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Exploring newer avenues in diagnostic utility of saliva: A review article

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Abstract
This article explores the potential of saliva as a valuable diagnostic tool in healthcare. It highlights two key prerequisites for achieving non-invasive physiological state assessment and disease monitoring through saliva-based diagnostics the discovery of relevant biomarkers within saliva's complex components and the continuous advancement of technology to enhance biomarker sensitivity and specificity. The review can further update clinicians on recent advances in salivary biomarkers for diagnosing various conditions, such as autoimmune diseases, cardiovascular diseases, diabetes, HIV, oral cancer, caries, and periodontal diseases, highlighting their accuracy, efficacy, ease of use, and cost-effectiveness. The article anticipates that with the development of sensitive and specific salivary diagnostic tools and established guidelines, salivary diagnostics will become readily available for chair-side testing of both oral and systemic diseases in the near future. Certain salivary biomarkers can potentially be helpful in combination and singularly for the diagnosis of periodontal disease. However, further meticulously robust research is required to validate these biomarkers.

Keywords: Biomarker, saliva, diagnostic tool, oral disease, systemic disease, oral cancer

Introduction

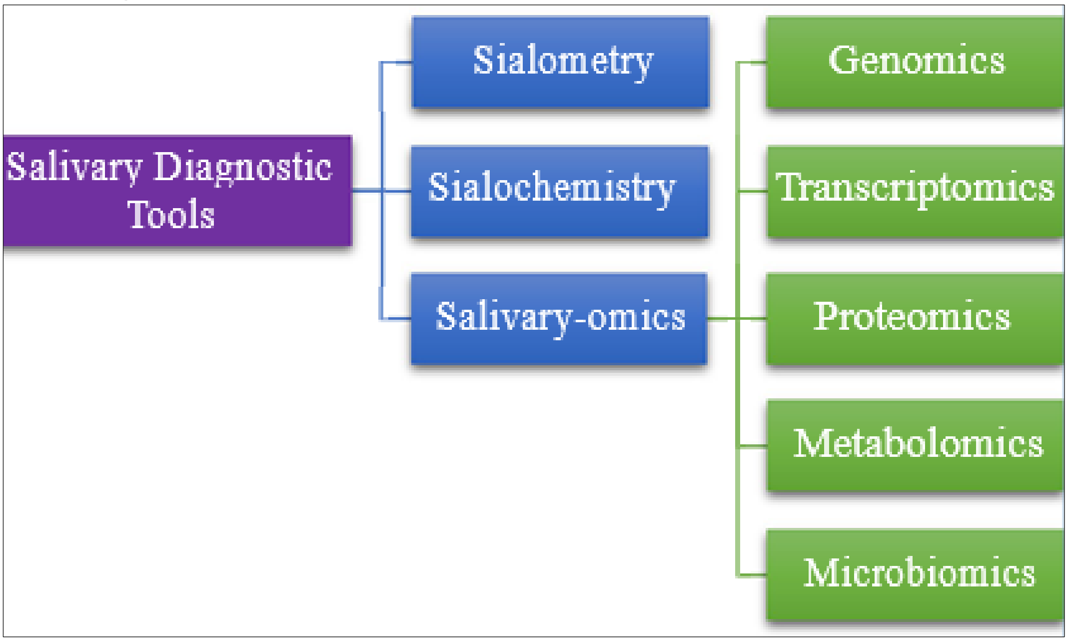
Saliva has been recognised as a diagnostic fluid for over two millennia and contains a diverse range of components, including hormones, antibodies, enzymes, and microbes that enter it through various mechanisms1. Salivary diagnostics holds immense potential for early disease detection, prognosis, and therapy monitoring in conditions like cancer, cardiovascular, metabolic, and neurological diseases [2]. Early detection is crucial for successful treatment and reducing disease severity and complications. Saliva's ease of access and rich biomarker content make it an attractive alternative to blood for various tests, including Alzheimer's and stress monitoring, especially in specific populations or emergency situations where blood collection is impractical. Differentiating between whole saliva and gland-specific saliva can aid in detecting gland-specific issues like infections or obstructions [3, 4, 5]. Out of various methods existing for the collection of whole saliva, common methods include the draining method, the spitting method, the suction method, and the swab method. Similarly, several commercially available devices and methods can be used to collect saliva from individual glands6. Saliva secreted from individual glands is less contaminated by food debris and micro-organisms, making it more suitable for diagnostic purposes [7].

