Salivary Biomarkers: The Current And Future Scenery Of Diagnostics

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Abstract: A successful treatment depends on the early and successful detection of the disease. A timely, cost effective diagnostic method is the most important part of the treatment plan. The discovery of saliva-based microbial, immunologic, and molecular biomarkers offers unique opportunities to bypass the short comings of other methods by utilizing oral fluids to evaluate the condition of both healthy and diseased individuals. Salivary diagnostics as a simple and non invasive method is of great value to periodontist for rapid screening and accurate evaluation. Hence, today in the era of nanotechnology and genomics, field of salivary diagnostics is promising a dramatic change in disease diagnosis and clinical monitoring.

Keywords: salivary biomarker, periodontal disease, diagnostics

I. INTRODUCTION

Early diagnosis of diseases is crucial to prevent complications that could have a negative impact on a patient's quality of life. despite the regular screenings and check-ups, many diseases are undetected until a late phase where morbid symptoms become apparent. To overcome this challenge, medical researchers are devoted to finding molecular disease biomarkers that reveal a hidden lethal threat before the disease becomes complicated. A biomarker, or biological marker, is in general a substance used as an indicator of a biological state. In oral diagnostics, it has been a great challenge to determine biomarkers for screening, prognosis and evaluating the disease activity and the efficacy of treatment. For the past decades saliva has evolved as an emerging landscape for the diagnosis of breast cancer, oral cancer, caries risk, salivary gland diseases, periodontitis, and various systemic disorders.

II. SALIVA AS A DIAGNOSTIC FLUID

Yoshizawa et al. notes that there are some clinical advantages to using saliva:

- ✓ Saliva collection is undemanding: procurement of saliva does not require highly trained personnel, and can be performed easily and readily, in contrast with blood sampling. To obtain sample saliva, expensive tools are not necessary.
- ✓ Saliva collection is noninvasive: Individual patients are usually more comfortable with saliva sampling, and are more likely to participate.
- ✓ Saliva samples are easier to handle and store: secretions in saliva that are not present in serum or plasma help decrease the risk of HIV transmission, and saliva does not clot.

Ultimately, the selection of saliva when compared to other diagnostic biofluids will be based on both the specific molecular constituents that are targeted and the practicalities of sample collection and processing.

LIMITATIONS

✓ Levels of certain markers in saliva are not always a reliable reflection of the levels of these markers in serum.
✓ Salivary composition can be influenced by the method of collection and degree of stimulation of salivary flow.
✓ Changes in salivary flow rate may affect the concentration of salivary markers and also their availability due to changes in salivary pH.
✓ Variability in salivary flow rate is expected between individuals and in the same individual under different conditions.
✓ In addition, many serum markers can reach whole saliva in an unpredictable way (*i.e.* gingival crevicular fluid flow and through oral wounds). These parameters will affect the diagnostic usefulness of many salivary constituents.
✓ Furthermore, certain systemic disorders, numerous medications and radiation may affect salivary gland function and consequently the quantity and composition of saliva.
✓ Whole saliva also contains proteolytic enzymes derived from the host and from oral microorganisms. These enzymes can affect the stability of certain diagnostic markers. Some molecules are also degraded during intracellular diffusion into saliva.

III. SAMPLE COLLECTION AND PROCESSING

After ensuring that the saliva may be useful for the diagnosis of the disease, collection of saliva is done. It can be collected with or without stimulation. Un-stimulated whole saliva is commonly collected by the `draining' method where the subject's head is tilted forward so that saliva moves towards the anterior region of the mouth and the pooled saliva is drooled into a wide-bore sterile vessel. Stimulated whole saliva is generally obtained by masticatory action (i.e., from a subject chewing on paraffin) or by gustatory stimulation (i.e., use of citric acid or sour candy drops on the subjects tongue) and is expectorated into a tube.[3]

PARAMETER	DESCRIPTION
Subject Status	Prior to collection of samples, study researchers should prescribe either fasted or unfasted states to patient cohorts. It has been observed that saliva in a fasted state may lead to differences in composition of saliva .
Sample Collection Time	When instructing patients on sample collection, it is necessary to specify a window of time that the patient may be allowed to contribute their saliva to a sample collection instance. These windows are important precautions against sample degradation if the time is long, and also allow adequate time for saliva to be collected with biomarker content.
Sample Collection Volume Requirement	Typically, running biomarker identifications or bioassays on a salivary sample will require a specific volume that must be collected for running tests. If the subject has a pathology that severely limits the flow of saliva to the oral cavity, it may be necessary for the study to have modifications made to account for the reduced volume that may be achievable.
Sample Collection Method	A multitude of different saliva collection methods can be used for testing. Typical collection protocol used at facilities such as UCLA involves the usage of traditional falcon tubes on ice, but saliva collectors have also been explored for collection . This method can be designated as "unstimulated" since it uses saliva that has naturally pooled in the mouth. This is differs from the class of "stimulated" collection, where samples of saliva are attained through methods such as absorbent pads or chewing on parafilm . The methodsused must be appropriately identified, as results of analysis may differ depending on the saliva collection method.
Sample Processing and Storage	Collections of saliva must be properly optimized based on desired targets to be tested for. The inclusion of constituents in the saliva such as epithelial cells may contribute background that may hinder assessments of whether molecular targets are truly in the saliva. For this reason, centrifugation may be considered for removing cells and creating cell-free saliva. Stabilizing agents may be necessary for preservation of samples, depending on the target.

