Saliva as a Diagnostic Medium: Biomarker Identification, Genetic Analysis, and Disease Association

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Abstract

Background: Saliva, an exocrine secretion, plays a vital role in maintaining oral and systemic health through its diverse molecular composition, including proteins, nucleic acids, and hormones. As an easily accessible and non-invasive biological fluid, saliva has emerged as a promising tool for diagnostic purposes, offering a potential medium for biomarker identification in various diseases. Methods: This review synthesizes current research on the use of saliva as a diagnostic medium, focusing on its molecular composition, including DNA, RNA, proteins, and noncoding RNAs, and their applications in disease diagnosis. We explore the use of salivary biomarkers in the detection of oral and systemic diseases, such as oral squamous cell carcinoma (OSCC), diabetes mellitus, cardiovascular diseases, and genetic disorders. The review also discusses the role of genetic polymorphisms in salivary proteins and their association with disease risk, as well as the potential of saliva in detecting infectious diseases like COVID-19, dengue, and HIV. Results: Salivary biomarkers, including DNA, mRNA, noncoding RNAs, and proteins, have shown promise in diagnosing a range of diseases. Studies have identified specific salivary mRNAs, such as IL-6 and NGFI-A binding protein 2, as potential biomarkers for OSCC. Noncoding RNAs, including miRNAs and piRNAs, have been implicated in various malignancies and systemic diseases. Proteomic analyses of saliva have revealed disease-specific protein signatures, providing insights into conditions such as Sjögren's syndrome, graft-versus-host disease, and diabetes. Furthermore, genetic polymorphisms in salivary proteins have been associated with oral diseases, such as dental caries and periodontitis, as well as systemic conditions. Conclusion: Saliva represents a valuable and underutilized diagnostic medium with the potential to revolutionize disease detection and monitoring. The identification of salivary biomarkers and genetic polymorphisms linked to disease risk underscores the importance of saliva in personalized medicine. However, further research is needed to standardize saliva collection and processing methods to enhance its diagnostic accuracy and reliability. The integration of salivary diagnostics into clinical practice could significantly improve patient outcomes through early detection and personalized treatment strategies.

Keywords: Salivary biomarkers, Genetic analysis, Non-invasive diagnostics, Oral diseases, Systemic health

Significance | Saliva acts as a non-invasive, cost-effective medium for diagnosing diseases, enabling biomarker identification, genetic analysis, and monitoring systemic health.

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Introduction

Saliva, an exocrine secretion of the salivary glands, contains a diverse range of molecules, including polypeptides, proteins, nucleic acids, electrolytes, hormones, and growth factors. These

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molecules play crucial roles in maintaining oral health, which in turn supports overall systemic health. Research into disease biomarkers has highlighted saliva as a promising medium for biomarker identification due to its accessible and non-invasive nature. As a form of liquid biopsy, saliva can be easily collected, is cost-effective, and does not require trained medical personnel. This allows for repeated sampling at different time points, with minimal risk of cross-contamination, and offers more manageable shipping and storage compared to serum.

Biomarkers in saliva can be found in various forms, such as DNA, coding and noncoding RNA, lipids, metabolites, and proteins. Given the complex interactions among salivary proteins, developing a comprehensive panel of biomarkers is essential for accurate disease detection (Buzalaf et al., 2020). Saliva's significance as a biological fluid for biomarker identification stems from its ability to reflect biomolecules from systemic sources that enter the oral cavity through different pathways, thereby mirroring tissue fluid levels of hormonal, immunological, and toxicological molecules (Zimmermann et al., 2007). The importance of salivary analysis lies in its origin, composition similar to serum, and its interactions with other bodily organs (Lima et al., 2007). For effective diagnostic use, saliva collection and processing protocols must be standardized based on the specific disease being studied.

However, one limitation of using saliva for routine diagnostics is that the concentration of its components is often lower than in serum or other biological fluids. Despite this, saliva testing is valuable for diagnosing oral conditions such as caries, periodontal diseases, and oral malignancies, as well as systemic illnesses like diabetes mellitus and cardiovascular diseases. Additionally, it plays a role in drug detection and forensic studies, offering substantial potential for advancements in medicine.

