ORIGINAL RESEARCH PAPER

Dental Science

SALIVA AND SALIVAOMICS IN THE DIAGNOSIS OF ORAL AND SYSTEMIC DISEASES: A REVIEW

KEY WORDS: Biosensors, Proteomics, Saliva, Salivaomics

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ABSTRACT

The quest for novel strategies in early disease detection and response to therapy is an essential ongoing process in health care setups. Along with other body fluids such as blood, mucus, urine, semen and vaginal fluids; saliva can also be considered for the detection of the disease. The Salivary diagnostics is a dynamic field that is being incorporated as part of disease diagnosis, clinical monitoring and for making important clinical decisions for patient care. This review presents the translational value of saliva as a credible clinical diagnostic biofluid in detection, early detection of the various diseases and response to treatment.

INTRODUCTION

Mandel (1990) eloquently stated: "saliva is not one of the popular bodily fluids. It lacks the drama of blood, the sincerity of sweat and the emotional appeal of tears". However, this unpretentious secretion is a perfect medium that can be explored for health and disease surveillance.

The idea of using saliva in diagnostics was made in the second half of the 20th century with the two main objectives: early detection of certain diseases and monitoring the disease course in conjunction with treatment. A third objective can be implicated in the detection of addictive drugs or substances.[1]

Physiology and composition of saliva.

Saliva is a watery substance produced in the oral cavity of and constitutes about 98 % of water, while the remaining 2 % are electrolytes (Na, K, Ca, Mg, hydrogen, carbonates), glycoprotein, antibacterial component such as certain types of immunoglobulins and lysozyme, and various enzymes like α-amylase, lingual lipase.

However, the oral fluid is a complex mixture of primarily the secretory products from both major and minor salivary glands and also the secretions from the oropharynx, aerodigestive tracts and gingival sulcus admixed with food and blood derived compounds.[2]

The submandibular gland produces 70 % of the overall volume, the parotid gland 25 %, and the sublingual gland about 5 % with insignificant contribution from the minor salivary glands. Normal unstimulated whole saliva secretion ranges from 800- 1500 ml/day or 1.0 to 3.0 ml/minute with a pH of 6-7.[3]

Saliva plays a vital role in maintaining the oral health via lubrication, digestion, physical protection, cleansing, antibacterial action, gustation, buffering, maintenance of pH and tooth integrity (Table1).

Table 1: Functions and composition of saliva.

Function	Components
Lubrication: Assists air flow, speech and swallowing **Physical protection:** Coats, protects against mechanical, thermal, chemical irritation.	Mucin, glycoproteins
Cleansing: Moistening assists mastication, clearing food and swallowing	
Ionic reserve: Modulates demineralization and remineralization of teeth	Calcium phosphate, statherins, proline-rich proteins
Buffering: Modulates pH of biofilm and buffering capacity of Saliva	Bicarbonates, phosphates, urea
Antibacterial action: Immunological agents and non-immunological agents help control oral microflora	IgA, IgG, IgM proteins, mucins, peptides and enzymes (lactoferrin, lysozyme, peroxidase)
Pellicle formation: Proteins form a protective layer on the teeth	Macromolecular proteins, statherins, histatins, cystatins, proline-rich proteins
Digestion: Enzymes in saliva begin the breakdown of starch and fat	α-amylase, lingual lipase
Gustation: The solvent action and hypotonicity of saliva enhance tasting capacity by allowing interaction between nutrients and taste buds	Protein, gustin, zinc

Why saliva can be used as a diagnostic fluid?

