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Review Article

Salivary Assessment in Dental Practice

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Abstract

This brief article reviews some of the recent advancements in the use of saliva to identify a number of systemic diseases, viral infection, oral cancer, and periodontal disease. Saliva, like blood and urine, contains a wealth of constituents that can serve as potential biomarkers of disease including DNA and RNA fragments, proteins, proteomes, hormones, and antigens. The development of 'point-of-care' devices that can detect well defined biomarkers reflecting disease will provide immediate feedback in the clinical, research, or public health setting. This represents the future of salivary diagnostics.

Keywords: *Salivary Assessment; Dental Practice; 'Point-of-Care' Devices*

Introduction

One million dollars was recently awarded (April, 2017) in the QualComm XPrize competition for advancement of medical diagnostics to a group of scientists out of Taiwan who developed a portable lab using biomarkers that is able to identify 13 systemic diseases using blood and urine fluid (https://tricorder.xprize.org/) [1]. Like these biologics, saliva is also on the cusp of becoming an important diagnostic tool for the assessment of oral as well as systemic diseases. The potential for saliva as a diagnostic aide was recognized in 2002 by the US based National Institute of Dental and Cranio-facial Research (the NIDCR). Consequently, research groups throughout the US and other countries have initiated studies that seek to elucidate the diagnostic possibilities associated with saliva assessment [2-4]. This review article briefly outlines some of the more interesting innovations in salivary biomarker research and comments on the future of this diagnostic in the context of Dentistry.

Saliva constituents useful as salivary markers

A number of salivary molecular biomarkers listed in figure one have the potential to be indicators for systemic conditions such as cancer (including oral cancer), periodontal disease, autoimmune disease, viral and bacterial disease, and cardiovascular disease. Gene testing can reveal abnormal nucleic acids and proteins in oral bacteria and existing within and between cells that are found in saliva. Saliva also contains hormones, antigens, and antibodies. What makes saliva such a valuable target for molecular diagnostic research is that it is easy to retrieve and collection does not involve discomfort.

- DNA, ctDNA, RNA, meta-RNA, RNA fragments
- Minor proteins
- Nucleic acids
- Peptides
- Proteomes (the entire protein complement expressed by a cell)
- Hormones, Antigens, Antibodies, Drugs

Figure 1

Recent Studies Supporting Saliva Diagnostics

Brain research

As reported May 6-9, 2017 in an abstract presented at a Pediatric Academic Societies Annual Meeting in San Franciso, and subsequently in the Journal of Neurotrauma [5], micro-RNA fragments found in saliva were found to be predictive of pediatric brain concussion in children with traumatic brain injury. Micro-RNA fragments are short (length: 19 - 24 nt) non-coding molecules that are important in regulating numerous cellular processes including those involving the brain [6]. This preliminary research suggests that salivary microRNA (miRNA), easily acquired and measured in the clinical setting, may be useful for identifying pediatric traumatic brain injury (TBI). As the authors indicate, concentrations of miR-320c (one of many fragments) were directly correlated with child and parent reports of attention difficulty. The test was shown to be 90 percent accurate. This is in contrast to a concussion survey now commonly used that has less than 70 percent accuracy. However, additional studies assessing the influence of orthopedic injury and exercise on peripheral miRNA patterns are needed in adults and children to further understand the potential utility of this strategy for diagnosing concussion.

Systemic Diseases

Researchers have identified potential salivary transcriptomic profiles for other diseases such as acute myocardial infarction, diabetes mellitus, Sjögren's syndrome, cystic fibrosis, and Parkinson's disease in addition to breast, pancreatic, ovarian, gastric and lung cancer and melanoma [7].

- Acute MI
- Diabetes mellitus
- Sjögren's syndrome
- Cystic fibrosis
- Parkinson's disease
- Cancers: breast, pancreatic, ovarian, gastric, lung, and melanoma

Figure 2: *Diseases with Identified Salivary Transcriptomic Profiles.*

A good salivary assay for identifying systemic disease should have high sensitivity, specificity, and functionality. The literature reflects an optimism that these fundamentals will be met in the future but much work remains to be done before assessment of salivary proteins and nucleic acids yields meaningful diagnostic capability. Nonetheless, research to date has revealed some interesting results with respect to salivary proteomes and some systemic diseases.