Saliva as a source of genetic material

The oral cavity could be a non-invasive source of genomic material. In recent years, saliva has emerged as a new tool for genetic testing due to its minimal invasive approaches. In a genetic epidemiologic study of type 1 diabetes mellitus with Norwegian children, DNA was extracted from buccal swabs and human leukocyte antigen HLA-DQA1 and -DQB1 allelic polymorphisms were determined by polymerase chain reaction which resulted in comparable results with previous studies (Witsø et al., 2002). In another study conducted by Adriaanse *et al.*,., 2016, DNA isolation using buccal swabs yielded a good quality and quantity of DNA to perform HLA-DQ typing in children for celiac disease which could reduce the need for current venipuncture (Adriaanse et al., 2016). Saliva is also a source of extracellular or cell-free DNA that can be used in forensic case studies (Vandewoestyne et al., 2013). DNA and RNA could also be isolated from saliva and salivary RNA analysis was done using microarray to understand neonatal development (Maron et al., 2010). Salivary mRNA could be a potent biomarker for early oral squamous cell carcinoma (OSCC) diagnosis and a study done by Oh *et al.*,.,2020 showed that mRNA levels of six genes (NGFI-A binding protein 2 (NAB2), cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1), nuclear pore complex interacting protein family, member B4 (NPIPB4), monoamine oxidase B (MAOB), sialic acid acetyltransferase (SIAE), and collagen, type III, alpha 1 (COL3A1)) were significantly lower in the saliva of OSCC patients (Oh SY (Maron et al., 2020). Salivary interleukin-6 (IL-6) mRNA expression was significantly higher in patients with OSCC and could be considered as a potential biomarker of OSCC (Márton et al., 2019). Exosomes have been successfully isolated from saliva and salivary exosomes could be useful tools for omics analysis due to lipids, proteins, and nucleic acids in exosomes (Adeola (Márton et al., 2020). The study by Zhong *et al.*,., 2005 investigated the expression of telomerase in saliva and it was detected positively in 75% of patients with OSCC and suggested that the telomerase in saliva could be used as an assistant marker for the disease (Zhong *et al.*,., 2005). Mitochondrial DNA mutations are useful targets to detect head and neck cancer and by sequencing alone, the study by Fliss *et al.*,.,2000 was able to detect mtDNA mutations in 67% of saliva samples (Fliss MS *et al.*,.,2000).

Noncoding RNAs as potential disease biomarkers

In addition to mRNA, noncoding RNAs such as microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), circular RNA (circRNA), and piwi-interacting RNAs (piRNAs) are present in saliva and are emerging as potential disease markers (Wong et al., 2015). The short size of these molecules makes them stable in different body fluids including saliva and is less susceptible to degradation by ribonucleases (RNases) (Majem et al., 2015). In a study performed by Zahran et al.,., 2015, miRNA was isolated from saliva and three salivary miRNAs (miRNA-21, miRNA-184, and miRNA-145) were showed as possible markers for malignant transformation in oral mucosal lesions (Zahran et al., 2015). It was identified that miRNAs (mmu-miR-140-5p, hsa-miR-374, hsa-miR-222, hsa-miR-15b, hsa-let-7g, and hsa-miR-132) were differently expressed between saliva samples of patients with a malignant tumor and benign parotid gland tumor (Matse et al., 2013). The differential expression of salivary miRNAs from Head and neck squamous cell carcinoma (HNSCC) in the Ecuadorian population was studied using PCR Arrays which identified miR-122-5p, miR-92a-3p, miR-124-3p, miR-205-5p, and miR-146a-5p were most associated (Salazar-Ruales (Matse et al., 2018). Bahn *et al.*,., 2015 compared >90 RNA-sequence data sets of different origins and observed that piRNAs were higher in cell-free saliva compared to other body fluids and miRNA expression profiles were similar to those in serum and cerebrospinal fluid (Bahn et al., 2015). piRNAs are found to be highly exclusive to saliva with very low abundance in blood or cerebrospinal fluid and indicate that salivary piRNAs might have been generated from cells in the oral mucosa or salivary glands, rather than circulating from systemic organs via blood (Lin et al., 2015).

Proteome of saliva

Protein components present in saliva include proline-rich proteins, α-amylases, mucins, salivary ("S-type") cystatins, histatins, statherin, lipocalin, and P-B peptide and are secreted from three major glands, parotid, sub-mandibular, and sub-lingual (Castagnola et al., 2017). Proteins in the whole saliva have been identified using large-scale mass spectrometry-based technologies and many of these proteins are also found to be present in the human plasma proteome, indicating that salivary proteins may also circulate and be indicators of systemic health (Griffin 2015) (Table 1). Using mass spectrometry analysis, salivary proteome was analyzed, and a set of 139 proteins along with their proteotypic peptides were identified which could serve as a reference of secretory markers for clinical applications in oral malignancies (Sivadasan et al., 2015).

