# Saliva Testing: A New Tool added to the Pathologist's Armamentarium

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## Abstract

Saliva harbors a wide spectrum of proteins/ peptides, nucleic acids, electrolytes, and hormones, that originate from multiple local and systemic sources. Research has identified many of these biomolecules as disease specific, which, along with recent availability of highly sensitive detection technology, has become the driving force for the development of saliva based diagnostic tools.

Although saliva offers many advantages over blood, including ease of collection and minimal risk of contracting infections, there are some limitations in its application in clinical setting. These include diurnal variation in salivary composition, influenced by the method of collection, and 1000 folds less concentration of salivary biomolecules as compared to blood etc.

Studies have shown mixed results regarding the clinical relevance of analytical tools developed so far. There is a need for further round robin testing, validation studies for reproducibility, sensitivity and specificity and clinical trials on large number of patients. The development of specific and standardized analytical tools, establishing reference ranges, individual cut-off values, and standardization of collection devices and methods will be other major challenges.

In future we are likely to see the increased utilization of saliva as a diagnostic fluid in routine clinical practice and it may become the first choice over blood, especially in some specific situations such as in obese and hemophilic patient.

***Keywords:*** Saliva, Biomarker, Biomolecules, Diagnostic tool, Non-communicable diseases, Infections.

## Introduction

The world is facing a dual challenge of several non-communicable diseases (NCDs) and pre-existing as well as newly emerging infectious diseases.[1] There is an urgent need than ever before to develop rapid, cost effective, non-invasive and technically simple tests for early diagnosis.

Saliva is a very suitable fluid for such a test with a non-invasive approach to sample collection and availability of several biomolecules associated with a healthy or diseased state. Till recently, saliva based testing has been limited to the diseases of oral cavity. Recently, there has been an increased understanding of the role of salivary biomarkers in disease etiology and progression, and the discovery of whole proteomics in saliva, some of which have relevance to specific diseases.[2-5]

Moreover, we now have the appropriate technologies to detect these biomarkers. There is ongoing development of high-sensitivity detection systems that include high-performance liquid chromatography (HPLC) and 2-D electrophoresis, mass spectrometry, multi-dimensional protein identification technology (MudPIT), enzyme-linked fluorescence technique, Western blot assays and polymerase chain reaction.[6-7]

All this offers a vast potential of developing non-invasive diagnostic tests for local and systemic diseases using human saliva which can be sensitive and specific and may be as an alternative choice to blood based tests in special circumstances.[8-13]

The present paper reviews emerging diagnostic applications of saliva and discusses challenges that will need to be overcome, before accepting it as a reliable diagnostic fluid.

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The aim is to raise the awareness of current status and encourage further research to develop clinically useful salivary biomarkers for laboratory and point of care testing (POCT) for non-communicable and infectious diseases.

## Composition of Saliva

Saliva contains many proteins, hormones, enzymes, nucleic acids, electrolytes, antibodies, chemicals, and drugs synthesized in the salivary glands as well as originating from local non-salivary sources (gingival crevicular fluid, mucosal transudate, bronchial and nasal secretions) and systemic sources. It also contains viral, bacterial, and fungal organisms from oral cavity.[2-4] These biomarkers and infectious agents can be detected in saliva of diseased patients and reflect an individual's health status at the moment of collection ("real-time" fluid).[2,8,13]

## Saliva as a non-invasive Diagnostic Tool

Blood and urine are well established diagnostic media. Saliva offers some distinct advantages over these in terms of collection, storage and transport.

Saliva collection methods are simple, non-invasive, do not require any special equipment and therefore can be collected by a non-skilled or semi-skilled person. Repeated and voluminous sampling can be done at short intervals. This is of particular importance in NCDs where repeated testing is required over a long period of time. It poses minimal risk of contracting infectious diseases such as Hepatitis or Human Immunodeficiency Virus (HIV) and is safe for both the operator and the patient.

