, immunochromatographic strips, and spectroscopic techniques, enhance the reliability of saliva as forensic evidence, even in degraded or trace amounts.⁴ Salivary biomarkers can be used for a wide range of oral diseases. Certain systemic conditions show distinct alterations in salivary constituents, including cytokines, enzymes, antibodies, and specific mRNA/microRNA signatures.²² This reinforces the role of saliva as a mirror of systemic and oral health. Thus, saliva is not only a key medium in disease biomarker research but also a critical tool in forensic investigations, bridging the gap between medical diagnostics and legal science. The ongoing advancements in salivary diagnostics and forensic detection technologies promise greater accuracy, speed, and non-invasiveness, making saliva an indispensable resource in both clinical and forensic domains.

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