Another mass spectrometry analysis of the proteome of the saliva of chronic graft-versus-host-disease (cGVHD) revealed reduction of salivary lactoperoxidase, lactotransferrin, and several proteins included in the cysteine proteinase inhibitor family suggesting impaired oral antimicrobial host immunity in cGVHD patients (Bassim et al., 2012). To identify disease-related markers in type 1 diabetes, with and without microvascular complications, the salivary proteome and peptidome profile were carried out using iTRAQ-based quantitative approach which revealed that bactericidal/permeability-increasing protein-like 1, pancreatic adenocarcinoma, alpha-2- macroglobulin, defensin alpha 3 neutrophil-specific, leukocyte elastase inhibitor, matrix metalloproteinase-9, neutrophil elastase, plastin-2, protein S100-A8, and protein S100- A9 were related with microvascular complications such as retinopathy and nephropathy (Caseiro et al., 2013).

Salivary secretions and associated diseases

Salivary analysis has become one of the important resources for monitoring health and the disease state due to its origin, composition similar to serum, and interactions with other organs. The main innate defense factors present in saliva are the peroxidase systems, defensins, lysozyme, lactoferrin, and histatins and the interactions between these factors result in synergistic inhibitory effects on bacteria and prevent the development of bacteria mediated oral diseases such as dental caries and periodontitis. There was an increase in sodium, total protein, albumin, immunoglobulin (Ig)A, IgG, IgM, amylase, lysozyme, IL-2, IL-6, and neural growth factor (NGF) in the saliva of burning mouth syndrome patients and these salivary changes were found to be associated with inflammation, dry mouth, and taste alterations in burning mouth syndrome (de Souza et al., 2015). Xerostomia occurs when the unstimulated whole saliva flow rate falls by 40-50% of its normal value and may result from changes in salivary composition or function, particularly of lubricating mucins (Pedersen et al., 2018). Sjögren's syndrome is characterized by dysfunction and destruction of the salivary and lacrimal glands and their secretory fluids, saliva and tears, reflect the pathophysiology of the disease.

The protein signature of this syndrome comprises secretory proteins, enzymes, calcium-binding proteins, abundantly expressed immune-related molecules such as β-2-microglobulin, cathepsin-D, α-enolase, cystatins, defensins, and Ig γ-light chain (Katsiougiannis et al., 2016). Sialadenitis and sialadenosis are common causes of submandibular gland swelling and include reduced salivary secretions and duct obstruction (Adhikari, Soni et al., 2020). Various cytokines such as IL-6, IL-8, IL-1a, IL-1b, TNF-a were found to be higher in oral cancer and these cytokines are proinflammatory and proangiogenic, which could be indicators of carcinogenic transformation from premalignant oral disorders (PMOD) to oral cancer (Khurshid et al., 2018).

The levels of salivary 8-hydroxydeoxyguanosine (8-OHdG) as a potential DNA damage biomarker in PMOD and OSCC were assessed and salivary 8-OHdG levels showed significant differences between cases and healthy controls indicating that salivary 8-OHdG can be used as a novel biomarker of DNA damage to assess disease progression from PMOD to OSCC (Nandakumar et al., 2020). When saliva of Down Syndrome patients was analyzed, the concentration of acidic proline rich proteins and S cystatins were found significantly reduced and levels of the antimicrobial α-defensins 1 and 2 and histatins 3 and 5 were significantly increased in the whole saliva of older Down syndrome subjects whereas S100A7, S100A8, and S100A12 levels were significantly increased in the whole saliva of Down syndrome subjects (Cabras et al., 2013). SAPHO syndrome is a rare disease characterized by synovitis, acne, pustulosis, hyperostosis, and osteomyelitis and there was a significant reduction in salivary proteins cystatin S1 and SN, histatins, the major acidic proline rich proteins, P-C and P-B peptides in SAPHO subjects (Sanna et al., 2015).

Genetic variant analysis of salivary secretions

The study by (Badea *et al.*, 2013) analyzed the genetic polymorphism of the IL-1 gene from oral swabs and the salivary level of the 8-OHdG biomarker and demonstrated that IL-1 gene polymorphism and level of 8-OHdG can be used in the evaluation of the oro-dental status of patients with aggressive periodontitis.

