Review Article

Saliva: An Emerging, Non-Invasive Tool for Detection of Diseases

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ABSTRACT

Saliva in human is an important biological fluid which is secreted by three major as well as many minor salivary glands. Apart from its major role in digestion of food and antibacterial properties, it can be used as a diagnostic tool in many oral as well as systemic diseases. It can be collected noninvasively from individuals without any special equipment for collection and storage. Now a day's saliva is used as a biomarker for many diseases. Analysis of biomarkers and altered level of mRNA in saliva by various sophisticated methods is useful in diagnosis, monitoring and prognosis of many autoimmune, infectious, systemic, oral and malignant disorders. Saliva also plays an important role in the monitoring of drug abuse such as cocaine, heroin, amphetamine, barbiturates etc. Thus saliva can be used as a non-invasive, emerging diagnostic tool for detection of various diseases.

Keywords: Saliva, Polymerase chain reaction, Point of care testing.

INTRODUCTION

Saliva is a biological fluid which is clinically informative, for clinical diagnosis and for monitoring, management of patients with both oral & systemic diseases. Saliva is considered as mirror of body health.

It is secreted by three major salivary glands i.e. (parotid, submandibular, sublingual) and the numerous minor salivary glands.

It can be collected noninvasively from individuals. It does not require special equipment for collection and storage and unlike blood, as saliva does not clot.

Now a day's saliva is used as a biomarker for many diseases. Saliva had been used for the monitoring of drug abuse such as cocaine, heroin, amphetamine, barbiturates etc.

Moreover salivary testing has been largely performed for the diagnosis of HIV infection.

Analysis of salivary parameters such as salivary flow rate, pH, buffer capacity, lactobacillus, and yeast content, presence of IgG, IgM and anti-La auto antibodies and raised protein levels such as that of lactoferrin and cystatin C as has been proposed for the diagnosis of Sjogren's syndrome.

Altered levels of certain mRNA molecules have been detected in saliva in oral cancer patients and certain proteins in several cancers. This also helps in diagnosis of many diseases.

Saliva also contains multiple biomarkers which make it useful for multiplexed assays that are being developed as point of care (POC) devices, rapid tests for centralized clinical laboratory is being incorporated as a part of disease diagnosis, clinical monitoring and for making important clinical decisions for patient care.

The diagnostic application of saliva is used for hereditary disorders, autoimmune diseases, malignant & infectious diseases & endocrine disorders as well as the therapeutic levels of drugs and monitoring of illicit drugs use and also for forensic evidence & diseases like periodontal diseases, dental caries and systemic disease like diabetes, osteoporosis, and cardiovascular disease.

Composition and Properties

Saliva is mixed fluid that is derived from major and minor salivary glands. It contains gingival crevicular fluid (GCF), mucosal transudate, expectorated bronchial and nasal secretions. Saliva is composed of 99.5% water and 0.5% solid material which are inclusive of organic and inorganic constituents. The inorganic constituents are made of sodium, potassium, chlorine, bicarbonate, magnesium, calcium, phosphate, thiocyanate, fluoride, lead, cadmium, copper, nitrite and nitrate.

Sodium, potassium and chlorine contribute to the osmolarity of saliva and their concentration give diagnostic information related to the efficiency of ductal transport system. Bicarbonates are the most important buffer present in saliva resisting changes in salivary pH. Calcium, phosphate and fluoride have anti-caries activity. Thiocyanate acts as an anti-bacterial agent. Nitrate estimation in saliva also predicts the future development of carcinoma.

The organic constituents are made up of proteins which include mucins and proline rich proteins which have lubricating properties, amylase and lipase with digestive properties, proteins such as sialoperoxidase, lysozyme[1], lactoferrins, chitinase, cystatins, histatins, defensins,

salivary leukocyte proteinase inhibitors, calprotectin, peroxidase, acid phosphatase, chromogranin A, sialin, agglutinin which have anti-microbial properties.[2] Other proteins include statherin, mucoproteins and glycoproteins. The rest of the organic constituents is made of blood group substances, enzymes such as aldolase, β-glucoronidase, succinic dehydrogenase, kallikrein, hormones, carbohydrates, lipids, nitrogen containing compounds, vitamins, oral microorganisms, various gases like oxygen, nitrogen and carbon dioxide, growth factors like epidermal growth factor and cells of desquamated epithelium[3].