Saliva like serum also contains the biochemical substances such as hormones, enzymes, antibodies, growth factors and many of which enter the saliva from blood through passive diffusion, active transport and extracellular ultrafiltration. Further it has the ease of collection which is inexpensive and non-invasive and multiple samples can be obtained with minimal risk of cross-contamination. Manipulation required

during diagnostic procedures is also would be less as compared to serum and it does not clot unlike blood. Saliva provides real-time diagnostic values and is more economical in terms of sampling, shipping and storage.[4]

Collection of the Saliva

Successful measurement of salivary parameters requires proper preparation of the subject and storage protocols as some of the important pre analytical steps. Various methods of collection of the saliva/ oral biofluid include:

- Draining, Spitting, Suction and Swab method for Whole saliva
- Suction and Cannulation method for Parotid saliva
- Suction, Cannulation and Segregator method for Submandibular /Sublingual secretion

Recent techniques of collection of saliva are mostly modifications of the expectoration methods of which Oragene, Saligene, Oracol and Verofy are a few[3]

Salivaomics for different diseases:

Identification and application of saliva-based "omics" biomarkers may overcome painful invasive procedures currently being used for the diagnosis of oral cancer. The term "Salivaomics" was coined in 2008, to indicate the research on the constituents of saliva which could be used as salivary biomarkers.[6] (Table 2) Salivaomics includes five diagnostic alphabets: proteins, messenger RNAs, micro-RNAs (mi-RNAs), metabolic compounds, and microbes which offers substantial advantages for salivary diagnostics.[5]

Table 2: Salivary Biomarkers and their applications.

Saliva/Oral biofluid Biomarkers	Applications
DNA	In Standard genotyping, Identifying Bacterial infection, Diagnosing carcinomas of the head and neck, In Forensics for personnel identification
RNA	Viral/bacterial detection & identification & hence diagnosing the underlying disease, Diagnosing Carcinomas of the head and neck
Proteins	In Diagnosing carcinomas of the head and neck, diagnosing periodontitis, Detecting dental caries
Mucins/glycoproteins	In Diagnosing carcinomas of the head and neck, Detecting dental caries
Immunoglobulins	In Diagnosing viral infections (HIV, hepatitis B and C)
Metabolites	In Diagnosing periodontitis
Drugs and their metabolites	In Monitoring drug abuse, Detection of drugs in the body

Genomics

The saliva DNA yield (7.8 g/0.5 mL saliva sample) using the manual purification method was comparable to the DNA yield from blood by the salt precipitation method (7.4 ug/0.5 mL blood sample).[7] Moreover, samples could be stored long-term without significant degradation. Salivary genetic and epigenetic analyses can provide gene-transcription profiles that could reflect abnormal pathological genetic processes.

Transcriptomics

The human salivary transcriptome consists mainly of mRNA and microRNA (miRNA). Other extracellular RNA molecules that function as salivary biomarkers include piwi interacting RNA (piRNA), circular RNA (circRNA), and small nucleolar RNA (snoRNA).[8] These salivary RNA biomarkers have been used to detect periodontal disease, Sjögren's syndrome, oral cancer, lung cancer, breast cancer, ovarian cancer, and pancreatic cancer.

Salivary mRNAs, as well as endogenous miRNAs, are protected from ribonucleases by exosomes in the saliva and are therefore quite resistant to degradation. However, research into salivary miRNAs has become an emerging field because these miRNAs are more stable than mRNAs, and it is relatively easy to distinguish the miRNA expression levels between healthy and abnormal cells. The five most abundant salivary miRNAs are miR223, miR-191, miR-16, miR-203, and miR-24.[9]

Proteomics

More than 3000 proteins have been detected in human saliva, and many have been used as biomarkers for disease detection. Elashoff et al. identified several proteomic biomarkers for oral cancer detection, including IL-1 beta and IL-8.[10] Rai et al. showed that adenosine deaminase might be used as a diagnostic tool for early detection of squamous cell carcinoma of the tongue.[11] Sweet et al., demonstrated calprotectin might aid in the diagnosis of oral candidiasis.[12] In 2004, a multi-institutional, multidisciplinary research consortium was initiated and funded by the National Institute of Dental and Craniofacial Research (NIDCR) to generate a complete catalog of all salivary secretory proteins (Human Salivary Proteome Project). This collaborative endeavor yielded 1,166 identifications in ductal fluid: 914 in parotid and 917 in submandibular/sublingual saliva.[13]The typical protein concentration of saliva is 0.7–2.4 mg/mL.[14]