Processing and storage of saliva in the laboratory is easy and less expensive, because it does not clot. Saliva samples are stable for 2 weeks when stored at 4 degrees temperature with protease inhibitors.[2,14]

There is good compliance because saliva sampling is stress free for the patients and is particularly advantageous in infants, disabled or anxious patients. Several biomarkers can be monitored in infants, children, elderly individuals, and, uncooperative patients, as well as in conditions where blood and urine sampling is not possible. It offers a cost effective approach for mass screening, early detection, monitoring of progression and predicting the prognosis of various diseases especially NCDs.[2,15,16]

## Saliva Collection Methods

Whole saliva is representative of complete oral environment, contains serum constituents and can be readily collected, therefore it is most frequently used for diagnosis of systemic diseases. Gland-specific saliva is obtained directly from individual glands for diagnosis of its specific pathology.[13]

Unstimulated saliva can be collected by draining or passive drooling method, spitting method, swabbing method and suction method. Stimulated saliva samples are collected either after masticating a piece of paraffin or after applying a drop of citric acid to tongue. Sample is then centrifuged to remove the solid contaminants (desquamated oral epithelial cells, keratin debris, blood cells, bacteria and food residues, if any) and stored in a frozen state until further analysis.[13,17]

Some presently available commercial saliva collection devices are Salimetrics oral swabs, OraSure and CoZart. An all-in-one system OMNIgene®•DISCOVER is used for the collection and stabilization of microbial DNA and RNA. Research is ongoing to produce standard collection devices for saliva.[18-20]

## Emerging Diagnostic Applications of Saliva

In recent times, the use of saliva as a diagnostic test has been explored and proposed in many conditions as discussed below:

### Infections

**Viral:** Antibodies against many viruses such as HIV, Hepatitis A & C, Epstein Barr, Measles, Rubella, Dengue, and Cytomegalovirus have been detected in saliva and correlate with serum levels. Hepatitis B surface antigen has been detected in saliva with high sensitivity and specificity. [21-27]

Four saliva tests, cleared by the US Food and Drug Administration (FDA), are commercially available. Orasure and OraQuick detect the presence of HIV antibodies in saliva. OraRisk test identifies Human papilloma virus (HPV) genotypes HPV 8, 11, 16, and 18. Other commercial non FDA approved tests are also available.[20]

For newborn infants, the salivary IgA response was found to be a better marker of rotavirus (RV) infection than the serum antibody response. This

could be used to monitor the immune response to vaccination and infection with RV.[27] PCR based identification of virus DNA in saliva is a useful method for the early detection of HSV-1 reactivation in patients with Bell's palsy.[28]

**Bacterial and Fungal Infections:** Causative organisms involved in dental caries, gingivitis, periodontal infections and oral candidiasis can be isolated from patient's saliva. PerioPath is a DNA test that determines the risk of periodontal infections by detecting bacterial pathogens in saliva.[29]

Detection of pneumococcal C polysaccharide in saliva has the potential of diagnosis of pnumococcal pneumonia. Helicobacter pylori (H. pylori) binds to salivary mucins MUC5B and MUC7. Higher levels of salivary MUC5B and MUC7 could be used as an indicator for infection with H. pylori. Mycobacterium tuberculosis can be detected in the saliva by polymerase chain reaction during the acute phase of the disease, when the bacterial load is high. The alterations in the salivary proteins, like immunoglobulins, Hsp70, calprotectin, histatins, mucins, basic proline rich proteins, Interleukins-1,6&8 and peroxidases also have diagnostic value in these infections.[2,13,30-34]

**Parasitic infections:** Specific antibody to *Tenia solium* larvae in serum, demonstrated greater sensitivity than antibody in saliva, for identification of neurocysticercosis. However, considering the simple and non-invasive nature of saliva sampling, saliva could be used in epidemiologic studies of this disease. Non-invasive diagnosis of amebic liver abscess is challenging, detection of E. histolytica DNA in saliva by real time PCR assay could be used as a diagnostic tool for amebic liver abscess.[30-32]