Table 1: Considerations regarding the collection and sample processing steps of saliva

Over the last few years other promising devices have emerged that are based upon modifications to the traditional expectoration technique.

ORAGENE

It is a more sophisticated way to collect saliva into a vessel to which is attached a screw-on cap containing a mixture of preservative buffers. Upon completion of the expectoration process, the cap is screwed onto the device releasing the preservative buffer, which drops into the saliva, is mixed by shaking and then acts to protect the integrity of the sample until processing and extraction can take place. It is the most widely used collection device.[3]

SALIGENE

It is an alternative "spit-in-a-cup" technology, which has additional application as a collector for stool or swab specimens (when coupled with specific extraction kits for these alternate specimen types). In the Saligene device, subjects expectorate into a modified collection tube until a pre-determined volume has been reached. A screw-cap with attached plunger is screwed in place and the plunger when depressed causes a preservative/lysis buffer to flow into the collected saliva specimen. The sample of mixed preservatives and saliva is gently shaken then sent to a laboratory for further processing.[3]

ORACOL

This test kit consists of an absorbent foam swab (designed to collect up to 1 ml of saliva), centrifuge tube and cap. It is supplied sterile in batches of 500. This kit is universally used to collect data on measles, human immunodeficiency virus (HIV), hepatitis A and B, mumps and rubella.[3]

VEROFY

Verofy is a unique platform technology that incorporates rapid and standardized saliva collection with high quality immune chromatographic test strips providing a system for delivery of immediate results in field or point-of care. Verofy collects saliva from under the tongue by means of a proprietary absorbent material connected to either one or two immune chromatographic test strips located in the device housing and in fluid communication with the test strips. After approximately 1 to 2 minutes of saliva collection time, a sample volume adequacy indicator built into the device changes appearance, signifying that sufficient sample has been collected for testing. The device can then be removed from the mouth and allowed to run for an additional time (3-15 minutes depending upon the specific test). As for standard immune chromatographic tests, a line or series of test lines will appear on the test strips depending on the diseases or analytes being tested. In addition a control line will appear confirming the validity of the test and the appropriate function of reagents used in the tests. If a positive test result is obtained and a confirmation specimen is required, this can be collected by squeezing the absorbent collection pad through a plastic compression tube provided and into a standard 2 ml eppendorf centrifuge (or equivalent) collection tube. Once collected this sample is capped then sent to a laboratory for suitable confirmation testing.

IV. SALIVA AS A DIAGNOSTIC FLUID

Autoimmune disease[4]

disease	Biomarker
Multiple sclerosis	IgA level
Sjogren's syndrome	Alpha-amylase, Kallikrin
Sarcoidosis	Interleukin-2, Interleukin-6

Hereditary diseases[4]

disease	Biomarker
Cystic fibrosis	Cathepsin-D, Sodium, potassium, chloride, calcium, magnesium, urea, uric acid and total protein.
Ectodermal dysplasia	Total protein
21-Hydroxylase deficiency	17-hydroxyprogesterone (17-OHP)

Infections[4]

disease	Biomarker
Bacterial	*H.Pylori* DNA, Pneumococcal pneumonia C polysaccharide, anti-shigatoxin antibody, *Mycobacterium tuberculosis*, MUC5B and MUC7
Viral	HIV-1, HIV-2antibodies, Salivary proteins
Fungal	Candidiasis immunoglobulins, Hsp70 and calprotectin, histatins, mucins, basic proline rich proteins

Malignancy[4]

disease	Biomarker
Head and Neck cancer	Mrnaof specific proteins, p53 antibodies
Breast cancer	Elevated levels of tumor markers c-erbb-2 (erb) and cancer antigen 15-3 (CA15-3), Elevated salivary levels
Others	Lncrna, mirna, CCNI, EGFR, FGF19, FRS2 and GREB1, AGPAT1, B2M, BASP2, IER3 and IL1B, FGF2,PSA,Cortisol, LDH
Renal diseases	Cortisol, nitrite, uric acid, sodium, chloride, pH, amylase and lactoferrin
Psychological research	Salivary amylase, cortisol, substance p, lysozyme and secretory Iga.

