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Saliva and viral infections

PAUL L. A. M. CORSTJENS, WILLIAM R. ABRAMS & DANIEL MALAMUD

Overview of viral infections

There is no single infectious route used by *all* viruses. Human viral infection and transmission can occur through multiple paths, such as fecal–oral, ingestion of contaminated food and drinks, sexual contact, exposure to infected blood, exchange of saliva or by aerosols generated by sneezing or coughing. Common examples of viruses isolated from the oral cavity include rotavirus, norovirus, HIV, hepatitis C virus, herpes simplex viruses 1 and 2, Epstein–Barr virus and influenza viruses.

Influenza and the common cold viruses are among the most frequent types of human viral infections. The common cold is generally not life threatening and usually resolves without medical intervention. As it is caused by a group of highly contagious airborne viruses for which no vaccine is available, the best protection is to avoid close proximity to individuals who are infected. By contrast, influenza results in many fatalities, particularly in the elderly and in individuals with suppressed immune systems. Annually modified influenza vaccines available in the western world were initially offered to individuals at risk but are now available to everyone. However, it should be noted that (87) 'The influenza vaccine was only 62% effective among people who did not receive influenza vaccination in the prior year. In comparison, vaccine effectiveness among those who did get influenza vaccination in the previous year was substantially lower.' Occasionally, a new influenza variant may cause a pandemic as a result of zoonosis (i.e. transmission of an animal flu virus to humans) or 'genetic recombination' (more precisely, reassortment of gene segments) of a human virus with a nonhuman form that crosses species. Well-known recent examples of these are swine flu and avian or bird flu, which is sometimes confused with the zoonotic severe acute respiratory syndrome virus (a corona virus).

Worldwide policies to reduce or eliminate the risk of epidemics have had some success, and several infectious diseases have been largely eradicated or are under control in the western world as a result of national vaccination programs. The most successful example of a human-driven eradication is that of the smallpox virus following implementation of a worldwide vaccination strategy. This approach succeeded because the smallpox virus (variola) is essentially comprised of a single strain and does not have an animal vector. The approach to measles is another example demonstrating the success of vaccines. Measles is a highly contagious infection of the respiratory system that spreads through aerosol transmission or contact with nasal and oral fluids. Effective vaccines are available and in the USA measles was declared eliminated in 2000. In 2010, at the 63rd World Health Assembly, a global goal was proposed to eliminate the disease with a target of a 95% reduction in mortality by 2015. Unfortunately, there has been a recent measles outbreak in the USA (288 cases), mostly in unvaccinated individuals (www.CDC.gov/measles/).

As a result of new vaccines and vaccination policies, and also improved medical care and development of novel drugs, worldwide infectious disease fatalities have not increased in the past two decades. However, a significant decrease worldwide can be expected only when the equivalent of western world resources become available in resource-poor areas. The global approach to eradicate polio is another example, with currently up to 80% of the world's population living in polio-free regions and a 'collaborative strategic endgame plan' in place to eradicate the last reservoirs of polio (the Global Polio Eradication Initiative). The list of viral infections and viral diseases is extensive and the eradication of one pathogen may provide a niche for another pathogen to become more virulent. Naturally acquired immunity through exposure to the pathogen is likely to provide the best protection against recurring infection.

Viral infections detectable using oral fluid samples

The presence of viable virus in oral fluid specimens is typically demonstrated by screening for viral nucleic acids. Initially, the viral nucleic acid is isolated from the oral fluid sample directly or after centrifugation performed to concentrate cell-associated virus. This is usually followed by PCR amplification of a virus-specific viral nucleic acid fragment or by using a signal amplification technology. For HIV viral-load analysis, a frequently used commercial assay, based on viral nucleic acid amplification, is the COBAS® Amplicor HIV-1 Monitor Test (Roche Diagnostics, Indianapolis, IN, USA) and an example of a commercial assay based on signal amplification is the VERSANT® HIV-1 RNA Assay (Siemens AG, Munich, Germany).

Viral nucleic acid-based methods are more sensitive and considered less contagious because pathogens in the sample are inactivated during the initial viral nucleic acid isolation protocol. However, amplification-based detection of viral nucleic acid is relatively expensive, requires trained personnel and specific equipment and may not always be proof of an active infection. Ultimately, a cultivation confirmatory test may be needed to obtain a definitive diagnosis. Detection of viral antigens by indirect fluorescent antibody labeling or electron microscopic techniques can be used to detect the presence of intact viral particles. Note that the presence of microscopically intact viral particles in a specific biological fluid/matrix cannot distinguish viable/infectious virus from noninfectious virus.