## Muscle and Joint Inflammation

Elevated levels of inflammatory biomarkers such as interleukins (IL-1β, IL-6, IL-8), tumor necrosis factor-α (TNF-α), matrix metalloproteinases (MMP-8 and 9), TIMP-1, and lysozyme are seen in saliva of these patients and have diagnostic potential.[2, 13-15, 32-34]

## Cardiovascular Disease

Salivary markers of myocardial infarction include C-reactive protein (CRP), myoglobin (MYO), creatinine kinase myocardial band (CKMB), cardiac troponins (cTn), myeloperoxidase, salivary lysozyme and ICAM-1. Floriano et al. and Denver et al. have demonstrated that salivary endothelin and natriuretic peptide levels are useful in heart failure.[35-37]

## Cancer

Application of salivary markers in early diagnosis, monitoring and prognosis of various malignancies of oral cavity, head and neck, breast, ovary, lung and prostate is under focus of extensive research. Some of the findings of genetic and epigenetic analysis are shown below.[34,38-41]

Promoter hypermethylation of three tumor suppressor genes (*DAPK1*, *RASSF1A* and *p16*) is found in saliva of head and neck cancer patients. Other tumor markers such as soluble CD 44, cytokeratin 19, tissue polypeptide antigen, CA-125, enolase 1, and p53 antibody were found significantly elevated in the saliva of oral squamous cell carcinoma (OSCC) patients. Salivary nitrate and nitrite, lactate dehydrogenase and cortisol levels and adenosine deaminase activity are all higher in OSCC. POST-C is a new test for suspected oral cancer being developed by PeriRx in UK.

Long non-coding RNA (lncRNA) are detectable in saliva due to aberrant expressions and are associated with oral, lung, breast, and prostate carcinomas. Dysregulation of short non-coding RNA molecules (miRNAs) was found in the saliva of oral cancer patients which could be used as an adjunctive tool to its diagnosis.

Salivary mRNA biomarkers can aid in the diagnosis of cancer such as CCNI, EGFR, FGF19, FRS2, and GREB1 for lung cancer and AGPAT1, B2M, BASP2, IER3, and IL1B for ovarian cancer.

**CA 15-3** in the saliva is useful to monitor advanced and metastatic cases of breast cancer. Elevated levels of *c-erb-2* were found in saliva of patients diagnosed with breast cancer when compared to patients with benign lesions and healthy controls. **CA 125** is found to be elevated in the saliva of oral, breast, and ovarian cancer patients.

Fibroblast growth factor 2 and its receptor 1 are significantly elevated in saliva of patients with salivary gland tumors. Salivary prostate specific antigen (PSA) levels correlate with serum PSA levels in patients with prostate adenocarcinoma. Other successfully validated proteins in various malignancies include M2BP, MRP14, CD59, profilin 1, and catalase.

### Diabetes Mellitus

A recent systematic review reported a significant relationship between salivary glucose concentration and associated glycemia/ HbA1c values in type 2 diabetes mellitus. In another study the salivary expression of pIgR, Arp 3, CA VI, and IL-1Ra was downregulated, whereas PLS-2, LEI, and IGJ chain appeared to be upregulated in diabetes mellitus. Studies have shown that reduced levels of salivary epidermal growth factor in diabetic patients may contribute to the development of oral and systemic complications of diabetes.[42]

### Autoimmune Diseases

Evaluation of reduced salivary secretion and measuring IgA autoantibody in Sjogren syndrome can serve as a diagnostic marker. Increased levels of lactoferrin, beta 2 microglobulin, lysozyme C, cystatin C and decreased levels of salivary amylase and carbonic anhydrase have been reported in Sjogren syndrome. Anti Ro60 and Ro52 antibodies have been detected in saliva of Sjogren syndrome patients with specificity above 90%. Measurement of salivary IgA antibodies can be useful in screening for celiac disease.[43]

