Review Article

Salivary Assessment in the Diagnosis of Oral and Systemic Disease

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Received Date: September 11, 2020
Publication Date: October 01, 2020

Abstract

Recent scientific advancements have been made in the use of saliva to identify several systemic diseases, oral cancer, periodontal disease, and viral infections - including Covid-19. Saliva contains a wealth of constituents that can serve as potential biomarkers of disease including DNA and RNA fragments, proteins, proteomes, hormones, and antigens. The future of saliva disease assessment (salivary diagnostics) will depend, ultimately, on the development of 'point-of-care' devices that can provide immediate feedback in the clinical, research, or public health setting. This represents the future of salivary diagnostics and in some cases, that future is now.

Introduction

The potential for saliva as a diagnostic aide was recognized in 2002 by the US-based National Institute of Dental and Craniofacial Research (the NIDCR). In light of the current pandemic, research groups throughout the US and other countries have initiated studies that seek to elucidate the diagnostic possibilities of using saliva to detect the Sars-CoV-2 virus (the virus that causes Covid-19) but it should be appreciated that before this current interest there has been much research assessing saliva as a potential diagnostic aid for several diseases.**(**1**)** This review article briefly outlines some of the more interesting innovations in salivary biomarker research and comments on the future of salivary diagnostics.

Saliva Constituents useful as Salivary Markers

Salivary molecular biomarkers that have the potential to be indicators of systemic conditions such as cancer (including oral cancer), periodontal disease, autoimmune disease, a viral and bacterial disease, and cardiovascular disease include DNA, ctDNA, RNA, meta-RNA, RNA fragments, minor proteins, nucleic acids, various peptides, proteomes (the entire protein complement expressed by a cell), hormones, antigens, antibodies, and drugs. What makes saliva such a valuable target for molecular diagnostic research is that it is easy to retrieve and collection does not involve discomfort.

Some of the more recent studies underscoring the promise of saliva in the diagnoses of the disease include those in the fields of brain research, systemic diseases (including Sjögren's syndrome), oral cancer and other cancers, periodontal disease viral infection.

Brain Research

As reported on May 6-9, 2017, in an abstract presented at a Pediatric Academic Societies Annual Meeting in San Francisco, and subsequently in the Journal of Neurotrauma **(**2**)**, micro-RNA fragments found in saliva were found to be predictive of pediatric brain concussion in children with traumatic brain injury. Micro-RNA fragments (mRNA) are short (length: 19–24 nt) non-coding molecules that are important in regulating numerous cellular processes including those involving the brain **(**3**)**. This preliminary research suggests that salivary mRNA fragments easily acquired and measured in the clinical setting, may be useful for identifying pediatric traumatic

brain injury (TBI). As the authors indicate, concentrations of mRNA-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 mRNA 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, Sjogren's syndrome, cystic fibrosis, and Parkinson's disease in addition to breast, pancreatic, ovarian, and gastric and lung cancer as well as melanoma. **(**4**)**

A good salivary assay for identifying systemic disease should have high sensitivity, specificity, and functionality. The literature reflects optimism that these fundamental research parameters, although in some cases not currently at acceptable levels, will be improved in the future. Consequently, much work remains to be done before the measurement of salivary proteins and nucleic acids yields meaningful diagnostic capability. Nonetheless, research to date has revealed some interesting results concerning salivary proteomes and some systemic diseases.

Sjogren's Syndrome

Saliva constituents appear to be altered in Sjogren's syndrome (SS), 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 SS 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 the disease. **(**5**)** In another study 15 proteins in the whole saliva differentiated non-SS subjects from those with SS. **(**6**)** There is also evidence that the microRNA profiles of minor labial salivary glands differ between normal subjects and those with SS.**(**7**)** These accumulated studies suggest that specific proteomic salivary biomarkers may prove useful

in the development of a diagnostic panel that can be used on-site to identify patients with Sjogren's syndrome.

Oral Squamous Cell Carcinoma (OSCC)

In recent years there has been a focus on micro RNA fragments (mRNAs) in saliva as biomarkers of oral cancer because these cellular constituents are known to affect cell growth, proliferation, and apoptosis and also appear to serve as oncogenes within different cancer types. **(**8,9**)** Multiple studies suggest that mRNAs may prove useful in early detection of oral cancer and could potentially lead to changes in how oral cancer is treated.**(**10**)** Yoshizawa and Wong provide a good overview of oral cancer detection and salivary mRNAs.**(**11**)** As they state: "Combined with transcriptomic and proteomic approaches, mRNA represents the third diagnostic alphabet (found) 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. **(**12**)** 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 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 against patients with oral OSCC from healthy controls.

Other studies have identified additional proteomic salivary biomarkers potentially useful in detecting OSCC.**(**13**)** In a comparison of sera with saliva in patients with OSCC, three tumor markers: specifically Cyfra 21-1, tissue polypeptide antigen (TPA), and a cancer antigen CA125 were found to be significantly more elevated in the saliva of diseased subjects.**(**14**)** And results of an additional study suggest that five salivary proteins (M2BP, MRP14, profilin, CD59, and catalase) can discriminate oral cancer with 90% accuracy.**(**15**)**

Additional research will help to fully elucidate candidate proteins that can be used to indicate the presence of OSCC.**(**16, 17**)** Whether the 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. Arellano, et al.(18) and Sannam, et al.(19) provide excellent reviews covering the advancements in the identification of OSCC via salivary proteomics.