Sjögren's syndrome

Saliva constituents appear to be altered in Sjögren's syndrome, an autoimmune disorder that causes, among other symptoms, dry mouth and dry eyes. In one preliminary study, twenty five proteins in whole saliva, including α-enolase, carbonic anhydrase I and II, and salivary α-amylase fragments in patients with Sjögren's Syndrome were found to be up-regulated and sixteen proteins expressed by the acinar cells of the glands, including lysozyme C, polymeric immunoglobulin receptor (pIgR), and calgranulin A were down-regulated as compared with the salivary proteomes of individuals without disease [8]. In another study 15 proteins in whole saliva differentiated non-SS subjects from those with SS [9]. There is also evidence that the microRNA profiles of minor labial salivary glands differ between normal subjects and those with SS [10]. These accumulated studies suggest that proteomic salivary constituents (biomarkers) may prove useful in the development of a diagnostic panel that can be used on site to identify patients with Sjögren's syndrome.

Oral Squamous Cell Carcinoma

In recent years there has been a focus on miRNAs in saliva as biomarkers of oral cancer because these cellular fragments are known to have an effect on cell growth, proliferation, and apoptosis and also appear to serve as oncogenes within different cancer types [11-13]. Multiple studies suggest that miRNAs may prove useful in early detection of oral cancer and could potentially lead to changes in how oral cancer is treated [12]. Yoshizawa and Wong provide a good overview of oral cancer detection and salivary miRNAs [15]. As they state: "Combined with transcriptomic and proteomic approaches, miRNA represents the third diagnostic alphabet in saliva".

In a study funded, by the 'Early Disease Research Network' a working group within the National Cancer Institute, salivary biomarkers were assessed for their utility in discriminating patients with oral cancer from healthy subjects [13]. In this case controlled study utilizing two independent laboratories, 395 subjects from five independent cohorts were assessed. The result was that seven mRNAs and three proteins were found to be increased in oral squamous carcinoma cancers (OSCC) versus controls in all the cohorts. Specifically, the increase in two markers: IL-8 and SAT demonstrated good sensitivity and specificity in predicting disease. These biomarkers were found to effectively discriminate patients with oral OSCC from healthy controls.

Other studies have identified additional proteomic salivary biomarkers potentially useful in detecting OSCC [16]. In a comparison of sera with saliva in patients with OSCC, three tumor markers: specifically Cyfra 21-1, tissue polypeptide antigen (TPA), and cancer antigen CA125 were found to be significantly more elevated in the saliva of diseased subjects [17]. And results of an additional study suggest that five salivary proteins (M2BP, MRP14, profilin, CD59, and catalase) can discriminate oral cancer with 90% accuracy [18].

Additional research will help to fully elucidate candidate proteins that can be used to indicate the presence of OSCC [19-22]. Whether assessment of salivary biomarkers will be helpful in also defining oral cancer disease severity and progression remains unclear but the discovery of specific biomarkers that can be used to diagnose cancer presents a real step forward in salivary diagnostics.

Other Cancers

A number of cancers, including those involving the ovaries, endometrial tissues, fallopian tubes, the pancreas, stomach, esophagus, colon, liver, and breast demonstrate an elevation of the cancer antigen 125 (CA 125) in blood. This protein biomarker has also been found in at least one study to be elevated in the saliva of individuals with malignant ovarian tumors. Saliva CA 125 levels were correlated with serum levels in subjects with ovarian cancer in terms of sensitivity and specificity (81.3 and 93.8 respectively). And in patients with endometriomas and pelvic tuberculosis the false positive rate was significantly lower for saliva CA 125 than serum CA 125 (13.6, 10% versus 72.7, 80%) [23] suggesting that saliva CA 125 may have better diagnostic value for these conditions than CA 125 found in serum. However, subsequent research results are in conflict with the above findings and suggest a lack of relationship between CA 125 levels and epithelial ovarian cancer and benign gynecologic conditions [24]. More recent research suggests that better specificity and sensitivity of CA125 as a tumor biomarker may occur when the assay is combined with another biomarker HE4 [25] and the presence of microRNAs may also help in determining therapeutic response [26]. The introduction of FDA-approved algorithms is reported to have improved the ability to assess risk of ovarian cancer from sera of patients with a pelvic mass. The extent to which this applies to the same markers found in saliva remains unclear. Arellano., *et al*. [27] and Sannam., *et al*. [28] provide excellent reviews covering the advancements in the identification of OSCC via salivary proteomics.