Metabolomics

Cells produce a multitude of metabolites as the final products of cellular biochemical processes like gene transcription, mRNA translation, protein synthesis, and metabolic enzymatic reactions. The comprehensive identification, quantification, and analysis of these metabolites are known as "metabolomics." Techniques such as the combination of capillary electrophoresis (CE) and a time-off light mass spectrometer (TOFMS), allow the detection of various metabolites and only require a small sample. Studies of the oral metabolome using CE-TOFMS may provide researchers and clinicians with insights regarding the diagnosis of oral diseases in specific sites as well as the clinical effects of various therapeutics in the oral cavity.[15]

Exosomics

Exosomes are 30–100nm cell derived vesicles released by most cells in the body upon the fusion of multivesicular bodies (MVBs) with the plasma membrane. These vesicles have essential biological functions, including intercellular signaling and macromolecular trafficking. The content of these microvesicles includes RNAs, lipids, proteins, immunoglobulins, enzymes. Recent studies suggest that salivary exosomes play important role in oral cancers and have the potential of becoming biomarkers.[16] This may obviate the limitations of the whole saliva, including sample contamination and the presence of proteins such as amylase that can mask other proteins with low expression.

Salivary biomarkers for Specific Diseases

Table 3: Salivary biomarkers for various diseases

Diseases	Salivary markers
Dental Caries and Demineralization Diseases	↓ salivary flow rate ↓ buffering capacity ↓ sugar clearance rate ↓ pH.
Periodontal Diseases and Peri-Implantitis	↑ Matrix metalloproteinase-8 (MMP-8), ↑ Interleukin-1 beta (IL-1 beta), and interleukin-6 (IL-6), ↑ Macrophage-activating factor (MAF), ↑ Macrophage inflammatory protein 1 alpha (MIP-1 alpha), ↑ Myeloperoxidase (MPO) and lactoferrin, ↑ C-reactive proteins (CRP), ↑ RANKL/ OPG ratio

Myocardial infarction	↑ Cardiac troponins I (TnI) and T (TnT), ↑ Creatine kinase-MB, ↑ C-reactive proteins (CRP), ↑ TNF-α, ↑ MMP-9 and ↑ Myeloperoxidase ↑ Myoglobin
Oral Cancers	Oncogenes (C-myc, C-Fos, C-Jun), Cytokines (TGF-Beta, IL-8, IL-1 Beta), Extracellular Matrix Degrading Protease (MMP-1, MMP-2, MMP-3, MMP-9), Hypoxia Markers (HIF - Alpha, CA - 9), Epithelial Tumour Factors CYFRA 21–1(a fragment of cytokeratin 19), Cytokeratin (CK 13, 14, 16), Micro RNA Molecules, Hypomethylation of cancer-related genes (p16, DAP - K), salivary defensin-1, etc. have been found to contain individual histone and miRNA alterations that are HNC specific ↓ miR - 125a, miR – 200 (down regulation)
Risk of oral mucositis in HNC patients	↑ EGF (epidermal growth factor), ↑ CRP (C-reactive protein), ↑ TNF-alpha (tumor necrosis factor alpha), and ↑ ESR (erythrocyte sedimentation rate)
Breast cancer	Overexpression of Glycoprotein CA 15-3
Lung cancer	Overexpression of HP, AZGP1, human calprotectin
Gastric cancer	Expression of Proteins 6556.81 Da and 7081.17 Da
Oral Lichen Planus	↓ Cystatin SA ↑ Chain C of the complement system and Chain B found in D -a fragment of fibrinogen
Sjögren's Syndrome	↑ Actin, alpha-actin-1, Ig gamma-1 chain C region, B2-microglobulin, Ig receptor polymeric salivary amylase, lysozyme C, carbonic anhydrase VI, cystatin C, polymeric ↑ Ig receptor, prolactin-inducible protein, cystatin SN, calgranulin A and B, fatty acids protein binding, anti-transglutaminase, anti-histone, anti-SSA and anti-SSB. Sodium, chloride, IgA, IgG, lactoferrin, albumin, soluble IL-2 receptor
C liac disease	Tissue anti-transglutaminase (TTG) antibodies
Cystic fibrosis	↑ Prostaglandin E2, ↑ Lipids, Electrolytes, Urea, Uric acid, Total protein (from the Submandibular salivary gland). Unusual form of EGF ↑ Calcium and Phosphate levels (In children)
Bone turn over markers	↑ Salivary osteonectin, ALP activity
Forensic evidence	Blood group antigens, DNA testing
Cushing's Syndrome	Cortisol is elevated
Diabetes mellitus	↑ Melatonin, ↑ Oxidized glutathione, Cysteine glutathione disulphide, ↑ Amino acids, ω-3 fatty acid (docosapentaenoate) and ω-6 fatty acids (linoleate and arachidonate), ↑ Glucose, ↑ α-hydroxybutyrate
Stress	↑ Salivary α -amylase