Salivary samples should be snap frozen in liquid nitrogen after collection in ice-cooled vessels, with storage at -80 °C or below for prolonged periods8. When designing a protocol for handling salivary samples, it is crucial to consider the intrinsic properties of the biomarker and the diagnostic purpose [9].

Salivary Diagnostic Tools

Saliva is increasingly recognized as a mirror of the body, displaying nearly 1000 proteins and 19,000 unique peptide sequences. It has been used as a liquid biopsy for detecting oral diseases like dental caries, gingivitis, periodontitis, Bechet disease, and systemic diseases like breast cancer, diabetes, and HIV. Various tools for saliva diagnostics are available [10].

Sialometry is a vital tool for volume measurement in saliva, detecting hyposalivation, a common disorder affecting 20% of the general population. It is the first tool to detect this disorder, and conventional therapies for reduced saliva flow are limited.



Sialometry can be used to assess salivary flow in standard conditions, with techniques such as collecting whole saliva or saliva from specific glands. The choice of technique depends on the patient's condition and the presence of stimuli [11].

Sialochemistry examines salivary electrolytes and proteins, revealing abnormal functions of salivary glands, gland dysfunction, and homeostatic fluctuations. Salivary analysis can be used to monitor patient compliance with medications, monitor anti-epileptic drugs, and assess antibodies to HIV. Both sialometry and Sialochemistry are complementary tests for assessing patients with primary Sjogren's syndrome in clinical practice. However, their diagnostic value has been criticised for its specificity and lack of standardization [12, 13].

Salivaomics explores various molecules in saliva, including proteins, mRNAs, miRNAs, metabolic compounds, and microbes. Salivary biomarkers are classified based on their mechanism of action, offering potential for presymptomatic diagnosis and continuous assessment of disease status [14].

- **Salivary Genomics and Epigenomics:** The salivary genome consists of both human and microbial DNA. A proportion of roughly 30% originates from the oral microbiota (and viruses, if any). Saliva samples yield sufficient DNA for sequencing arrays and polymerase chain reaction (PCR) assays; and the quality of salivary DNA is comparable to that in blood [13, 15].
- **Salivary Transcriptomics:** The salivary transcriptome, composed of coding and noncoding RNAs, provides valuable diagnostic information. Over 1,000 miRNAs and 3,000 species of mRNAs are found in saliva, with miRNAs playing a crucial role in cancers like lung, pancreatic, and breast cancer [16].
- **Salivary Proteomics:** Salivary proteomics is the study of the entire protein content of the oral cavity, comprising over 2000 proteins and peptides involved in various biological functions. Proteomic analysis in saliva offers advantages over blood due to the even distribution of distinct salivary peptides. The presence of salivary proteomic markers in low concentrations plays a major role in disease diagnosis. The analysis of salivary proteomes may reveal morbidity signatures and monitor disease progression [13-17].
- **Salivary Metabolomics:** The metabolome validates the parallel assessment of a group of endogenous and exogenous metabolites, including lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols, and carbohydrates, and is a valuable tool for discovering biomarkers, monitoring physiological status, and making proper treatment decisions [13, 16].
- **Salivary Microbiomics:** It is a crucial aspect of oral health and is influenced by the presence of 700 to 1,200 bacterial species in the mouth. NGS suggests that this number could reach 10,000, providing insights into disease pathogenesis, diagnosis, monitoring, and prognosis. Early detection of disease-specific microbiological signatures can aid in therapeutic interventions, potentially inhibiting pancreatic cancer progression. The combination of multiple biomarkers, including nucleic acids, proteins, and metabolites, can enhance diagnostic accuracy and improve therapeutic outcomes [13, 16].

Liquid biopsy

1. It is a non-invasive method used to diagnose physiological situations and inflammatory processes, providing a valuable tool for detecting molecular biomarkers in cancer patients. It is a complementary or alternative method to surgical biopsy, overcoming challenges in clinical evaluation due to tumour tissue accessibility and clonal heterogeneity.
2. Circulating tumour cells (CTCs) are cancer cells that break away from the primary tumour and enter the bloodstream, providing a powerful tool for detecting and monitoring cancer and metastases. Cell-free tumour DNA (ctDNA) represents the tumour in its entire heterogeneity, is easily obtainable from a blood sample, and is repeatable. CtDNA offers information on the genetic profile of the primary lesion and metastases and

is detectable in blood, urine, saliva, breast milk, and synovial fluid [17].

3. Extracellular Vesicles (EVs) and molecules, DNA, and RNA are potential analytes for liquid biopsies18. These fluids can provide dynamic assessments of tumour genomes and provide personalised snapshots of primary and metastatic tumours at successive time points. Recent studies have explored the potential of saliva as an additional medium for detecting tumor biomarkers, offering a personalized snapshot of primary and metastatic tumors at successive time points and providing a molecular profile for each patient [13, 16].