Oral disorders4

disease	Biomarkers
Dental caries	*S.Mutans* and Lactobacillus counts, pH, Buffering capacity
Periodontal diseases	Aspartate aminotransferase, alkaline phosphatase, uric acid, albumin, Arp3, CAVI, IL-1Ra, PLS-2.
Bone tumor markers	Osteocalcin (OC) and Pyridinoline (PYD)

Occupational/Environmental disorders4

disease	Biomarkers
Acute stress	Lead (Pb) and cadmium (Cd) poisoning
Chronic stress	Saliva chromogranin A and alpha-amylase, increased levels of salivary cortisol and decreased level of salivary igaand Lysozyme.
Cardiovascular markers	Alpha amylase salivary activity, salivary lysozyme, salivary TC, TGL, HDLC and VLDLC

SALIVARY BIOMARKERS IN THE DIAGNOSIS OF PERIODONTITIS

DENTAL BIOFILM	INFLAMMATORY	COLLAGEN BREAKDOWN	BONE REMODELLING
Immunoglobulins (IgA, IgM, IgG)	β-glucuronidase	α2-macroglobulin	Alkaline phosphatise
Mucins	C- reactive protein	MMP8	Osteoprotegerin
Lysozyme	IL 1β	MMP9	Osteocalcin
Lactoferrin	IL 6	Aspartate aminotransferase	SPARC/osteonectin
Histatin	MIP 1α	Alanine aminotransferase	RANKL
Peroxidase	Tumor necrosis factor-α	TIMP	β C-terminal type I collagen telopeptide
			C-telopeptide pyridinoline cross-links of type I collagen

IL: Interleukin; MIP: Macrophage inflammatory protein; MMP: Matrix metalloproteinase; RANKL: Receptor activator of NF-κB ligand; SPARC: Secreted protein, acidic, rich in cysteine; TIMP: Tissue inhibitors of metalloproteinase.
Table 2: Demonstrates various salivary biomarkers of periodontal disease

V. RECENT DISCOVERIES AND PROGRESS

Advancements in analytical techniques have enabled scientists to discover the specific biomarkers associated with human diseases. Salivary genome and epigenome, transcriptome, proteome and metablome projects and nanobiochip technology have given greater dimensions to saliva as a diagnostic tool.

NANO-BIOCHIP TECHNOLOGY

The tools of nano materials and microelectronics for the practical implementation of miniaturized sensors are suitable for a variety of important applications. There are two types of systems been created, the first is based on a micro bead array, wherein micro-pits within a silicon wafer are populated with a variety of chemically sensitized bead `microreactors'. The development of a point of care (POC) device that contains a modular and miniaturized sensor system, universal analyzer with functional integrated mechanical/optical interfaces, and flexible microchip architecture can service the future needs of clinicians and the research communities.[5] In this POC device, saliva (100–300 µl) is placed into the salivary collection/delivery module, and then delivered into the Nano-Biochip. The injection-molded cartridge is `credit card' size and encloses the array Nano-Biochip where complex fluorescent immunoassays are performed.

All processing steps are conducted within the micro fluidic network of the biochip via actuation inside the analyzer without human intervention. These features eliminate the need for external fluidics, such as pumps, tubing and connectors. Therefore, the integrated system has the potential to reduce cost and reduce the risk for leaks and contamination. The assay is processed entirely through a 5–15 min sequence that is programmed in the main controller board. The flexibility of the control software allows for modifications to be made through an assay builder interface. Control over the flow rate, incubation time and reagent wash, is achieved by the actuation of stepping motors that direct the fluid flow through the depression of the fluid pouches. The sample is directed to an on-chip waste reservoir, which provides a safe containment of bio-hazardous fluids. The entire biochip can be discarded as solid waste after the assay, facilitating biohazard waste management. Together, these essential features serve to facilitate the transition from chips-in-alaboratory to a lab-on-a-chip, and offer significant opportunities for POC technology needs.[5]

The wide continuum of molecules present in saliva provides valuable information for clinical diagnostic applications in clinical utility for followings:
✓ Proteomic analysis
✓ Genomic analysis
✓ Transcriptome analysis

