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SALIVARY GLANDS AND SALIVA
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Saliva as a diagnostic fluid

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In the last 10 years, the use of saliva as a diagnostic fluid has become somewhat of a translational research success story. Technologies are now available enabling saliva to be used to diagnose disease and predict disease progression. This review describes some important recent advances in salivary diagnostics and barriers to application and advancement. This review will also stimulate future research activity.
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Introduction

Saliva is a unique fluid and interest in it as a diagnostic medium has advanced exponentially in the last 10 years. In the US, the need for further research in salivary diagnostics has been emphasized by federal action plans emanating from the Office of the Surgeon General [Health and Human Services (HHS), 2000] and the National Institute of Dental and Craniofacial Research (NIDCR, 1999). The literature is replete with articles, 2500+ since 1982, describing the use of saliva, gingival crevicular fluid, and mucosal transudates for drug monitoring and for the detection of various oral and systemic maladies.

Advances in the use of saliva as a diagnostic fluid have been tremendously affected by current technological developments. For example, the ability to measure and monitor a wide range of molecular components in saliva and compare them to serum components has made it feasible to study microbes, chemicals and immunologic markers (Slavkin, 1998). As a consequence, these advances in technology have helped to move saliva beyond measuring oral health characteristics to where it now may be used to measure essential features of overall health. The primary purpose of this review is to summarize some important recent applications of saliva-based diagnostics.

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Considerations in the development of salivary diagnostic testing

The major advantages for using saliva in diagnosis rather than blood (easy access, non-invasive collection) have been described in depth earlier (e.g. Ferguson, 1987; Mandel, 1990, 1993a, 1993b; Malamud, 1992; Slavkin, 1998). Similarly, considerations for selecting the type of saliva, i.e. mixed vs individual glandular (Mandel, 1980; Sreebny and Zhu, 1996), the specific collection methodology to be used (Navazesh, 1993) and the physiological factors affecting salivary collection (Dawes, 1993) have also been reviewed in depth. Consequently, more attention will be given here to fundamental issues involved in the development of a saliva-based diagnostic test, with examples as well as possibilities.

The analysis of saliva, like blood-based analyses, has two purposes: the first, to identify individuals with disease and second, to follow the progress of the affected individual under treatment (Copeland, 1974; Aguirre *et al*, 1993). There exists an hierarchical model for diagnostic technology assessment (Fryback and Thornbury, 1991) which consists of five basic levels of analysis at which the effectiveness of any diagnostic test should be evaluated: (1) the analytic (precision and accuracy); (2) diagnostic (sensitivity and specificity); (3) patient outcome efficacy (medical decision-making); (4) operational (predictive value and efficiency); and (5) cost/benefit (societal efficacy). The cost/benefit analysis would provide the practical value of the information derived from the diagnostic test in patient management (Zweig and Campbell, 1993) and the primary utility of a screening test is in its ability to alter the pretest probability of disease (Sox *et al*, 1998). Collectively, a diagnostic test should satisfy all the aforementioned requirements in order to reach the optimum goal, which is to reduce the morbidity and mortality of the diseased or symptomatic population (Goodman, 1993).

Meeting all of these requirements, while achievable, is formidable. The process is typically long in duration and extremely expensive. Many existing tests are accurate and satisfy most or all five described criteria, however, this in itself does not guarantee that the test actually will lead to a reduction in morbidity and mortality of a given disease. The latter must be shown – even for well-known and often-used diagnostic tests. For example, mammography and the detection in serum of prostate specific antigen, which have been in use for nearly two decades, are still undergoing such assessments.

The following sections review representative and important reports concerning saliva-based diagnostics according to disease category. Additionally, an evaluation of each test is provided with respect to whether it has satisfied the above five levels of effectiveness (see summary Table 1).

Autoimmune disorders

Sjögren's syndrome is a chronic, autoimmune disorder characterized by salivary and lacrimal gland dysfunction, serologic abnormalities, and multiple organ-system changes (Daniels, 1996; to be reviewed in a subsequent volume of this series).

