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Salivary Proteomics: Current Status & Future Perspectives

[1] Saroj Kumar Rath
[2] Lt Co (Dr) Manab Kosala
[3] Brig S K Ray
[1] Professor
[2] Senior Lecturer
Dept. Of Periodontics
[3] Professor, Dept. Of Oral & Maxillofacial Surgery
Army Dental Centre, Delhi Cantt
Address For Correspondence:
Col S K Rath
Army Dental Centre,
Research And Referral Delhi Cantt 110010
EmailID : drrathfamily@yahoo.com
MobileNo : 08800507126
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Abstract
Analysis of saliva may offer a cost-effective approach to assessment of oral and systemic diseases . Apart from proteomic markers, saliva is a pool wherein various genomic and microbiological markers are also found. Currently, inflammatory mediators and other host-derived enzymes hold the greatest promise as salivary diagnostic tests for oral diseases. Long-term longitudinal studies, however, are required to quantify the relationship between specific markers with progression of any oral disease. The biomarkers can be obtained from blood components such as: serum or plasma. However because of it's being an invasive procedure other body fluids such as saliva and GCF are being considered for potential source of biomarkers. The simple and non- invasive nature of saliva collection and its high sensitivity assay development has led to the salivary biomarkers being a promising future for periodontal diagnosis. Further technological advancement and identification of robust and discriminatory sets of salivary biomarkers is necessary to fulfill all requirements for being regular diagnostic tool for the everyday clinical practice. Despite limitations we experience today, the use of saliva for diagnostic purposes becomes increasingly popular, and as a result, more and more diagnostic tests become commercially available, and are currently used by clinicians and researchers.

Key Words
Biomarkers, Saliva, Proteomic

Introduction

Saliva is simple, non-invasive, readily available body fluid and easily collected without specialized equipment or personnel. For the past two decades, saliva has been increasingly evaluated as a diagnostic fluid for detecting breast cancer, oral cancer, caries risk, salivary gland diseases, periodontitis, and systemic disorders such as hepatitis C and the presence of human immunodeficiency virus (HIV). It may reflect levels of therapeutic, hormonal, and immunologic molecules and can yield diagnostic markers for infectious and neoplastic diseases. Various mediators of chronic inflammation and tissue destruction have been detected in whole saliva of patient with oral diseases.

Proteomics, an emerging field of biochemistry, is the study of the complete set of proteins in an organism, tissue or cell and the interactions between these proteins. The first step in this process is the separation and identification of the thousands proteins in the sample. Proteomics then focuses on determining the structure, function and interactions of between them. Comparative studies can lead to useful observations on diseases and to targeted new treatments.

The word "proteome" is derived from "protein" and "genome", and was pioneered by Marc Wilkins in 1996[1]. The term "proteomics" was first coined in 1997[2] to make an analogy with genomics, the study of the genes. In simple terms, proteomics is defined as the study of all proteins including their relative abundance, distribution, post translational modifications, functions and interactions with other macromolecules, in a given cell or organism within a given environment and at a specific stage in the cell cycle.

Saliva collection is far more practical and safe compared with invasive methods of sample collection, because of the infection risk from contaminated needles during, for example, blood sampling. Furthermore, the use of saliva could increase the availability of accurate diagnostics for remote and impoverished regions. However, the development of salivary diagnostics has required technical innovation to allow stabilization and detection of analytes in the complex molecular mixture that is saliva. The recent development of cost-effective room temperature analyte stabilization methods, nucleic acid pre-amplification techniques and direct saliva transcriptomic analysis have allowed accurate detection and quantification of transcripts found in saliva. Novel protein stabilization methods have also facilitated improved proteomic analyses. Although candidate biomarkers have been discovered using epigenetic, transcriptomic, proteomic and metabolomic approaches, transcriptomic analyses have so far achieved the most progress in terms of sensitivity and specificity, and progress towards clinical implementation.

The post genomic era provides opportunities for simple and parallel approaches to genomics and proteomics. There has been progress made in the field when saliva is used as a body fluid within portable devices[3],[4]. In this review, we summarize current knowledge about the salivary proteome using mass spectrometry (MS) based technologies and future perspectives.

Analysis of the Saliva Proteome

Human saliva contains a plethora of compounds that can be informative for monitoring overall health and well being, disease pathogenesis, and oral health out of which protein plays a very important role. Comprehensive analysis and identification of the proteomic content in

human saliva targets at identifying the proteins that display biological activity at the glandular level and/or under various pathological conditions. The biochemical techniques such as Liquid Chromatography (LC), gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, Mass Spectroscopy (MS), immunoassay, and lectin probe analysis [5],[6] have been widely used in saliva proteome work.