Viral Disease

With advancement of methodological techniques able to identify viral DNA, RNA, microRNA proteins or salivary antibodies, a number of viruses can now be identified in saliva. These include norovirus, rabies, human papillomavirus (HPV), Epstein-Barr virus, herpes simplex viruses, hepatitis C virus, cytomegalovirus (CMV), and HIV [29]. With the Morbillivirus virus that causes measles infection, the presence of salivary antibodies demonstrates 97% sensitivity and 100% specificity [30], for Paramyxoviridae virus that causes mumps 94% sensitivity and 94% specificity [31], and for the Togaviridiae virus that causes rubella, 98% sensitivity and 98% specificity [32].

Antibodies used to diagnose HIV infection are also found in saliva and salivary assays have been shown to be as accurate as those associated with serum, particularly when plasma virus exceeds 50 copies/mL (when there is active disease) [33]. A commercial product called OraQuick has been FDA approved and is available for assessing HIV antibodies in saliva [34,35]. The testing kit contains a collection stick, test tube, and testing information/directions. It is reported to be able to detect antibodies to HIV-1 and HIV-2 within 20 minutes [36,37].

Periodontal disease

In a move towards establishing more precise diagnosis of periodontal disease a number of salivary constituents have been considered as potential biomarkers including DNA from specific bacteria, inflammatory cytokines that are host-derived, cell death host-derived proteins, and enzyme, protein, or calcium derived factors from bone destruction [38]. Figure three lists the many biomarkers associated with periodontal disease.

- Bacteria-derived DNA salivary biomarkers (*Porphyromonas gingivalis, Prevotella intermedia*, and *Tannerella forsythia*)
- Host-derived inflammatory biomarkers (inflammatory cytokines (IL-1β and MIP-1α)
- Host-derived biomarkers associated with soft tissue destruction (MMP-8, MMP-9, HGF, lactate dehydrogenase, aspartate aminotransferase, and TIMP-2)
- Host-derived biomarkers associated with bone destruction (alkaline phosphatase, osteonectin, RANKL, and calcium)
- Among the various salivary biomarkers listed, *P. gingivalis* has been shown to satisfy all of the requirements for an ideal biomarker of periodontitis

Figure 3: *Validated Salivary Biomarkers for Periodontal Disease.*

Presently periodontal disease remains a clinical diagnosis established through visual examination, periodontal probing of the gingival sulcus, and evaluation of radiographic imaging to detect bone loss. For public health and research purposes, the Community Periodontal Index (CPI) that includes a periodontal 'probe' and rating system defining pocket depth was developed and adopted for use by the World Health Organization [39]. Elements of the rating instrument, including a version of the probe, currently represent the standard of care in clinical practice in establishing a diagnosis of periodontal disease and its severity [40]. Tracking periodontal disease progression, however, remains an elusive problem in clinical care.

Recent advances in salivary research suggest that the diagnosis of periodontal disease and its progression may be effectively tracked via integration of biologic measures (e.g. the presence of specific biomarkers in saliva) with standard clinical and radiologic measures [41]. Ebersole., *et al*. in a case controlled study of 209 subjects, evaluate a specific kit (the Milliplex Map Kit – EMD Millipore, Billerica, MA, USA) to detect saliva analytes related to the biological processes of periodontitis. IL-1β and IL-6 (both cytokine inflammatory signals), MMP-8 (a primary collagenase), and MIP-1α (also known as CCL3 -a chemokine macrophage inflammatory protein) isolated alone or in combination, were found to distinguish healthy subjects from those with gingivitis and periodontitis. Their findings suggest that the salivary level of MIP-1α could have clinical utility as a screening tool for identifying moderate to severe periodontal disease and that sensitivity, specificity, and accuracy may be improved by exploring combinations of the identified biomarkers.

Discussion and Conclusion

Other salivary constituents including the enzymes aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase (LD), and alkaline phosphatase have also been considered useful as biomarkers for diagnosing and screening periodontal disease [42]. Given that lactate dehydrogenase is related to epithelial cell breakdown, a 'kit' allowing quick assessment of this enzyme was tested on 70

healthy volunteers against standard periodontal examination. Authors Ekuni D., *et al*. report that the salivary LD level was positively correlated with bleeding on probing and that the sensitivity and specificity of the kit was 0.89 and 0.98 respectively, at a cut-off value of 8.0 for LD level. Although the study was limited because it was cross sectional, it is concluded that the evaluated 'kit' could have utility in the early detection of gingivitis [43].