Salivary biomarkers for oral diseases

Dental Caries: Caries risk assessment can be done by two methods:

1. Analysing the Levels of Cariogenic Bacteria in Saliva;
2. Caries Risk Assessment by Analysing Host-related Factors in Saliva, Like Salivary Flow Rate, Saliva pH and Buffering Capacity, Salivary Electrolytes, and Salivary Proteins (Proteomes) [19, 20].

Periodontitis: Six clusters of markers released during the immune response may be eligible as biomarkers for periodontitis, which include host-derived enzymes, tissue breakdown products, host response modifiers, cytokines, oxidative stress markers, and specific and non-specific salivary proteins. Biomarkers for peri-implantitis include pro-inflammatory cytokines, anti-inflammatory cytokines, osteoclastogenic cytokines, antioxidant proteins, and chemokines. Early diagnosis and follow-up using salivary biomarkers could help prevent and treat the disease [21].

Oral Potentially Malignant Disorders

The direct contact of saliva with oral cancer lesions, in addition to the presence of fallen cells of the oral cavity in saliva, makes salivary analysis the optimal diagnostic tool for searching for and identifying possible biomarkers for oral cancer detection [22].

- The combination of multiple mRNAs and miRNAs could be regarded as more dependable than just a single biomarker. A group of overexpressed miRNAs (miRNA-181b, miRNA-21, miRNA-345, miRNA-518b, miRNA-140b, miRNA-184, miRNA-520 g, and miRNA-649) were proposed to denote early events in oral carcinogenesis [21].
- Salivary levels of phenylalanine, valine, and lactic acid were reported to be the best forecasters for distinguishing oral cancer from healthy controls and for distinguishing oral cancer from premalignant lesions such as leukoplakia. Moreover, the mixture of these three metabolites could discriminate oral leukoplakia and oral cancer from controls with high sensitivity and high specificity [21].
- Nucleatum and Porphyromonas gingivalis, HPV, and EBV have been reported in salivary samples of the OSCC patients, indicating that analysis of these salivary biomarkers could be a useful diagnostic and prognostic indicator of oral OPMDs and cancer.

Oral Squamous Cell Carcinoma

The biological entities expressed in the serum or saliva that consequently lead to oral cancer may serve as biomarkers for oral cancer detection. The direct contact between saliva and oral cancer lesions makes the measurement of tumour markers in saliva an attractive alternative to serum and tissue testing. The DNA, RNA, and protein molecules derived from living cancer cells can be conveniently obtained from saliva [23].

These molecular markers for diagnostic purposes can be determined at three levels changes in the cellular deoxyribonucleic acid (DNA), which results in altered mRNA transcripts, followed by alterations in protein levels [24].

Mutations of p53, DNA mutations, cyclin D1 gene amplification, microsatellite alterations of DNA, Loss of heterozygosity (LOH), The presence of HPV and EBV genomes is a recognised possible DNA biomarker that could be used for the diagnosis and prognosis of oral cancer [21].

Salivary levels of miR-125 and miR-200 (tumour suppressors) were shown to be reduced in patients with oral cancer compared to healthy individuals. Several salivary proteomics, such as transforming growth factors, interleukins, Ki67, matrix metalloproteinase (MMP 2, 9), transferrin, cyclic D1, Cyfra 21.1, tumour necrosis factor-α, α amylase, and catalase, have been detected in oral cancer in several investigations. Metabolites like cadaverin, alanine, choline, taurine, piperidine, piperolic acid, alpha-aminobutyric acid, etc. were found to be potential salivary biomarkers for oral squamous cell carcinoma [25].

Recently, advancements have been reported in biomarker detection, such as the detection of cancer-derived exosomes. The cancer-derived exosomes are reported to represent the tumour microenvironment and, thus, can effectively be used as a biomarker for tumour detection.

The combination of multiple biomarkers could include nucleic acids, which are highly discriminatory, proteins, and small molecules like metabolites. With the advent of these salivary diagnostics, it has been an effective and promising modality for early diagnosis, prognostication, and post-therapy status monitoring in oral cancer [26].