For identifying the proteins present in parotid glandular saliva, Hardt et al. [7] used 2D SDS-PAGE to separate proteins before MS analysis. These methods have been used to identify peptides in the ranges of 1–6 k Da (histatins, cystatins, and PRPs) as well as proteins with middle to high relative molecular mass.

When combining LC-MS with 2Dimensional – MS (2D-MS), researchers have identified more than 1050 proteins in whole saliva [5]. This combination is particularly suitable for the separation and identification of proteins and peptides of low relative molecularmass [8]. Investigators conducting proteomicstudies of human saliva have characterized 4major types of salivary proteins: PRPs, statherins, cystatins,and histatins [9]. These proteins play major roles in maintaining the integrity of tooth structures in the oral cavity, particularly by controlling the equilibrium between demineralization and re mineralization of the tooth enamel [10].

Global analysis of human whole saliva (WS) [5],[11] as well as saliva from individual glands, has revealed protein profiles indicative of each gland type [7], [12]. WS was the focus of many of the early proteomic studies. Schipper et al. identified approximately 1100 proteins (approximately 650 in parotid / submandibular and approximately 50 in sublingual saliva), and Denny et al. identified approximately 1166 proteins in WS (914 in parotid and 917 in submandibular/sublingual saliva) [12]. Recent work by many laboratories has catalogued a total of 2290 proteins in WS, and approximately 27% of plasma proteins are found in human saliva.

Recently, advances in MS technologies and high resolution nanoflow LC have produced MS-based proteomics known as shotgun proteomics. This strategy involves protein digestion before or after purification by chromatography [13],[14],[15]. More recently, MS-based methods combined with multi dimensional chromatography have been applied in the study of extremely complex protein/ peptide mixtures, including cancer tissues and blood [16],[17] Wilmarth et al. reported the successful use of applied two-dimensional liquid chromatography (2DLC)[18] and combined capillary isoelectric focusing/nano-RPLC [19], [20] to greatly extend the human saliva proteome.

More recently, three groups based at the University of California San Francisco, University of California Los Angeles and the Scripps Research Institute/University of Rochester, became involved in a salivary proteome consortium to build a human salivary protein catalogue, funded by the National Institute of Dental and Craniofacial Research (NIDCR). The three groups independently studied the proteome using different samples, sample preparation methods and MS-based analysis [21],[22]. The huge amount of data can be accessed from the Salivary Proteome Knowledge Base (PSKB, http://hspp.dent.ucla.edu/cgibin/ spkbcgi-bin/main.cgi) as a saliva diagnostic atlas.

Quantitative Proteomics of the Saliva

Quantitative protein profiling of relative protein abundance between different samples is an important technology in determining candidate disease biomarkers and to increase our knowledge in systems biology. Generally, three major methods have been used in quantitative proteome profiling.

The first method is 2DE based, in particular, 2D difference gel electrophoresis (2DDIGE), [23] which is a powerful tool in quantitative proteome profiling. Fleissing and co-workers reported the salivary protein profile in patients with Sjogren's syndrome and healthy control subjects using this method [14].

The second method is a label–free direct semi-quantitation method, based on LC-MS chromatogram data. The mass peak intensity difference between two different saliva samples such as oral cancer [24] is used to quantify the relative protein abundance. Most recently, Rao and coworkers applied this label–free method to determine salivary protein biomarkers in human type 2 diabetes [25], [26].

The third method is an isotope labeling strategy, based on stable isotope labeling by metabolic labeling, specific chemical probes and enzymatic transfer of the 18O atom from heavy water to the C-terminus of peptides. Unfortunately, the SILAC method is not applicable for use in biological fluids, including blood, plasma and saliva.

In contrast, isobaric labeling methods, such as the isobaric peptide Tags for Relative and Absolute Quantification (iTRAQ) reagent,[27] are based on differential labeling of N-termini and lysine residues from digested peptides. iTRAQ labeled peptide produces specific fragment ions (reporter ions) , which have a different mass; using MS/MS, relative peptide abundance can be compared via the intensity of reporter ions. Large scale analysis of the abundance of low molecular weight peptides (<10 kDa) secreted from human parotid saliva during the day were analyzed using iTRAQ technology [28] The peptides were categorized into up–or down–regulated groups as the concentrations oscillated throughout the day. The study also identified and measured the relative abundance of a small peptide derived from histatin.

Recently, breast cancer related proteins were discovered from differential whole saliva proteome analysis between healthy women and those diagnosed with a benign breast tumor, using the iTRAQ method [29], [30].