Attempts have been made to use saliva for the conclusive diagnosis of Sjögren's syndrome (Fox and Spreight, 1996; Sreebny and Zhu, 1996; Rhodus *et al*, 1998; Streckfus *et al*, 2001). With the exception of sialometry (salivary flow rate determination), most salivary function tests must be conducted in special laboratories or clinics. Included among these tests are sialography, salivary scintigraphy, biopsies, and serological tests. While these tests are helpful, they are invasive, expensive, and not always conclusive (Daniels, 1996). Sreebny and Zhu (1996) proposed a panel of salivary determinants that could be used clinically for the diagnosis of Sjögren's syndrome. These include flow rate, pH, buffer capacity, lactobacillus, and yeast concentration. Sreebny and Zhu (1996) utilized the panel because many of the individual tests had been approved by the US Food and Drug Administration (FDA). They suggest, but do not unequivocally prove, that these tests, when performed on whole saliva, can provide compelling evidence for the presence of Sjögren's syndrome. Additional benefits of these are that they are non-invasive and may be conducted in any dentist's or physician's office (Sreebny and Zhu, 1996).

Other investigators have measured specific cytokine concentrations in the saliva in Sjögren's syndrome patients for their possible diagnostic utility (e.g. Fox, 1996; Rhodus *et al*, 1998; Streckfus *et al*, 2001). The results of these studies suggest that salivary IL-2 and IL-6 concentrations are significantly elevated among individuals suffering from Sjogren's syndrome. Interestingly, the levels of these cytokines appear to be lowered with the systemic administration of pilocarpine (Rhodus *et al*, 1998) or with mucosally administered interferon (Streckfus *et al*, 2001). Thus, alterations in salivary cytokine profiles may be useful for both Sjögren's syndrome diagnosis and progression, although, as indicated in Table 1, considerably more study is needed.

Table 1 Summary of the efficacy achieved in saliva-based clinical diagnostic testing*

Condition analysis	*Level I Analytic analysis*	*Level II Diagnostic analysis*	*Level III Patient outcome*	*Level IV Operational analysis*	*Level V Cost/benefit analysis*
Autoimmune disorders	Sreebny and Zhu (1996); Fox (1996); Rhodus *et al* (1998); Streckfus *et al* (2001)	■	■	■	■
Cardiovascular diseases	Adam *et al* (1999); Chatterton *et al* (1996)	■	■	■	■
Endocrine	Aardal and Holm (1995); Filaire and Lac (2000); Choe *et al* (1983); Heine *et al* (1999); Schramm *et al* (1990); Schramm *et al* (1992); van Honk *et al* (1999); Halpern *et al* (1998); Granger *et al* (1999); Odber *et al* (1998); Barrou *et al* (1996); Castro *et al* (1999); Raff *et al* (1998); Lu *et al* (1999); Bettendorf *et al* (1998); Heine *et al* (1999); McGregor *et al* (1995); Voss (1999); http://www.salivatest.com/journals/saliva_ref.html			■	■
Infectious diseases – viral diseases	Scully (1997); Malamud (1997); Emmons (1997); Martinez *et al* (1999); Nishanian *et al* (1998); Grant *et al* (1996); Lucht *et al* (1998); LaDuca *et al* (1998); Pozo and Tenorio (1999); Bello *et al* (1998); El-Medany *et al* (1999); Elsana *et al* (1998); Crowcroft *et al* (1998)			■	■
Infectious diseases – bacterial diseases	Reilly *et al* (1997); Kountoruras *et al* (1998); Reilly *et al* (1997); Kountoruras *et al* (1998); Jiang *et al* (1998); Stecksen-Blicks (1985); Billings (1993); Larmas (1993); Togelius *et al* (1984); Lenander-Lumikari and Loimaranta (2000); Lendenmann *et al* (2000); Rudney (2000)			■	■
Renal	Lloyd *et al* (1996)	■	■	■	■
Cancer	Boyle, 1994; Tavassoli *et al* (1998); Jenzano *et al* (1986, 1987, 1988); Di-Xia *et al* (1990); Navarro *et al* (1997); Streckfus *et al* (1999, 2000a, b, 2001).		■	■	■
Pharmacologic	Siegel (1993); Jusko and Milsap (1993); Schramm *et al* (1992), (1992) (1993); Smolle *et al* (1999)				■
Psychiatric	Aura *et al* (1999); Yamada *et al* (1998)	■	■	■	■