Other Cancers

Several 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%) (20) suggesting that saliva CA 125 may have better diagnostic value for these conditions than CA 125 found in serum. However, subsequent research results conflict with the above findings and suggest a lack of relationship between CA 125 levels and epithelial ovarian cancer and benign gynecologic conditions so the issue of its potential diagnostic use remains cloudy. (21) 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 (22) and the presence of mRNAs may also help in determining therapeutic response. (23) The introduction of FDA-approved algorithms is reported to have improved the ability to assess the 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.

Viral Disease

With the advancement of methodological techniques able to identify viral DNA, RNA, mRNA proteins, or salivary antibodies, several 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), HIV, and now COVID-19. (24)

In April, the FDA authorized, by prescription only, the use of a saliva test for diagnosing Covid-19. Multiple institutions including Yale University have assessed saliva testing capability. In a letter to the editor of the NEJM, August of 2020, it is reported that a Covid-19 saliva test was used on 70 patients who had already tested positive for Covid-19 (confirmed by positive nasal swab). The author, Anne Wyllie, notes that a higher percentage of saliva samples were positive for SARS-CoV-2 at 10 days than the nasopharyngeal swab samples. She reports that at 1 to 5 days after diagnosis, 81% (95% CI, 71-96) of the saliva samples were positive, as compared with 71% (95% CI, 67-94) of the nasal specimens, suggesting that both tests were relatively equivalent in terms of sensitivity (detection of the disease). What remains a concern for both nasal swab and saliva tests, however, is the relatively low specificity (detection of actual disease) and their relative specificity (their ability to detect whose individuals who do not have the disease) Nonetheless, the initially reported results related to use of saliva are quite promising for its use in detecting Covid-19 disease. **(**25**)**

With the Morbillivirus virus that causes measles infection, the presence of salivary antibodies demonstrates 97% sensitivity and 100% specificity **(**26**)**, for the Paramyxoviridae virus that causes mumps 94% sensitivity and 94% specificity **(**27**)**, and for the Togaviridiae virus that causes rubella, 98% sensitivity, and 98% specificity. **(**28**)**

Antibodies used to diagnose HIV infection are also found in saliva and salivary assays are as accurate as those associated with serum, particularly when plasma virus exceeds 50 copies/mL (when there is an active disease). **(**29**)** A commercial product called OraQuick has been FDA approved and is available for assessing HIV antibodies in saliva. **(**30**)**, 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. **(**31**)**

Periodontal Disease

In a move towards establishing a more precise diagnosis of periodontal disease, many 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. **(**32**)** Figure three lists the many biomarkers associated with periodontal disease. The validated biomarkers include bacteria-derived DNA salivary (porphyromonas gingivalis, prevotella intermedia, and tannerella forsythia) host-derived inflammatory mediators (inflammatory cytokines (IL-1β and MIP-1α), host-derived

markers associated with soft tissue destruction (MMP-8, MMP-9, HGF, lactate dehydrogenase, aspartate aminotransferase, and TIMP-2), and host-derived markers 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

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. **(**33**)** 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. 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 the specific biomarkers in saliva mentioned above) coupled with standard clinical and radiologic measures. **(**34**)** 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.

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.**(**35**)** 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.

Salivary LD level was positively correlated with bleeding on probing and 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 above study was limited because it was cross-sectional, the author concluded that the evaluated 'kit' could have utility in the early detection of gingivitis. **(**36**)**

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 a study by Syndergaard, et al., mean biomarker concentrations were found to decrease in the gingivitis groups following a dental prophylaxis. However, certain markers, specifically MIP-1α and PGE2, remained significantly higher in the healthy group. **(**37**)** Based on these results, the authors conclude that relative change in the assessed biomarkers could prove helpful in identifying diseased patients who, despite prophylaxis, might be at risk for continued chronic gingival inflammation and the development of the more destructive periodontal disease.

For 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.

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). **(**38**)**

The Future of Salivary Diagnostics

The emergence of Covid-19 and the worldwide pandemic underscores the need for non-invasive tests offering immediate diagnostic results. And recent developments affirm that saliva can be used to diagnose this disease as well as many others.

Developments in the field of salivary diagnostics should lead to significant advances in point-of-care precision medicine and dentistry. The benefits of using saliva as a biomarker for the disease are multiple.

The collection of fluid is non-invasive, simple, and easily accomplished by the patient or in the clinical setting.

Relative to the 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. **(**39**)** Salivary diagnostic assessment will also allow for the screening of patients outside the clinical setting, for purposes of treatment monitoring, and 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". **(**40**)** Further, the use of salivary diagnostics may increase access to treatment for identified at-risk individuals not aware of developing the disease and also help in containing community spread of viral infections such as Covid-19. Kaczor-Urbanowicz and colleagues provide a nice overview of salivary diagnostics, the current views and directions. **(**41**)**

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Volume 1 Issue 2 October 2020