Table 1. Important salivary proteins and their functions

Salivary Proteins	**Function**
Mucins	Glycoproteins that protect tooth surface from demineralization, aids in lubrication and prevents bacterial adhesion
Lysozyme	Antibacterial enzyme that lyse bacterial cell wall
Lactoferrin	Iron binding glycoprotein that has bacteriostatic and bactericidal activity
Peroxidase	Eliminates hydrogen peroxide
Histatin	Inhibits bacterial enzymes
Defensins	Small cationic proteins with antimicrobial activity
Immunoglobulins Predominant is IgA	Inhibition of bacterial adherence, inactivation of bacterial enzymes and toxins
Metalloproteinases	Breakdown proteins such as collagen
Proline rich proteins	Calcium homeostasis
Statherin	Inhibits precipitation of calcium phosphate in saliva and also inhibit the growth of anaerobic bacteria
Cystatin	Protease inhibiting proteins
carbonic anhydrase VI	pH control

3 has two common haplotypes located at three sites, two in the promoter region and one in the signal peptide domain that considered which might affect the detectability of viral RNA in the saliva, such as the timing and method of sample collection, the choice of transport medium, storage, and transport temperatures (Czumbel et al., 2020).

Using salivary samples, it was possible to diagnose Dengue IgG antibody with high sensitivity and specificity (Banavar et al., 2014). HIV antibodies can be detected in saliva providing an alternative to blood to diagnose HIV infection (Balamane et al., 2020). However, the viral load could be lesser compared to blood but methods are being carried out to increase the accuracy of detection. The examination of the saliva of oral cancer patients has gained interest because of the direct contact with cancer lesions and also contains fallen cells making it a prime choice for screening. A study conducted by Dhanya & Hegde 2016 showed an increase in the level of fasting salivary glucose and a correlation between salivary glucose and serum glucose in diabetic patients and the study concluded that fasting salivary glucose level could be used as a noninvasive diagnostic and monitoring tool to assess the glycemic status of type II diabetes mellitus patients (Dhanya , Hegde et al., 2016).

Saliva could be used to monitor drug levels. Salivary therapeutic drug monitoring was investigated and levels of antiepileptic drug, perampanel, in saliva was studied which showed that perampanel concentration in saliva correlated with that in plasma (Kim et al., 2020). A meta-analysis by Rapado-González *et al.*,., 2020 showed that salivary biomarkers may be potentially used for the non-invasive diagnosis of malignant non-oral tumors and several biomarkers detected in saliva were able to discriminate cancer patients from healthy individuals with a significant degree of sensitivity and specificity. Higher levels of *c-erb-2*, a receptor tyrosine kinase, were found in the saliva of patients with breast cancer when compared with patients with benign lesions (Streckfus et al., 2000).

Conclusion and Future Perspective

Saliva, with its rich and diverse molecular composition, has emerged as a valuable tool in disease diagnostics and biomarker discovery. Its non-invasive collection method, cost-effectiveness, and ease of handling offer significant advantages over traditional diagnostic fluids like blood. The presence of various biomarkers in saliva—ranging from proteins and nucleic acids to noncoding RNAs—provides a comprehensive picture of both oral and systemic health. Saliva's potential extends beyond mere diagnostics, encompassing genetic analyses, disease monitoring, and even forensic applications.

Recent advancements in salivary diagnostics highlight its role in detecting a wide array of conditions, including oral diseases, systemic illnesses, and even cancers. For instance, salivary DNA and RNA analyses have demonstrated their efficacy in identifying genetic predispositions and early biomarkers for diseases such as oral squamous cell carcinoma and type 1 diabetes. Similarly, the proteomic and genetic profiling of saliva has uncovered novel biomarkers and insights into various health conditions, from Sjögren's syndrome to cancer.

Despite its advantages, the use of saliva in routine diagnostics faces challenges, primarily due to the lower concentration of some biomarkers compared to other biological fluids. Nonetheless, ongoing research and technological advancements are addressing these limitations, enhancing the sensitivity and specificity of salivary tests. Saliva's role in therapeutic drug monitoring and infectious disease detection, including its potential use in diagnosing COVID-19, further underscores its versatility and promise.

In conclusion, saliva stands as a powerful, non-invasive medium for disease diagnosis and health monitoring, with the potential to revolutionize the field of medical diagnostics. Continued research and development will likely expand its applications, making it an indispensable tool in modern medicine.

Author contributions

U.S. led the research conceptualization, design, and analysis. H.V.S. played a pivotal role in data collection, interpretation of results, and manuscript drafting. S.K.S., L.S. contributed significantly to the methodological framework and critically reviewed the manuscript for important intellectual content. All authors actively participated in discussing the results, refining the manuscript, and approved the final version for submission.

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Competing financial interests

The authors have no conflict of interest.

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