*The Table depicts the relative levels of efficacy achieved with saliva-based diagnostic tests for specific clinical problem areas. The references shown (see reference list for complete citation) are representative. See text for additional details

Cardiovascular diseases

Cardiovascular disease is a major cause of death worldwide. Markers in saliva may be useful in postoperative follow up among patients undergoing cardiovascular surgery. For example, determinations of total serum amylase and salivary amylase activity have been made before and 6 h after cardiovascular surgery. The results indicated that if salivary amylase levels were low in preoperative patients with ruptured aortic aneurysm, there was an associated increase in mortality (Adam *et al*, 1999). Furthermore, salivary α-amylase appears to be a more direct and simple end point of catecholamine activity than changes in heart rate when evaluating patients under a variety of stressful conditions (Chatterton *et al*, 1996). Such assessments are in the initial stages of development and require considerable further research to determine their clinical utility, if any (Table 1).

Endocrinology

Many clinical assessments of endocrine function require the temporal monitoring of plasma steroid levels. Standard plasma sampling techniques or urine analyses do not necessarily provide the optimal or routine sampling conditions required in this type of monitoring (Quissell, 1993). Saliva levels of steroid hormones reflect the free, and thus active, level of these hormones while most blood measurements reflect the total level, i.e. free and bound. Consequently, the use of saliva for monitoring of steroid hormone levels has increased (Read, 1989).

Currently, the following steroids can be accurately assessed in saliva: cortisol (Aardal and Holm, 1995), dehydroepiandrosterone (Filaire and Lac, 2000), estradiol (Choe, Khan-Dawood and Dawood, 1983), estriol (Heine, McGregor and Dullien, 1999), progesterone (Schramm *et al*, 1990), and testosterone (Schramm *et al*, 1992a). These assays can be useful in evaluations of mood and cognitive emotional behavior (Van Honk *et al*, 1999), to predict sexual activity in adolescent males (Halpern, Udry and Suchindran, 1998), to study child health and development (Granger *et al*, 1999), in considerations of premenstrual depression (Odber, Cawood and Bancroft, 1998), and to screen for Cushing's syndrome (Barrou *et al*, 1996; Raff, Raff and Findling, 1998; Castro *et al*, 1999). Further, salivary steroid hormone levels can also be used to assess ovarian function (Lu *et al*, 1999), to monitor full-term and preterm neonates (Bettendorf *et al*, 1998), and to evaluate risk for preterm labor and delivery (McGregor *et al*, 1995; Heine *et al*, 1999; Voss, 1999). A comprehensive review of the literature concerning salivary testing and its use for assessing endocrine function can be found on the Internet (http://www.salivatest.com/journals/saliva_ref.html).

Infectious diseases

Viral diseases

Testing for the human immunodeficiency virus (HIV) is an excellent example of the potential usefulness of saliva in infectious disease diagnosis. The development of antibodies directed toward specific viral protein epitopes, and the development of technologies capable of measuring these proteins, have facilitated the use of testing for HIV infection (Scully, 1997; Emmons, 1997; Malamud, 1997). For example, when testing saliva for HIV using an enzyme-linked fluorescence technique in combination with Western blot assays, saliva was superior to serum and urine with regard to both sensitivity and specificity (Martinez *et al*, 1999).

Another test which has proven highly effective and reliable for use in community outreach activities and surveillance studies, utilizes saliva in a self-contained kit that does not require trained laboratory personnel (Schramm *et al*, 1999). Additionally, saliva can be used to measure beta2 microglobulin and/or soluble tumor necrosis factor α-receptor levels, and thus assess the disease activity in patients with HIV infection or other chronic inflammatory disease states (Grant *et al*, 1996; Nishanian *et al*, 1998). The general commercial and clinical success of such HIV tests have shown the opportunity that exists for using saliva in infectious disease diagnosis.