Normal daily salivary flow range from 0.5 to 1.5 L with average volume of 1ml at any given time. It is slightly acidic with pH of 6.35 to 6.85, with specific gravity from 1.002 to 1.012 and hypotonic to plasma.

Regulation of salivary secretion

Salivary secretion is only regulated by autonomic nervous system (ANS), through sympathetic and parasympathetic pathway.

Parasympathetic fibres arise from the superior salivary nucleus situated in the upper part of medulla oblongata. it supplies the sublingual and submandibular gland through facial nerve, and the glossopharyngeal nerve supply the parotid gland.

Sympathetic supply is through the fibres arising from first and fourth thoracic segments.[3]

Collection and storage of saliva

Whole saliva can be collected with or without stimulation. Stimulation can be performed with masticatory movements or by gustatory stimulation (citric acid). Stimulated saliva can be collected in larger quantities. Unstimulated saliva can be collected by spitting in a test tube or by leaving saliva drool from the lower lip and it is more often used for the diagnosis or follow up of systemic diseases.

The methods of saliva collection comprise of collection of whole saliva and collection of glandular saliva. Among these, whole saliva collection is easiest and most feasible method. Specific glandular saliva can be collected with less easy methods.

The common methods of resting saliva collection include draining method, spitting method, suction method and swab method.

Certain approaches to store saliva in order to prevent degradation of salivary compounds[4] Include;

❖ Immediate storage without any processing; if analysis is to be done within 30-90min

❖ Saliva can be stored at room temperature; for analysis after 3 to 6hrs from collection

❖ Storage is to be done at +4^{0}C and if analysis is to done after days to months after collection, storage is

to be done at -20^{0}C or still better at -80^{0}C.

❖ Snap freezing of saliva in liquid nitrogen

❖ Inhibition of enzyme activity in saliva by mixing with certain enzyme inhibitors

❖ Addition of sodium azide to retard bacterial growth.

❖ Addition of trifluor acetate to denature salivary enzymes that could degrade salivary compounds such as proteins and steroid hormones.

For salivary DNA analysis, pre-clearing of saliva before analysis is not recommended since the main source is from desquamated oral mucosal cells.

In appropriate buffers of DNA extraction kits, saliva can be stored at room temperature for atleast one year.

However before adding such buffer, saliva should be stored on ice to prevent microbial growth and to decrease salivary DNAase activity.

Saliva can also be frozen and stored at -20^{0}C or -80^{0}C before extraction or after extraction before use.[5]

For RNA analysis, saliva sample is usually centrifuged because the majority of RNAs in the centrifuged whole saliva are genuine human RNA's.[6]

For RNA studies focusing on oral microbiota, any kind of pre-clearing of saliva should be avoided.

In order to avoid destruction of salivary RNA, the use of RNAase inhibitors are recommended.

With the use of inhibitors, mRNA can be stabilized for longer run even in room temperature although cooling of saliva in ice is useful. RNA can be preserved in saliva by freezing at - 80^{0}C also.[5]

For saliva protein analysis, centrifugation and/or microfiltration can be done.

Pre-cleared saliva can be stored on ice without significant protein degradation for only few hours.

Addition of protease inhibitors is advantageous especially for time consuming analysis procedures. Samples can be stored for few days at -20^{0}C.

However prolonged storage at any temperature may lead to significant protein precipitation.

Technologies for saliva based diagnosis

Proteomics

The proteomics (large-scale study of proteins, particularly their structures and functions) has a great role in bodily fluids particularly accessing the sources of disease markers.

A brief analysis of the human salivary proteomes can provide a comprehensive spectrum of oral and general health and may unveil morbidity signatures in the early stage as well as monitor disease progression.[7]

The total amount of protein in whole saliva ranges between 0.5-3mg/ml.

Separation of proteins are usually done by using two dimensional polyacrylamide gel electrophoresis (2D-PAGE) and for low abundance proteins particularly integral membrane protein are done by Two dimensional high performance liquid chromatography (2D-HPLC). Once separated, proteins can be primarily identified by mass spectrometry (MS) or further characterized by ionization methods such as electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI).[7,8]

An alternative mass spectrometric based method for protein identification is nanoelectrospray in which extremely low levels of protein digest mixtures to be analysed at flow rated of approximately 20-50nl/min.

Transcriptomics

Salivary transcriptome diagnostics constitutes a novel clinical approach where a large panel of human RNAs can be readily detected in saliva.