Salivary Proteomic Analysis Human saliva is a plasma ultra-filtrate and contains proteins either synthesized in situ in the salivary glands or derived from blood and contains biomarkers derived from serum, gingival crevicular fluid, and mucosal transudate. To date, researchers have identified 2,340 proteins in the salivary proteome, of which 20– 30% are also found in blood[6], an encouraging indicator of the clinical utility of saliva as a diagnostic fluid. In contrast to the plasma proteome, in which 99% of the total protein content is contributed by 22 highly abundant proteins, the 20 most abundant proteins in WS constitute only 40% of the protein content[7]. This composition suggests that detecting biomolecules of clinical sensitivity and specificity in saliva should be practicable and easier than in blood. How molecules of blood transport in saliva may also be important for successful use of saliva as a diagnostic fluid. Lipophilic

molecules such as steroid hormones passively diffuse into saliva, while water and electrolytes pass through the pores of acinar cells. Various peptides in blood move through protein channels, and large proteins are transported via pinocytosis[8]

SALIVARY PROTEOMICS FOR EXISTING PERIODONTAL DISEASE (PD)

Interlekin-1β (IL-1β) is a proinflammatory cytokine that stimulates the induction of adhesion molecules and other mediators which in turn facilitate and amplify the inflammatory response. Its levels correlated significantly with periodontal parameters after adjusting for the confounders. Moreover, combined levels of IL1β and matrix metalloproteinase (MMP)-8 increased the risk of experiencing PD by 45 folds.[9] MMPs, MMP-8, a key enzyme in extracellular collagen matrix degradation, derived predominantly from PMNs during acute stages of PD. Its presence significantly increased the risk of PD (odds ratios in the 11.3-15.4 range). MMP-1 (interstitial collagenase) also appeared to be activated in periondontitis26. Additionally, higher levels of other MMPs, including MMP-2, MMP-3 and MMP-9, were also reported in the saliva of periodontitis patients.[9]

VI. SALIVARY TRANSCRIPTOME ANALYSIS

The Salivary Transcriptome (ST) offers an additional valuable resource for disease diagnostics. The first report of the ST demonstrated that the normal ST consists of about 3,000 mRNAs. Of particular importance is that of the 3,000 mRNAs, 180 are common between healthy subjects, constituting the normal salivary transcriptome core (NSTC)[10] . To demonstrate the diagnostic and translational potential of the ST, the UCLA group profiled and analyzed saliva from patients with oral cancer. Four genes from the NSTC (IL-8, ornithine decarboxylase, spermidineacetyltransferase and IL-1) were able to discriminate and predict, whether the saliva sample was from a patient with cancer or from a healthy subjects, with a sensitivity and specificity of 91%. The behavior of these ST biomarkers is consistent and their levels are significantly higher in saliva of patients with oral cancer compared to control subjects.

VARIOUS PRODUCTS AND THEIR USES FOR MEASURING SALIVARY BIOMARKERS

PRODUCT NAME	PURPOSE
My PerioID	Identifies the type and concentration of specific bacteria
My perio path	Determines the cause of periodontal bacteria
Oral fluid nano sensor test	Simultaneous and precise detection of multiple salivary proteins and nucleic acid.
Electronic test kit	Detects salivary biomarkers for early diagnosis of periodontal disease
Ora quick	Detects HIV1 and HIV2

Integrated microfluidic platform for oral diagnostics	rapidly measures the concentration of MMP8 and other biomarkers in small amount of saliva

Bacterial enzymes &host enzymes	BANA periodontal test	Ora Tec Corporation Manassas (USA)	It utilizes the BANA test for bacterial trypsin like proteases
	Periocheck	CollaGenex Pharmaceuticals, Newtown, PA	Detects presence of neutral proteinases i.e. Collagenase
	Perioscan	Oral B Laboratories	Detects enzymatic activity of Aggregatibacteractinomycetemcomitans, T forsythus,P gingivalis
Immunological identification	Evalusite	Kodak Eastman Company (Switzerland)	Immunological detection of antigens of Aggregatibacter actinomycetemcomitans, P intermedia, P gingivalis using antibodies (ELISA
Biochemical identification	Prognostic	Dentsply	Aids in detection of serine proteinases and elastases
	Biolise	SLT-Labinstruments, Crailsheim, Germany	Aids in detection of elastase
	Periogard	Colgate	Detects the presence of AST
	Pocket watch	SteriOss®, San Diego, CA, USA	Detects aspartate aminotransferase through colorimetric detection
	TOPAS	Affinity Labelling Technologies (USA)	Detects toxins derived from anaerobic metabolism

VII. CONCLUSION

Diagnostic tests are routinely used in evaluation of many diseases. Saliva-based diagnostics present incomparable opportunities for research and commercialization opportunities because of increased understanding of genomics, transcriptomics and proteomics. The use of proteomics and gene expression will advance the diagnosis and treatment of various oral pathological conditions. It is clear that no single marker will fulfill all the criteria necessary for assessment of the clinical state of the periodontium, The development of a wide spectrum of marker factors will be a primary goal of periodontal research.

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