As a result of methodological developments, there are many tools available for measuring a variety of viruses in saliva. An excellent example of such a development is the polymerase chain reaction (PCR) which is being widely used to measure many viruses, e.g. human herpes virus 8 levels in salivary (and nasal) secretions. The presence of viral particles in these body fluids has led to the realization that both fluids could be potential sources of non-sexual transmission of this virus (Blackbourn *et al*, 1998). PCR is also being used to measure the shedding of cytomegalovirus and herpes viruses 6, 7, and 8 in the saliva of HIV infected patients (LaDuca *et al*, 1998; Lucht *et al*, 1998). A new multiplex nested PCR technique can be used with saliva to detect and type lymphotropic herpes viruses including Epstein-Barr, cytomegalovirus, human herpes virus 6, 7 and 8 (LaDuca *et al*, 1998; Lucht *et al*, 1998; Pozo and Tenorio, 1999). PCR has also been used to facilitate diagnosis of human rabies using saliva (Crepin *et al*, 1998). These PCR-based salivary applications are in their infancy, but they may prove to be valuable future diagnostic aids (Pozo and Tenorio, 1999).

In addition to the above determinations, saliva has also been used for the measurement of Hepatitis C, a leading cause of cirrhosis (Bello *et al*, 1998; Elsana *et al*, 1998; El-Medany *et al*, 1999). Furthermore, a new Epstein-Barr virus (EBV) capsid antigen antibody capture radioimmunoassay with saliva is apparently useful in epidemiological studies of EBV in school children (Crowcroft *et al*, 1998).

Bacterial infections

Recently, there has been interest in using saliva for the diagnosis of *Helicobacter pylori* infection, which is the critical pathogen associated with peptic ulcer (Reilly *et al*, 1997; Kountoruras, 1998). For example, a nested PCR assay is available to detect *H. pylori* DNA in saliva and confirm the presence of *H. pylori* infection in

patients (Jiang *et al*, 1998). Also, a relatively new immunologic assay that reportedly can detect *H. pylori* antibodies in saliva may be valuable for predicting risk for gastric adenocarcinoma (Vaira *et al*, 1999).

There is a large and long-standing literature concerned with the use of saliva for the detection of dental plaque-induced diseases, i.e. dental caries and gingivitis (Togelius *et al*, 1984; Stecksen-Blicks, 1985; Billings, 1993; Larmas, 1993; Lenander-Lumikari and Loimaranta, 2000; Lendenmann, Grogan and Oppenheim, 2000; Rudney, 2000). The major research emphasis has been on developing convenient oral diagnostic aids to measure the two bacteria most frequently associated with dental caries *(Streptococcus mutans* and *Lactobacillus acidophilus)*. Additionally, tests have been developed to conveniently measure *Porphymonas gingivalis,* which is associated with periodontal disease.

In principle, this application of saliva-based diagnostics appears to be feasible. However, as Larmas (1993) points out, it may not be possible. Larmas (1993) states that, 'From the theoretical point of view, the first attempts to use diagnostic salivary tests could be criticized on the grounds of the following: (1) dental caries and periodontal diseases have not been found to be always specific diseases and they may be the outcome of infections by many individual microbial species and/or microbial combinations; (2) the working hypothesis has generally been that the greater the number of causative micro-organisms in the saliva, the higher the number of carious teeth or sites of periodontitis; however, from the diagnostic point of view, it is impossible to quantify the disease in an individual; and (3) both diseases are chronic and progressive, which means that the time elapsing from infection to clinical signs in the host may be a matter of years, with both active and inactive phases of progression'.

As a consequence of such assessments, salivary-based tests for dental plaque-related bacteria have diverted from being truly employed as diagnostic tests, to be instead, indicators of a patient's risk potential for either disease and the consequent need for aggressive preventive measures. Commercially, this approach has proved successful and salivary tests for caries susceptibility (Birkhed, Edwardsson and Andersson, 1981) are commonly used in Scandinavia and are now commercially available in the USA.