The large panel of human mRNA is determined by the use of microarray technology and after profiling, validation of transcriptome biomarkers will be done by quantitative real time PCR.

Sometimes multiplex reverse transcriptase PCR are used to overcome problems with quantitative real time PCR.[7]

Polymerase chain reaction (PCR)

PCR is a simple *in-vitro* method for amplification of specific short segments of DNA or cDNA reverse transcribed from RNA (RT-PCR).

The technique simplifies genetic analysis and permits the study of all types of clinical samples.

Quantitative PCR (qPCR) is used for accurate quantification using fluorescent labeled probes.

The visualization of amplification is done by agarose/ polyacrylamide gel electrophoresis, southern blot, dot blot and reverse blot assays.

Genomics

Genomic analysis is one of the recent advances in the diagnosis of oral cancer and considerable research is being performed in this field.

Plasma DNA were shown to exhibit tumor specific characteristics such as somatic mutations in tumor suppressor genes or oncogenes, microsatellite alterations, abnormal protein methylation, mitochondrial DNA mutations and presence of tumor related viral RNA.[9]

These DNA related changes were also found in saliva, the identification of which helps a great deal in diagnosis of oral cancer.

The techniques that are available for detection of genetic changes are discussed below.

Hybridization methods

Hybridization refers to the pairing of complementary DNA or RNA strands to produce double stranded nucleic acids.

This method uses a radio-labeled or fluorescence labelled DNA or RNA probe that binds to the target molecule of interest, permitting visualization.

The target nucleic acids can either be immobilized in a membrane (blotting) or examined in tissue sections (in situ)[10]

Flow cytometry

Flow cytometry is an important method used to analyse cell kinetics and protein expression in normal and tumor cells[10].

Point of Testing

Point-of-care testing (POCT), or bed-side testing is defined as medical testing at or near the site of patient care.

These tests require only a single drop of whole blood, urine or saliva, and they can be performed and interpreted by any general physician within minutes which allows for immediate clinical management decisions to be made.

POCT has become established worldwide and finds vital roles in public health.

Point of care microfluidic systems are being developed for diagnosis of oral and breast cancers.[11]

They permit concurrent detection of multiple salivary analytes including protein and nucleic acid.

Oral fluid nanosensor test (OFNASET) is a handheld, automated, easy to use integrated system that will enable simultaneous and rapid detection of multiple salivary proteins and nucleic acid targets.

Analysis of Saliva for Diagnosing Different Diseases

Table 1: Systemic& oral Diseases Affecting Salivary Glands and Saliva

Autoimmune disease	Graft-vs.-host disease
(Sjögren's syndrome,	Renal diseases
Rheumatoid diseases,	Disease of adrenal cortex
Myasthenia gravis)	Detection of drugs
Cancer	Dental caries
Cystic fibrosis	Gingivitis
Ectodermal dysplasia	Periodontitis
HIV infection	Oral squamous cell carcinoma
Hormonal disorders	adrenal-cortical disease,
Hypertension	diabetes mellitus,
Metabolic disturbances	thyroiditis, acromegaly
	Parkinsonism, Bell's palsy

Salivary biomarkers[12]

Table 2: Summary of the salivary biomarkers in various systemic diseases.