The clinical use of saliva testing occurred as early as 1836 in patients with bronchitis.[17] More recent studies have focused on detection of steroid hormones and antibodies in the saliva. Determining hormone levels, including estradiol (indicator of premature birth and low birth weight babies), progesterone and testosterone, DHEA in saliva is possible.[13] (Table 4)

Table 4: Detection of various drugs and medications in saliva

Substances	Characteristics of salivary detection
Drugs	
Alcohol	Salivary concentration of ethanol is 9% greater than in plasma.
Nicotine	Presence of metabolites: cotinine and 3-hydroxycotinine.
Cannabis	Salivary THC(Delta-9-tetrahydrocannabinol) is detectable very early and remains for up to 14h.
Cocaine	Salivary levels remain high up to 1hour after administering the drug. (Similar to blood levels).
Amphetamine	Detectable 10 minutes after administration for up to 72hours.
Methamphetamine	Detectable up to 8.1–11.1 hours after being inhaled, smoked and ingested.
Heroine	Salivary levels reach max concentration after 2 minutes (similar to blood levels).
Medication	
Barbiturates	Highest salivary concentration 1h after administration and remains stable for 50 hours.
Benzodiazepines	Nordiazepam, oxazepam, appears in saliva 45 minutes after administration.
Codeine	Detected in saliva 1 hour after being taken (concentration in saliva 3-4 times greater than in plasma)
Morphine	Detected in saliva soon after parenteral administration.

Saliva based biosensors

Biosensors are small, self-contained analytical devices used for the detection and measurement of a particular substance (analyte) of interest[18] Wide range of diseases can be diagnosed with different biomarkers and biosensors. (Table 5)

Table 5: Salivary biosensors for various diseases

Disease	Target/biomarker	Biosensor
Caries [19,20]	*Streptococcus mutans* Saa	Fiber-optic biosensor sAA biosensor
Periodontitis [21]	IL-1β MMP-8	Lab-on-a-chip multiplex biosensor
Oral cancer [22,23,24]	IL-8 EGFR mi-RNA	Optical protein sensor Nano-biochip cellular analysis sensor Electrochemical biosensor
Diabetes mellitus [25]	Glucose	Salivary nano biosensor
HIV [26]	Immunoglobulin	Electrochemical peptide-based sensors
Stress induced disorders [27]	Cortisol	Flow filtered ported SPR biosensor
Cardiovascular disease [28,29]	CRP Lactate	Microchip assay biosensor Electrochemiluminescence biosensor
Hyperphosphatemia in chronic renal failure [30]	Phosphate	Amperometric biosensor

Obesity [30,31]	Phosphate Uric acid	Amperometric biosensor Mouth guard biosensor
Breast cancer [32,33,34]	ATP6AP1 CA15-3, CEA CA125 HER-2/neu(c-erbB-2)	Quartz crystal biosensor Surface plasma resonance biosensor Multiplexed microfluidic biosensor