Salivary biomarker for diagnosis of systemic diseases

Systemic Malignancies

Pancreatic Cancer: The top 5 miRNA candidates (miR-17, miR-21, miR-181a, miR-181b, and miR-196a) were differentially expressed in the saliva samples of pancreatic cancer patients compared to controls and validated by qRT-PCR. In some studies, it was found that decreased abundances of N. Elongata as well as a significantly higher ratio of Leptotrichia to Porphyromonas were detected in the saliva of patients diagnosed with pancreatic cancer compared to healthy controls or those with other diseases. These reports open a new venue for salivary microbiota to serve as an informative source for discovering non-invasive biomarkers for systemic diseases [13].

Breast Cancer: In a recent article, salivary autoantibodies against both HER2 and MUC-1 have been shown to be useful candidates for early detection of breast mRNA (CSTA, TPT1, IGF2BP1, GRM1, GRIK1, H6PD, MDM4, S100A8) in saliva that can be used for the detection and identification of breast cancer 28.

Lung Cancer: Valuable markers LC have been discovered in saliva, including metabolic (catalase activity, triene conjugates, and Schiff bases), inflammatory (interleukin 10, C-X-C motif chemokine ligand 10), proteomic (haptoglobin, zinc-α-2-glycoprotein, and calprotectin), genomic (epidermal growth factor receptor), and microbial candidates (Veillonella and Streptococcus). In combination with each other and with other established screening methods, these salivary markers could be useful for improving early detection of the disease and ultimately improving the survival odds of lung cancer patients [29].

Ovarian cancer: The gold standard serum biomarker CA125, often called mucin 16 (MUC16), is the single most biomarker approved by the US FDA for early diagnosis of ovarian cancer and disease monitoring. This highly recommended serum biomarker is also found in saliva and gives a better diagnostic value with high specificity, diagnostic efficiency, and PPV than the serum CA125 assay in ovarian cancer patients. Therefore, the trend of evaluating CA125 levels from saliva through a non-invasive approach for the screening of ovarian cancer would be a great initiative in this direction [30].

Multiple Sclerosis: Diagnosing multiple sclerosis usually relies on clinical examination, MRI scans, evoked potential testing, and analysis of CSF31, 32. The concentration of HLA class II is significantly elevated in the saliva of patients with relapsing remitting MS (RRMS) compared to healthy controls, opening a possibility of non-invasive testing for the disease [33].

Cardiovascular Diseases

- Approximately 27% of the whole saliva proteins resemble those found in plasma, similar proteins present in both saliva and plasma. This will be very useful to facilitate monitoring of both disease progression and therapeutic treatments among these patients. Out of all these proteins, natriuretic peptides, C Reactive Protein (CRP), creatine kinase (CK), and cardiac troponin were included as commonly used cardiac biomarkers in acute cardiac care.
- Literature has been published on the importance of salivary biomarkers in the diagnosis of CVDs, which includes Myoglobin (MYO), cardiac Troponin-I (cTn-I), Creatine phosphokinase MB (CK-MB), Myeloperoxidase (MPO), Brain Natriuretic Peptide (NT-proBNP), Exosomal miRNA, C Reactive Protein (CRP), Matrix Metallo Proteinase-8 (MMP-8), and tissue inhibitor of MMP-8 (TIMP-1). Many inflammatory components and adhesion molecules have also been identified as salivary biomarkers for the diagnosis of AMI, including IL-6, MMP-9, and soluble intercellular adhesion molecule (sICAM-1), a soluble form of CD40 ligand (sCD40-L), CRP, cTnI, and adiponectin34.

Diabetes Mellitus

Various studies have concluded that the concentration of glucose in saliva increases with an increase in serum glucose concentration and vice versa. A significant correlation was seen between salivary and serum glucose levels in diabetic as well as non-diabetic subjects.

Levels of adiponectin, an anti-inflammatory adipokine that regulates glucose, in saliva and serum showed a significant correlation, confirming their association with each other. Data from this study suggest that salivary adiponectin may be combined with serum adiponectin in the field of research for clinical conditions such as obesity, insulin resistance, energy imbalance, etc.

A strong association between glycemic index and saliva levels of Alpha-2-macroglobulin in diabetes mellitus type 2, indicating that Alpha-2-Macroglobulin can be used as a diagnostic method for diabetes with high specificity. Other useful markers include leptin, CRP, 1, 5-Anhydroglucitol, IL6, etc. [35, 36].

Infectious Diseases

Bacterial Infections: For pneumococcal pneumonia, the detection of pneumococcal C polysaccharide in saliva by ELISA may offer a valuable complement to conventional diagnostic methods for pneumococcal pneumonia. Lyme disease is caused by the spirochete Borrelia burgdorferi. The detection of antitick antibodies in saliva serves as a screening mechanism for individuals at risk for Lyme disease [4].