Despite so many positive reports of salivary applications, importantly there are reports clearly showing that saliva is not the fluid of choice for certain infectious conditions. For example, salivary endotoxin levels are not an accurate predictor of sepsis in pediatric leukemia patients and do not correlate with oropharyngeal carriage of aerobic gram-negative bacilli in these patients (Millns, Martin and Williams, 1999).

Nephrology

There are few reports that employ saliva to screen for renal disease. However, there are some. For example, salivary creatinine concentrations show a high sensitivity and specificity for determining the presence of renal disease (Lloyd, Broughton and Selby, 1996). Much more research is required before any role for saliva-based diagnosis can be assigned in nephrology.

Oncology

Because of the anatomical proximity of saliva to both premalignant and malignant oral neoplasms, saliva seemingly would be ideal for the screening of these lesions. Several investigators have tested this hypothesis. For example, a study by Boyle *et al* (1994) examined the possible value of p53 in saliva as a marker for squamous cell carcinoma. Interestingly, they detected and identified tumor-specific mutations in p53 in preoperative salivary samples of individuals suffering from head and neck squamous cell carcinoma. Positive findings were observed in 71% of the patients studied. A somewhat related study found salivary antibodies to p53 elevated among patients with oral carcinomas (Tavassoli *et al*, 1998).

For sometime, there also have been numerous studies examining the utility of saliva for the detection of malignancies remote from the oral cavity. With current detection methods, investigators are able to readily measure proteins present at concentrations in the femtomolar level. Saliva, thus, may be useful as an adjunct diagnostic test for systemic cancers or saliva-based testing could supplant current diagnostic methodologies. For example, an inexpensive saliva test used in conjunction with imaging (e.g. mammography) may increase the overall diagnostic value of the latter test and reduce the number of false positives and negatives currently associated with imaging (Kerlikowske *et al*, 1995). This may allow a diagnosis of cancer to be made at an earlier stage, giving a patient more choice in various treatment options.

An early example of such testing comes from the work of Jenzano *et al* (1986a, b), Jenzano, Brown and Mauriello (1987), Jenzano *et al* (1988). They reported the use of saliva to detect kallikrein in patients with solid tumors that were remote from the oral cavity. Their results demonstrated that higher levels of salivary kallikrein were observed among patients diagnosed with malignant tumors as compared with those individuals diagnosed with benign tumors or those from a cohort of healthy controls. The diagnostic value of these observations has yet to be determined.

With respect to more commonly studied cancer antigens, Di-Xia, Schwartz and Fan-Qin (1990) found that saliva contained CA 125, a glycoprotein complex that is an often-used marker for ovarian cancer. In comparing salivary CA 125 concentrations among healthy controls, women with benign lesions, and those with ovarian cancer, found a significant elevation in salivary CA 125 concentration among the ovarian cancer. Their results suggested that the salivary CA 125 assay had a better diagnostic value than the comparable serum assay (Di-Xia *et al*, 1990).

Epidermal growth factor (EGF) has long been reported to be present in saliva. A study by Navarro *et al* (1997) showed that EGF concentrations were

higher in the saliva of women with primary breast cancer or a recurrence of breast cancer when compared with women without disease. The highest concentrations of EGF were found in the local recurrence subgroup, presenting potential for this marker of malignancy to be used in postoperative follow up (Navarro *et al*, 1997).

Studies with a quite different oncologic marker have also demonstrated that saliva testing may be useful in breast cancer detection. The protein product of the oncogene c-*erb*B-2, also known as HER-2/*neu*, is elevated in the saliva of women diagnosed with breast cancer (Streckfus *et al*, 2000a). These studies demonstrated that this salivary marker is reliable and may also be used in patient postoperative follow up (Streckfus *et al*, 1999, 2001). This same group also found the presence of CA 15-3, EGF receptor, cathepsin-D, p53, and Waf-1 in saliva and are investigating their collective utility in a diagnostic panel for cancer detection (Streckfus *et al*, 2000b). This potential test is currently undergoing evaluation by the US Food and Drug Administration.