### Hormones

Salivary hormone levels (both peptide and non-peptide) have been measured and have shown to be useful in endocrinopathies such as Cushing's syndrome.[2,13,33,34]

### Genetic Diseases

Cystic fibrosis (CF) occurs due to abnormality in Cystic fibrosis transmembrane conductance regulator (CFTR) protein caused by mutation in the CFTR gene. CFTR protein levels in saliva of CF patients are significantly higher than in healthy controls. 21-Hydroxylase deficiency is an inherited disorder of steroidogenesis which leads to congenital adrenal hyperplasia. Early morning salivary levels of 17-hydroxyprogesterone (17-OHP), determined by ELISA, is an excellent screening test for the diagnosis of non-classic 21-hydroxylase deficiency, since the salivary levels accurately reflected serum levels of 17-OHP. [2,13,33,34]

### Kidney Disease

Assessment of salivary creatinine levels can be used in diagnosis and monitoring of kidney failure. The various salivary markers associated with end stage renal disease included nitrite, phosphate, sodium, chloride, uric acid, cortisol, alpha-amylase, and lactoferrin.[44]

### Drugs Monitoring

Intake of Lithium and digoxin as well as substance abuse (alcohol, nicotine, cannabinoids, cocaine, phencyclidine, opioids, barbiturates, diazepines, and amphetamines) can be monitored using saliva. [2,13,33,34]

### Metabolic Bone Disorders

Alveolar bone loss has been reported to have a positive association with salivary concentrations of alkaline phosphatase, hepatocyte growth factor and interleukin-1 beta and a negative association with salivary osteonectin. Significant correlations have been found between age, body mass index, salivary levels of bone turnover markers [deoxypyridinium (D-PYR) and osteocalcin (OC)] and calcaneus T scores son bone matrix density test. This means that saliva can be used for assaying biomarkers of bone turnover. [2,13,33,34]

### Forensic Science

The salivary samples can be easily obtained from glasses, cigarettes, food products, envelopes, and other sources. Blood group antigens secreted into the saliva can be used for criminal identification and paternity determination. Identification of DNA in saliva by genetic profiling can be helpful in cases of sexual abuse. The foreign DNA tends to be present in the victim's saliva for as long as 60 minutes, providing a valuable piece of forensic evidence.[2,13,33,34]

### Occupational and Environmental Medicine

Measurement of heavy metals such as lead, mercury, and cadmium in saliva can be useful in monitoring environmental pollution.[2,13,33,34]

### Psychological Disorders

Increased levels of salivary amylase and cortisol and decreased levels of salivary IgA and lysozyme are markers of stress. Salivary testosterone levels have been correlated with the aggressive behavior and athletic activities.[2,13,33,34]

## Challenges

Expansion of salivary diagnostics is hampered by several factors as discussed below.[45-47]

### Mechanism of Salivary Diagnostics

The mechanism of how systemic diseases lead to the appearance of discriminatory biomarkers in saliva is largely unclear and need to be elucidated by more research.

### Lack of Baseline Reference Values

There is marked physiological variation in salivary biochemical substances and biomarkers due to diurnal rhythms, age, gender and genetic effects. The influence of diet, medication, smoking, and alcohol has to be ascertained. Enzymes derived from the host and oral bacteria can catalyze salivary proteins and change the composition and levels of biomarkers. The baseline reference ranges of salivary biomarkers need to be established within a healthy population to identify disease-specific changes.

### Correlation with Blood Levels

To be clinically useful, there must be reliable correlation between levels of the target substance in saliva and in blood or plasma. For example, glucose levels in saliva must correlate with blood levels, which is always not true in all studies.

### Low Concentration in Saliva than Blood

Some anylates in saliva are usually present at almost 1000-fold less concentration of that found in blood, therefore, very sensitive detection technology is required to develop effective tests. For instance IL-6 and IL-8 cytokines of potential clinical relevance are present at concentrations of only pg/mL. Tool kits and devices based on highly sensitive technology need to be developed, where keeping the cost to a minimum will be challenging.