Salivary Analysis for Pathological conditions

1. Hereditary diseases

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 [31].

2. Autoimmune diseases

Sjögren's syndrome is an autoimmune disease where serum chemistry can demonstrate polyclonal hypergammaglo bulinemia and elevated levels of rheumatoid factor, antinuclear antibody, anti-SS-A, and anti-SS-B antibody. In addition, increased concentrations of sodium and chloride, IgA, IgG, lacoferrin, and albumin, and a decreased concentration of phosphate were reported in saliva of patients with SS [32].

3. Malignancy

Salivary analysis may aid in the early detection, screening and monitoring of prognosis of certain malignant tumors.

p53: It is a tumor suppressor protein which is produced in cells exposed to various types of DNA-damaging stress which can be detected in the saliva of patients with oral squamous cell carcinoma (SCC).

Defensins: Elevated levels of salivary defensin-1 were found to be indicative of the presence of oral SCC [33].

CA15-3, c-erbB-2: Elevated levels of recognized tumor markers c-erbB-2 (erb) and cancer antigen 15-3 (CA15-3) were found in the saliva of women diagnosed with breast cancer [34].

CA 125: Itis a tumor marker and its elevated salivary levels were detected in patients with untreated breast cancer . A positive correlation was also found between salivary and serum levels of CA 125 [35].

Higher levels of salivary nitrate and nitrite, and increased activity of nitrate reductase, were found in oral cancer patients compared with healthy individuals [36].

4. Infectious diseases

Saliva contains immunoglobulins (IgA, IgM, IgG) that originate from two sources: the salivary glands and serum. Antibodies against viruses, bacteria, fungal and parasite can be detected in saliva and can aid in the diagnosis of infections.

Viral Diseases

HIV Antibody to HIV in whole saliva of infected individuals was detected by ELISA and Western blot assay, correlated with serum antibody levels [37]. Salivary IgA levels to HIV decline as infected patients become symptomatic. It was suggested that detection of IgA antibody to HIV in saliva may, therefore, be a prognostic indicator for the progression of HIV infection.

Orasure is the only FDA- approved, commercially available testing system. It detects antibodies against the p24 antigen of HIV. The applicator swab is gently rubbed along the outer gumsand inserted into a vial containing the developer solution that detects the antibody to p24 antigen of HIV [38]. In conclusion, collection and analysis of saliva offer a simple, safe, well-tolerated, and accurate method for the diagnosis of HIV infection.

Applicable for both clinical use and epidemiological surveillance Hepatitis Saliva was found to be a useful alternative to serum for the diagnosis of viral hepatitis. Acute hepatitis A (HAV) and hepatitis B (HBV) were diagnosed based on the presence of IgM antibodies in saliva. Hepatitis-B virus DNA revealed by PCR in saliva. Quantitative detection of DNA used to evaluate level of virus in the body copy for judgement of infection. It also point to the possible role of saliva as a source of HBV infection [39],[40]. Saliva has also been used for screening for hepatitis B surface antigen (HbsAg) in epidemiological studies.

Saliva may also be used for determining immunization and detecting infection with measles, mumps, and rubella [41]. For newborn infants, the salivary IgA response was found to be a better marker of rotavirus (RV) infection than the serum antibody response. Salivary antibodies could be used to monitor the immune response to vaccination and infection with RV [42]. Reactivation of herpes simplex virus type-1 (HSV-1) is involved in the pathogenesis of Bell's palsy and PCR based identification of virus DNA in saliva is a useful method for the early detection of HSV-1 reactivation [43]. 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 useful markers for infection [44].

5. Detection of drugs

Saliva has been proposed for the monitoring of systemic levels of drugs. Saliva is useful for the monitoring of anti-epileptic drugs and anti-cancer drugs [45].

Saliva sampling, handling and storage

Salivary proteins are synthesised in the serous and mucous acinar cells of salivary glands, where post-translational processing occurs, involving glycosylation, phosphorylation and sulfation [46]. These post-translational modifications are involved in many of the physiological functions of salivary proteins. Additionally, secretion of saliva into the oral cavity results in the exposure of the proteins to a high number of proteolytic and other enzymes originating from oral microbes, epithelial cells and from leukocytes, which enter the mouth via the gingival crevice [47]. These enzymes rapidly break down many of the salivary proteins into peptides, as well as deglycosylate and dephosphory late them. Such modifications may be particularly important, since they change the native salivary proteome after collection and therefore might also change the success of biomarker detection[47]. To avoid changes of proteins and peptides in salivary secretions, standardised salivary sampling protocols and storage conditions need to be applied as earlier emphasised for humans [47],[48],[49]. A study using porcine saliva showed that changes in protein composition occur, when saliva is stored at - 20 °C [50]. Therefore, working on ice for not more than 1 hand subsequent freezing of samples at - 80 °C for long-term storage are considered the safest and most practical handling protocol to date [50],[51],[52],[53]. Another issue in regard to biomarker search is the origin of the collected secretions, i.e. whether it is glandular or whole saliva and if stimulated or unstimulated saliva is being used [54], [55].