Additional studies suggest that assessment of salivary biomarkers may also help in determining treatment response, with some biomarkers more important than others in defining efficacy. In the study by Syndergaard., *et al*. mean biomarker concentrations were found to decrease in the gingivitis groups following dental prophylaxis. However, certain markers, specifically MIP-1α and PGE2, remained significantly higher in the healthy group [44]. Based on their results, the authors conclude that relative change in these biomarkers may prove helpful in identifying diseased patients who, despite prophylaxis, might be at risk for continued chronic gingival inflammation and the development of more destructive periodontal disease.

With respect to disease progression, the accumulated research evidence suggests that a panel of optimal biomarkers has to be carefully selected based on the pathogenesis of periodontitis. The biggest hurdle for the diagnosis and tracking of periodontitis progression using saliva may be validating specific disease related biomarkers as well as the efficacy of point-of-care devices within large diverse patient populations. Although necessary, this research has not been completed to date. Effort has been made to put together an organization, the International Consortium for Biomarkers of Periodontitis that could accumulate large amounts of data to identify, select, and validate salivary biomarkers. The use of biomarkers in saliva coupled with point-of-care devices and clinical examination represents an important step forward in the diagnosis of periodontal disease and assessment of treatment response.

There are currently two point-of-care devices that have been developed for the salivary diagnosis of periodontitis: one is called the Integrated Microfluidic Platform for Oral Diagnostics (IMPOD), the other is a lab-on-a-chip (LOC) system developed by the University of Texas. The IMPOD measures MMP-8 (a neutrophil collagenase, also known as matrix metalloproteinase-8), TNF-α (a tumor necrosis factor), IL-6, and CRP (C-reactive protein) in saliva and the LOC measures CRP, MMP-8, and IL-1β. The LOC has shown good comparative accuracy with the enzyme-linked immunosorbent assay (ELISA). The LOC device is currently undergoing clinical trials: (NCT02403297 at ClinicalTrials.gov) [45].

The future of Salivary Diagnostics

Developments in the field of salivary diagnostics should lead to significant advances in point-of-care precision dentistry. The benefits of using saliva as a biomarker for disease are multiple. Collection of fluid is non-invasive, simple, and easily accomplished by the patient or in the clinical setting. Relative to collection of serum it is inexpensive, and clotting is not a problem as it is with serum. Saliva contains physiological markers for many conditions, both systemic as well as those localized to the oral environment [46]. Salivary diagnostic assessment will also allow for the screening of patients outside the clinical setting, for purposes of treatment monitoring, and for epidemiological research or public health screening. Combining point-of-care devices such as those currently available or those being developed for assessment and monitoring of periodontal disease and oral cancer, as well as systemic diseases, coupled with improved medical and dental electronic software communication could lead to more accurate disease tracking of patients by providers via the internet and through digital charting. As pointed out by Yager., *et al*. "underserved communities and resource-limited areas may be accessed more efficiently than by current cumbersome and poorly utilized screening programs" [47]. And the use of salivary diagnostics may increase access to treatment for identified at-risk individuals not aware of developing disease.

However, current development of medical micro-devices utilizing saliva as a substrate for identifying disease continues to be limited by a number of factors: definitive molecular biomarkers for many diseases of interest are not available; fluid volumes remain a limiting factor; RNA fragment amplification remains a problem; and the sensitivity and specificity for many salivary markers needs confirmation. As Wong (monitoring editor) and Giannobile., *et al*. point out in an AADR research symposium on the subject in 2011, "Despite remarkable progress toward POC clinical assay systems, few complete working prototypes have emerged. Although promising starts have been made

with microfluidic LOC approaches, and important goals have been defined with the micro-total analysis system (μTAS) paradigm, the broad-scale release of workable devices has yet to be achieved" [48]. Nonetheless, there are six major clinical trials assessing a programmable bio-nano-chip (PBNC) that can assay nucleic acids, proteins, and cells in saliva specific to several pathologic conditions including cardiac heart disease, oral cancer, ovarian cancer, and prostate cancer and there is one device currently available for assessing periodontal disease. The future looks bright for salivary diagnostics [49-51].

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