SPR: Surface plasmon resonance, sAA: Salivary α-amylase, IL: Interleukin, MMP: Matrix metalloproteinases, EGFR: Epidermal growth factor receptor, mi-RNA: Micro-RNA, CRP: C-reactive protein, CEA: Carcinoembryonic antigen, CA125: Cancer antigen 125, HER-2: Human epidermal growth factor receptor-2

IL-1 β, MMP-8, TNF-α, IL-6, and C-reactive protein (CRP) are the biomarkers associated with periodontitis, for which saliva-based biosensors were developed by the University of Texas at Austin (2007). It is a Lab-on-a-chip (LOC) system that combines microfluidics and fluorescence-based optical system. In this sensor, sandwich immunoassays are performed on chemically sensitized beads.[21]

Another biosensor called the Integrated Microfluidic Platform for Oral Diagnostics was developed by Herr et al.[35] which can detect biomarkers such as MMP-8, TNF-α, CRP, and IL-6. This device incorporates photopolymerized gels for immunoassays, microfluid chip, optical elements, and data acquisition software.

An MMP-8 oral test became available commercially in 2010. This test has shown reasonable sensitivity and specificity in diagnosing active or stable periodontal lesions in patients who smoke, as well as in those who do not smoke.[36]

A surface-immobilized optical protein sensor has been used to detect IL-8 protein cancer marker. In this sensor, the analyte immobilized on the surface with the capture probe reacts with biotinylated monoclonal Ab. The emission light from fluorophore conjugated with the reporter probe is then used as the detection signal, and the optical noise is reduced using confocal optics.[22]

Diagnosis of infectious diseases at the bed side of a patient where there is no need of sending patient samples to sophisticated labs is called as Point of Care testing (POC). The need for non-invasive simple-to-use POC (point of care) diagnostic tools is particularly acute in the developing world where many health risks and illnesses remain poorly defined and receive inappropriate treatment. Rapid point of-care HIV tests utilize saliva, gingival crevicular fluid, or oral mucosal fluid to rapidly provide test results to patients.

Floriano et al. (2009) showed in their investigation that saliva-based nano-biochip tests together with an electrocardiogram could provide a prompt screening method for Acute Myocardial Infarction patients in the prehospital stage.[37]

Xiang J.;Yan M. et al. (2020) used lateral flow antigen assay as a Point-of-Care testing for COVID-19 diagnosis.[38]

In addition to the salivary biomarker initiatives, the UCLA School of Dentistry is also engaged in the parallel development of the saliva diagnostics technology platform. They invented the "Oral Fluidic Nano Sensor Test" (OFNASET).[39] The handheld, automated, easy-to-use, integrated system will enable simultaneous and rapid detection of multiple salivary protein and nucleic acid targets. OFNASET can be used to detect breast, pancreatic, and lung cancers.

Limitations for the use of saliva as diagnostic fluid [4]

Salivary composition can be influenced by the method of collection and the extent of stimulation of salivary flow. Changes in salivary flow rate (shows inter and intra individual variability) may affect the concentration of salivary markers and also their availability due to changes in salivary pH.

Levels of certain markers in saliva are not always a reliable reflection of the levels of these markers in serum. Some systemic disorders, medications and radiation may affect salivary gland function and consequently the quantity and composition of saliva. Also, many serum markers may reach whole saliva in an unpredictable way (i.e., gingival crevicular fluid flow and through oral wounds) and proteolytic enzymes derived from the host and oral microorganisms. These parameters will affect the diagnostic usefulness of many salivary constituents.

CONCLUSION

A drop of saliva harbors a world of diagnostic information, proteomically and genomically. Saliva is an epitome of a non-invasive, readily-available, easy to collect, transport, and store "biofluid." Since the emphasis is switching more towards high impact personalized medicine, pioneering "Point-of-care salivary technologies" are being developed. Although blood is the gold standard for the diagnosis of drugs and diseases, the success of saliva as a diagnostic media is guaranteed, particularly for substances that reflect or can be directly correlated with systemic analytes. It is almost certain, that in the forthcoming years, home testing kits incorporating oral fluid biosensors may begin to appear, outperforming the routine laboratory tests in the diagnosis of diseases.

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