S. No.	Diseases	Biomarkers level	Source of biomarkers
(1)	Autoimmune diseases		Saliva
	Sjogren's syndrome	↑lactoferrin, beta2 microglobulin, lysozyme C, and cystatin C and ↓levels of salivary amylase and carbonic anhydrase	
	Multiple sclerosis	↓ IgAproduction	
	Sarcoidosis	↓Alpha-amylase and kallikrein	
(2)	Bone turnover markers	Analysis of D-PYR, OC concentration, hepatocyte growth factor, interleukin-1 beta, salivary osteonectin, and ALP activity	Serum and saliva
(3)	Cardiovascular markers	Cardiac troponins, C-reactive protein, myoglobin, myeloperoxidase, ICAM-1, CD 40,MMP-9 and salivary lysozyme	Serum and saliva
(4)	Dental caries and periodontal diseases	Streptococcus mutans and lactobacilli count, aspartate aminotransferase, alkaline phosphatase,	Saliva
(5)	Diseases of the adrenal cortex	↑Salivary cortisol	
(6)	Drugs level monitoring	Nicotine, cannabinoids, cocaine, phencyclidine, opioids,barbiturates, diazepines, amphetamines, and ethanol	Serum and saliva
(7)	Forensic evidence	Blood group antigens and DNA testing	Saliva
(8)	Genetic disorders (1) Cystic fibrosis	↑ Cathepsin-D and ↓ sodium, potassium, chloride, calcium, magnesium, lactate dehydrogenase	Saliva
	(2) Ectodermal dysplasia	↑ inorganic constituents, total protein and ↓ alpha-amylase	
(9)	Infections		
	Viral infections	Measles virus- specific IgM, HIV(HIV-1 and HIV-2)-antibodies, salivary proteins	
	Bacterial infections	Mycobacterium tuberculosis detection by PCR. Helicobactorpylori- ↑Mucin (MUC5B and MUC 7)	Serum and saliva
	Fungal infections	Candidiasis- immunoglobulins, Hsp 70, and calprotectin, histatins, mucins, basic proline rich proteins, and peroxidises	
(10)	Malignancy		
	Lung, breast, and prostate cancer	Expression of lnc RNA-	
	lung cancer	mRNA biomarkers (CCNI, EGFR, FGF19, FRS2, and GREB1)	Serum and saliva
	Oral squamous cell carcinoma	MiRNAs and p53 antibodies	
	ovarian cancer	mRNA biomarkers (AGPAT1, B2M, BASP2, IER3, and IL1B)	
	Breast cancer	CA15-3 and c-erb-2	
(11)	Occupational and environmental medicine	Salivary cortisol, IgA, lysozyme, chromogranin, alpha-amylase, lead, and cadmium	Serum and saliva
(12)	Psychological research	Salivary amylase, cortisol, substance P, lysozyme, secretory IgG, and testosterone	Saliva
(13)	Renal diseases	Cortisol, nitrite, uric acid, sodium chloride, pH, alpha-amylase, and lactoferrin. Salivary phosphate, serum creatinine, and glomerular filtration rate	Serum and saliva

deoxypyridinium (D-PYR), osteocalcin (OC), **Hsp 70- Heat Shock protein** MMP- matrix metalloproteinase

Potential Use of Salivary Diagnostics

The wide spectrum of compounds present in saliva may provide information for clinical diagnostic applications.

Saliva is a good medium because its collection is noninvasive and the donation process is relatively stressed free, so that multiple collections can be performed without imposing too much discomfort on the donor. Saliva is easy to collect, store, and transport; it does not require highly trained personnel; and it is safer for hospital staff to handle compared to blood and other body fluids. In addition, saliva is a "real-time" fluid because the salivary glands are exocrine glands that produce protein profiles indicative of an individual's

health and well-being status at the moment of collection. These characteristics make it possible to monitor several biomarkers in infants, children, elderly individuals, and uncooperative patients, as well as circumstances in which blood and urine sampling are not available.

Both basic and clinical research on the development of methods to assay saliva is increasing. Whole saliva is most frequently used for diagnosis of systemic diseases, because it can be readily collected and, more importantly, it contains serum constituents. Some systemic diseases affect salivary glands directly or indirectly and may influence the quantity and composition of saliva. Human saliva proteins also have diagnostic value for systemic diseases. Although proteomic constituents are a logical first choice as salivary diagnostic analytes, genomic targets have emerged as highly informative and discriminatory biomarkers.

The future for saliva diagnostics relies on combinations of biomarker panels used as screening tools to improve on diagnostic accuracy and specific.

The use of combinations of biomarkers may provide additive and powerful diagnostic information. Thus far we have focused mainly on collating information on protein biomarkers and their potential utility in diagnosis, prognosis, and staging of diseases.

Drawbacks of salivas diagnostic tool

- ❖ Small specimen volumes confine the number of tests that can be performed

- ❖ Contamination of the mouth may affect drugs screening results or adulterate the samples[13]

- ❖ Difficult to collect from those abusing stimulants such as amphetamines and ectasy[14]

- ❖ Routine procedures are not always applicable to screening saliva

- ❖ Low concentrations make detection difficult and necessitate the use of expensive equipment

- ❖ The possibility of saliva being contaminated by drug residues in the oral or nasal cavities.[15]

Technically, saliva testing is more challenging than blood testing, limiting the number of laboratories that are capable of performing the tests.[16]

Foods, beverages such as coffee, and drugs taken just before collecting saliva can interfere with test results or cause a transient shift in hormone levels. Mucins in saliva can interfere with the test, causing spuriously high levels. Some chewing gums and cottons used for saliva collection contain substances that interfere with some saliva tests, resulting in erroneously high hormone levels.