However, as noted above for infectious disease diagnostic testing, saliva appears only useful for certain clinical oncologic assessments. For example, the measurement of salivary sialic acid initially appeared promising as an aid for detecting lung cancer. This has not been substantiated (Koc *et al*, 1996).

Drug monitoring

The use of saliva for drug monitoring, and the detection of illicit drugs, has grown remarkably (Slavkin, 1998). Currently, saliva can be used to detect and/or monitor cotinine, cannabinoids, cocaine, phencyclidine, opioids, barbiturates, diazepines, amphetamines, and ethanol (e.g. Schramm *et al*, 1992b, 1992c; Schramm, Smith and Craig, 1993; Smolle *et al*, 1999).

A key concern that for many years hampered routine salivary drug testing was associated with saliva collection (Jusko and Milsap, 1993; Siegel, 1993). Most drug tests utilize whole saliva. Whole saliva is a complex fluid consisting not only of the secretions from the three major pairs of salivary glands, the minor glands and gingival fluid, but it also contains other matter including bacterial products, sloughed epithelial cells, and food debris. These unwanted materials can make the specimen unstable and/or interfere with drug measurement (Jusko and Milsap, 1993; Siegel, 1993). These problems generally have been resolved by the use of improved saliva collection devices and preservatives that maintain the integrity of the saliva specimen (Jusko and Milsap, 1993; Siegel, 1993).

Psychiatry

Saliva may also be useful in providing objective outcome measures during psychiatric therapy. For example, saliva has been used to monitor therapeutic responses in the treatment of anxiety by measuring salivary levels of 3-methoxy-4-hydroxyphenylglycol (MHPG) (Yamada *et al*, 1998). Saliva also has been used to measure post-traumatic stress disorder associated with wartime (Aura *et al*, 1999). It would seem that potential applications of salivary monitoring in psychiatry are worthy of further exploration.

Areas of further research

As depicted in Table 1 the use of salivary diagnostics is just beginning (George and Fitchen, 1997). Considerably more research is needed simply to identify best candidate markers for use in monitoring specific oral and systemic diseases (NIDCR, 1999). This could reflect a broad range of possibilities as indicated by the examples provided above. Furthermore, advances in nanotechnology research will undoubtedly play an increasing role in all diagnostic monitoring (Kohli-Seth and Oropello, 2000).

A factor of primary importance in furthering clinical applications of salivary diagnostics is further clarification of the underlying physiological mechanisms by which these analytes enter the saliva (e.g. oncogenes, cytokines, etc.). Many of these are proteins that are extremely large, but appear in both whole and glandular secretions.

Barriers to the development of salivary diagnostics

As summarized at the 1999 US National Institute of Dental and Craniofacial Research workshop on development of new technologies for saliva and other oral fluid-based diagnostics, there are three general types of barriers to salivary diagnostics. The first is associated with research, the second with product development, and the third with third party acceptance and associated legal issues (NIDCR, 1999).

An example of the first barrier is the need to design and develop microsensors capable of accurate measurements in small volumes. This type of research requires a long-term investment, which may not be attractive to the private sector. Similarly, some sense of the degree to which collection methods require rigorous standardization, for any particular new assessment, must be developed.

Secondly, as noted, for any diagnostic test the cost of development is a serious issue, and may produce a significant barrier. This problem may require cooperative agreements between the government agencies, academia, and the private sector in order to succeed. Further, if mass production is envisioned, then cost effective manufacturing methods need to be developed.

Finally, a significant barrier for saliva-based testing is clinician and insurer acceptance of such a non-traditional diagnostic test. In particular, medical insurance companies will have to be convinced that saliva-based tests are highly accurate as well as cost-effective.

Conclusion

The study and use of saliva-based diagnostics have increased exponentially during the past 10 years. Saliva-based clinical testing shows much promise. There is,

however, a pressing need for much additional research in this area before the true clinical value of saliva as a diagnostic fluid can be determined.

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