Parasitic Infestations: For malaria, IgG antibodies directed against specific Plasmodium falciparum antigens can be detected in saliva and were found to correlate strongly with levels in plasma [37].

Fungal Infections: Saliva may also be used for the detection of oral fungi. In the case of oral candidiasis, salivary fungal counts may also reflect mucosal colonization. Alteration of the salivary proteome related to proteins showing antifungal properties like immunoglobulin, calprotectine, histatin-5, mucin peroxidases, basic prolin-rich proteins, molecular chaperone Hsp70, etc. may also have important diagnostic and prognostic value, especially in recurrent cases [38].

Viral Infections: Acute hepatitis A (HAV) and hepatitis B (HBV) were diagnosed based on the presence of IgM antibodies in saliva. Hepatitis B virus DNA was revealed by PCR testing of saliva.

Saliva may also be used for determining immunisation and detecting infection with measles, mumps, and rubella [37]

Salivary levels of anti-dengue IgM and IgG demonstrated sensitivity of 92% and specificity of 100% in the diagnosis of infection. So, detection of dengue-specific salivary IgG and IgM antibodies is a useful marker for dengue infection.

Salivary diagnostic tests are available for HPV, and essentially, they involve the use of PCR; thus, they are not POC tests. Investigators in the field have used oral swabs, expectorated saliva, or an exepctorated oral rinse with mouthwash. Sufficient evidence has been generated in the review related to salivary specimens in SARS-Cov-2 for viral replication, longevity, sensitivity, specificity with other related viruses, and practicality in the collection of specimens. Tracking viral load can be vital in monitoring SARS-CoV-2 infections, risk assessment for infectivity, morbidity, clinical prognosis, and mortality [3].

Therapeutic drug monitoring and hormone level monitoring saliva is an alternative matrix for the therapeutic drug monitoring of Carbamazepine, Phenytoin, Primidone and Ethosuximide because the concentrations of these medications in saliva reflect the concentrations of the drug in serum40. A fundamental prerequisite for this diagnostic application of saliva is a definable relationship between the concentration of a therapeutic drug in blood and the concentration in saliva. Only the unbound fraction of the drug in serum is available for diffusion into saliva. The unbound fraction of a drug is usually the pharmacologically active fraction. This may represent an advantage of drug monitoring in saliva in comparison with drug monitoring in serum, where both bound and unbound fractions of a drug can be detected [4].

Hormone monitoring Substrates monitored

Salivary androgens, salivary Progestins and Oestrogens, salivary Glucocorticoids, Aldosterone, Insulin, etc. The application of hormone monitoring is in fields like Psychobiology, Sports Medicine, Pharmacology studies, and Pediatric studies. In the diagnostic laboratory, salivary progesterone and oestradiol have been used for assessing ovarian function and 17a-OH progesterone for the diagnosis of congenital adrenal hyperplasia (CAH). Salivary cortisol is used for investigating adrenal function, and recently there has been considerable interest in the use of bedtime salivary cortisol levels as a screening test for Cushing's disease [41].

Salivary diagnostic test for forensic investigation

Saliva can be used for various forensic applications, like the analysis of drugs of abuse in saliva, cigarette consumption detection, organic substance intoxication detection, heavy metal intoxication detection, gender determination, bitemark analysis, blood group antigen detection, etc. [42].

Point of care testing

It is defined as medical testing conducted outside of a laboratory at or near the site of patient care, including the patient's bedside, the doctor's office, and the patient's home.

A self-monitoring blood glucose, coagulation (INR), and pregnancy testing kits using urine samples are well-known examples of PoCT and have become over-the-counter products to be sold on the market. Saliva is predicted to be a substitute for blood, collected non-invasively for the diagnosis of oral and systemic diseases. Thus, PoCT replaces the specialist testing centres by using samples other than blood and urine. For the development of PoCT devices, the minimum risk of infection with no mental or physical pain is of utmost importance to consider, in addition to automation, integration, multiplexed detection ability, quick analysis, a small sample size, and minimal training as the primary goals of modern medicine [43].

Conclusion

Salivary diagnostics is a rapidly advancing field with the potential to revolutionise disease diagnosis and monitoring. It offers the ability to assess disease status non-invasively, even before symptoms appears, making it a promising tool for healthcare. Salivary tests need to be cost-effective, easy to use, and have applications in clinical and basic sciences, including population-wide disease screening. Despite of many challenges, the emergence of sensitive and specific salivary diagnostic tools and established guidelines is expected to make salivary diagnostics a reality in the near future.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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