DISCUSSION

Saliva has the potential to become a first-line diagnostic sample of choice owing to the advancements in detection technologies coupled with combinations of biomolecules with clinical relevance.

In the last 10 years Saliva has been emerged as a diagnostic medium exponentially[17]. Saliva contains a wide spectrum of proteins/peptides, nucleic acids, electrolytes, and hormones that found from multiple local and systemic sources. Sensitive detection systems are thus needed before we can unveil the utility of saliva as a diagnostic medium.[18]

In this review, we revel the diagnostic potential of saliva with regard to the diseases that are prevalent within Western countries as well as in developing world, such as cardiovascular disease (CVD) and breast cancers (systemic inflammation), and oral cancers (local inflammation). The diagnostic utility of saliva to detect periodontal disease has been extensively reviewed by others[4] which is not included in our study. Before addressing the diagnostic capability of saliva and its potential clinical applications, salivary constituents are increasingly investigated to aid in the diagnosis of oral and systemic diseases. To date, saliva has been studied to detect caries risk, periodontitis, oral cancer, salivary gland diseases, and viral infections such as HIV and HCV. Another important development in oral disease diagnosis has been the identification of salivary markers for the detection of oral cancer. Based on levels of specific molecular markers, molecular staging profiles have been identified in saliva as objective prognostic indicators. Researchers have found that panels of salivary mRNA (messenger ribonucleic acid) and protein markers can serve as biomarkers for oral cancer detection. Advancements in nano scale technologies have allowed high-throughput biomarker screening studies to be performed, including RNA detection using microarrays. Protein detection using highly sensitive mass spectrometry (MS) technologies has allowed researchers to accurately monitor changes in biomarker proteins that are representative of disease.

Salivary diagnostic technology also solves the problem to detect and monitoring of periodontal disease which includes the assessment of molecules related to the host inflammatory response and the destruction of associated connective tissue, as well as detection of specific pathogenic bacteria and its products.

Saliva also provides clues to systemic diseases and disorders. For example, rapid point-of-care HIV tests utilize saliva, gingival crevicular fluid, or oral mucosal fluid. The simplicity of collecting saliva provides useful for determining hormone levels, including estrogen(estradiol), progesterone, testosterone and cortisol. This is particularly vital in the case of estradiol, where it can be an indicator of premature birth and low birth weight babies. A number of drugs are detectable in oral fluid.

Seven technology groups are developing point-of-care salivary diagnostic technologies. Three groups are working together toward deciphering the salivary

proteome[7]. These collective efforts and the convergence of salivary diagnostic technologies and the salivary proteome will present unparalleled opportunities to explore the diagnostic potential of saliva for oral and systemic diseases.[7]

The technology can also be used for therapeutic monitoring of drugs such as digoxin, methadone and some anticonvulsants.

Saliva diagnostics may also play a great role in the fight against bioterrorism. Determining the scope of exposure to toxins requires a technology that is fast, highly sensitive and specific, portable, user friendly and capable of screening for multiple agents simultaneously. Saliva and other oral fluids are well suited for this purpose because they can be collected under observation (with or without stimulation), and are less susceptible to adulteration.

CONCLUSION

Saliva has been viewed as an important diagnostic fluid for a very long time now. In recent times, due to improved efficiency of genomic and proteomic technologies, the use of salivary diagnostics in a clinical setting is becoming a reality. Salivary metabolomics (scientific study of chemical processes involving metabolites) is a new advancement in the field of salivary diagnostics which analyzes a large array of low molecular weight endogenous metabolites present in the saliva for the detection of diseases.

Another major improvement is the development of the Oral Fluidic NanoSensor Test (OFNASET) for the real-time, ultrasensitive, and ultra-specific detection of multiple salivary protein and nucleic acid targets in disease conditions. The greatest milestone in salivary diagnostics is to identify the disease biomarkers and to transfer it from the laboratory to the clinical practice. But the growth of salivary diagnostics has been limited because of lack of sensitive detection methods, lack of correlation between the biomolecules in the blood and saliva, and the circadian variations in saliva. However, unlike blood and other body fluids, salivary diagnostics offers an easy, inexpensive, painless, and stress free approach to disease detection.

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