### Preanalytical Variation and Need for Standardization

Proteins in saliva are more susceptible to biochemical processes and degradation as compared to serum and such changes can happen even during saliva collection and handling, which may compromise its clinical usefulness. Methodological variations in saliva collection and stimulation of salivary flow, handling, and storage conditions can influence the composition of saliva and thus, make the widespread adoption of salivary diagnostics, challenging. Standardization of methods, involved in collection, processing and storage of saliva, is essential, so that all the research findings among different research groups can be compared and validated. Commercial saliva collection devices available for diagnostic and research purposes need to be tested for their efficacy and sensitivity. For example, one study reported that the OraSure saliva collection device detects hepatitis C virus with greater sensitivity than the Salivette device.

### Contamination of Saliva

Samples with even a small amount of blood will lead to a false-positive result. In fact, bleeding after brushing or flossing occurs quite frequently and could contribute to high false-positive rates for salivary tests.

### Lack of Specific and Standardized Analytical Tools

The efficacy of various analytical tools may vary in detection of micro quantities of proteins, proteins of varying molecular weights in health and disease. The development of specific and standardized analytical tools is required in randomized controlled trials.

### Selection of Biomarker Panel

One biomarker alone may not be reliable to identify a disease. The use of a panel can provide high diagnostic sensitivity and specificity. Both, whole proteome-wide application and target specific biomarker discovery are required to determine such clinically relevant biomarker panels. The findings of different research groups sometimes concur and sometimes do not. Many validation studies require large number of subjects, to ensure that the most reproducible markers are developed.

## Future Perspective

Saliva based diagnostics have immense clinical potential with rapidly advancing technology and participation of all stakeholders.[48-50]

### Expansion of Diagnostic Spectrum

As our knowledge of the biomolecules present in saliva grows, its potential application for oral and systemic disease diagnosis will expand. It is hoped that more and more reliable, specific and sensitive salivary biomarkers may be found soon.

### Lab Chip Technology

Novel diagnostic devices based on nanotechnology (e.g., microfluidics-based chips), are being developed for simple and instant measurement of the biomarkers. It may become

possible to attach a tiny device to a patient's tooth, allowing personalized monitoring of medication levels and the detection of biomarkers for specific disease states. The fully integrated automated diagnostic systems will have the potential to measure several biomarkers in saliva instantly and making early detection of many diseases through saliva a reality in the near future.

### Point of Care Testing (POCT)

Currently, there are no saliva-based POCT devices that enable rapid diagnosis and/ or screening of diseases. Portable devices of the smallest size possible are being made that can be used as home testing kits or at the general practitioner's office and will provide immediate results to patients.

### Stakeholders' Responsibility

The parties responsible for translating research to clinical practice, including scientists, regulatory agencies, and third parties such as insurance companies, will need to work together to help adoption of new saliva diagnostics in the field.

## Conclusion

Current technological advances will provide fast measurement of a wide range of salivary biomarkers with high sensitivity and specificity. These can be used for detecting common cancers, viral, and bacterial diseases, cardiovascular and metabolic diseases, and general nutritional deficiencies. Early detection of diseases through saliva, especially for life threatening diseases, can improve survival and prognosis.

There is need to address a lack of standardization for saliva sample collection, processing, and storage; wide variability in the levels of biomarkers in both normal and diseased individuals. Once these challenges are met, saliva based diagnostics have the potential to become important component of routine health monitoring, early detection of diseases through population based screening programs, confirmatory diagnosis of diseases, risk stratification, determination of prognosis, and therapy response monitoring which is a highly desirable goal in healthcare management.

Saliva based tests are noninvasive, technically simple requiring minimal training, and inexpensive due to the low cost of collecting and processing samples. These are particularly useful for developing countries. Next few years will witness an evolving spectrum of salivary screening and POCT kits for home testing will begin to appear.

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