Conclusively, appropriate collection procedures need to be considered for the analysis of different types of salivary analytes. It is important to keep in mind that it may not be feasible to assume that saliva samples collected using one particular method can be assayed for multiple markers. Researchers with intentions of analyzing multiple biomarkers in saliva should conduct pilot testing to verify that different collection procedures do not interfere with anticipated assay procedures [56].

Salivary Proteomics for Periodontal Diseases

Saliva contains biomarkers specific for the unique physiological aspects of periodontitis, and qualitative changes in the composition of these biomarkers could be diagnostic. It contains a wide variety of periodontal proteomic markers from immunoglobulins to bone remodeling proteins.

Interleukin (IL) 1α is a proinflammatory cytokine and its levels correlated significantly with periodontal parameters after adjusting for the confounders. Moreover, combined levels of Il-1α and matrix metalloproteinase (MMP)-8 increased the risk of experiencing periodontal disease by 45 folds [57].

MMP-8 a key enzyme in extracellular collagen matrix degradation, derived predominantly from PMNs during acute stages of periodontal disease also correlated significantly with periodontal activity even after adjusting for the confounders, Moreover, its presence significantly increased the risk of periodontal disease (odds ratios in the 11.3-15.4 range) [57]. MMP-8 is an indicator of disease severity and activity. MMP-1 (interstitial collagenase) also appeared to be activated in periodontitis [57]. Higher levels of other MMPs, including MMP-2, MMP-3 and MMP-9, were also reported in the saliva of patients affected by periodontitis.

Immunoglobulin (Ig): Patients with periodontal disease are shown to have higher salivary concentrations of Ig A, Ig G and Ig M specific to periodontal pathogens compared with healthy patients[58]. Also the values decrease significantly post treatment.

Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP): A significant positive correlation between salivary ACP and calculus formation has been found[59]. It was found that mixed whole saliva of adult periodontitis patients revealed the highest enzyme activities [60] and the increase in salivary ALP activity in periodontitis can be associated with alveolar bone loss.

Esterase, Lysozyme, Lactoferrin: A statistically significant, positive correlation between salivary esterase and calculus formation was observed [59] and the esterase activity of whole saliva was higher in individuals with periodontal disease[60]. Moreover periodontal treatment reduced its levels. Hence the efficacy of periodontal treatment may be readily monitored by changes in levels of activity of specific enzymes like esterase in whole saliva [61].

Patients with low levels of lysozyme in saliva are more susceptible to plaque accumulation, which is considered a risk factor for periodontal disease. Lactoferrin is strongly up-regulated in mucosal secretions during gingival inflammation and is detected at a high concentration in saliva of patients with periodontal disease compared with healthy patients [62].

Conclusions and Perspectives

With advances in genomics, transcriptomics and proteomics of saliva, salivary testing in clinical and research settings is rapidly proving to be a practical and reliable means of recognizing several systemic and oral conditions. However, further detailed studies establishing the diagnostic value of saliva in comparison with that of other biomedia, (especially with blood) will be necessary to assess the detailed prognostic and diagnostic value of saliva. At this stage of knowledge, saliva seems to be a highly important possible tool for regular screening of larger populations. However, it may also turn out in many cases that saliva is as accurate (or even better) as blood in establishing a definitive diagnosis of certain disorders and monitoring disease progression. However, the road to practical and effective regular use of salivary diagnostics is expected to be promising, but long. For the regular clinical use the analysis should be highly automatized, and coupled with microfluidic technology, enabling a small sample size to be used, avoiding reagents' and waste's cost and allowing that types of assays that are impossible at the macroscopic level . The identification of biomarkers with a proper and definite sensitivity and specificity to as many disorders and conditions as possible is also a prerequisite. Thus, further technological advancement and identification of robust and discriminatory sets of salivary biomarkers is necessary to fulfill all requirements for being regular diagnostic tool for the everyday clinical practice. Despite limitations we experience today, the use of saliva for diagnostic purposes becomes increasingly popular, and as a result, more and more diagnostic tests become commercially available, and are currently used by clinicians and researchers. Taking together all these aspects, it can be concluded that there are rich possibilities in saliva-diagnostics already at present, and the immediate future of this area is even more promising.

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