A list of examples of viable viruses detectable in oral fluid samples is provided in Table 1. In contrast to serum or plasma, there is no standard protocol for saliva/oral fluid collection and thus saliva may refer to whole-mouth stimulated or unstimulated saliva or to a sample obtained with an oral swab or brush. Filtered or centrifuged saliva can be used to detect free virus, but the virus may be free or cell-bound when using whole saliva or oral swabs. Not all viruses are easily cultivated, and in contrast to bacteria they cannot be directly grown in culture media, but require specific living cells to support their replication. Human papillomavirus, hepatitis C virus, herpes simplex virus, human herpesvirus 8, coronaviruses and the rhinoviruses are examples of viruses known to be difficult to culture. Determining the presence of viable virus in oral fluid samples does not always indicate that the virus can be transmitted orally. In the case of cytomegalovirus, a member of the *Herpesviridae* family, it is known that transmission occurs via direct contact with body fluids. Although serology suggests a prevalence of cytomegalovirus of up to 80%, blood donors are not routinely tested for active cytomegalovirus infections because the virus in blood is cell associated and leucocyte-depleted red-blood-cell products are considered cytomegalovirus safe. However, the situation in saliva is probably different. The cytomegalovirus transmission route through

Table 1. Examples of viable (cultivable) virus isolation from saliva and oral swabs

Virus	Sample type*	Patient group	Reference
Cytomegalovirus	Saliva	Patients with AIDS	(64)
Ebola virus†	Saliva	Ebola hemorrhagic fever	(14)
Human herpesvirus 6	Saliva	Healthy adults	(58)
HIV	Saliva	HIV-infected patients	(26)
Herpes simplex virus 1	Whole saliva	Varied patient group	(117)
Herpes simplex virus 2	Mouth swab	Requested laboratory tests	(49)
Influenza virus A‡	Saliva	Recurrent parotitis	(123)
Mumps virus	Parotid saliva or swab	Clinical parotitis	(96)
Rabies virus	Saliva	Hospitalized patient	(107)
Tobacco mosaic virus§	Sputum	Pulmonary disease, smokers	(65)

*Sample description as indicated in the publication.
†Viable virus detected in the saliva of one out of eight individuals. Note that cultivation of Ebola virus is extremely high risk and requires a high-containment laboratory (BSL-4). Literature studies for this review were conducted up to 2014, before the World Health Organization reported the outbreak of the Ebola virus epidemic in West Africa (25 March 2014).
‡Also described cultivation of enterovirus, possibly Coxsackie virus B.
§Tobacco mosaic virus is a plant pathogen; the presence of viable virus in the clinical sample was verified by cultivation through inoculation of the leaves of a tobacco mosaic virus-sensitive host plant.

saliva is similar to that following infection with Epstein–Barr virus, a *Herpesviridae* member responsible for causing infectious mononucleosis ('kissing disease') and oral hairy leukoplakia.

In contrast, many studies focusing on HIV describe inactivation of the virus by salivary components and indicate that the likelihood of transmitting HIV by saliva is extremely low. A few reports mention the isolation of viable HIV from an oral fluid sample (13, 79); further analysis of these 'infective' saliva samples (26) showed that they lacked both specific anti-HIV immunoglobulins and nonspecific antiviral activity. In addition to reports of human viruses, some studies describe the presence of viable plant pathogens in human oral samples; for example, the tobacco mosaic virus was identified in sputum samples from smokers (7, 65). Although speculated, associations of this plant virus with specific communicable diseases have not been established.

Recent examples demonstrating viral nucleic acid in the oral cavity by amplification of specific viral nucleic acid fragments are shown in Table 2. Besides the currently widely utilized viral nucleic acid-based detection methods, immunologic-based diagnostics of pathogen-derived antigens are also used to demonstrate active infection. For example, detection of hepatitis B surface antigen is a common test amenable for salivary diagnosis (5) of hepatitis B virus. Other antigen assays demonstrating active viral infections using oral samples have been described for ebola (44), rabies (72) and HIV (62, 86). Pathogen-derived antigens (e.g. p24 for HIV) are often present at higher levels than the viral genome, and identifying such abundant viral proteins (>1,000 copies per

Table 2. Examples utilizing nucleic acid amplification to determine viral infection via oral samples

Virus	Sample*	Patient group	Reference
Cytomegalovirus	Oral swab	Newborns	(17)
Dengue virus	Saliva	Hospitalized febrile patients	(93)
Ebola virus	Oral swab	Suspected Ebola patients	(44)
Enteroviruses	Saliva	Beta-cell autoimmunity	(53)
Epstein–Barr virus	Whole saliva	HIV-infected individuals	(39)
Hepatitis A virus	Saliva	Exposed during hepatitis A virus outbreak	(1)
Hepatitis B virus	Saliva	Acute hepatitis case	(115)
Hepatitis C virus	Whole saliva	Gastroenterology patients	(112)
Human herpesviruses†	Stimulated saliva	Acute uncomplicated malaria	(23)
HIV	Whole saliva	Confirmed infections	(6)
Human papillomavirus	Saliva	Population screening	(21)
Influenza virus‡	Saliva and throat swab	Children up to 17 years of age	(99)
Measles virus	Saliva	Congenital measles	(50)
Mumps virus	Buccal swab	Clinical parotitis	(100)
Polyomavirus§	Saliva pellet	Healthy individuals	(98)
Rabies virus	Saliva	Hospitalized individuals	(82)
Rhinoviruses	Sputum	Acute respiratory infection	(102)
Rubella virus	Oral fluid	Clinical diagnosed cases	(60)
Severe acute respiratory syndrome coronavirus	Saliva and sputum	Confirmed infection	(41)
Tobacco mosaic virus¶	Saliva	Smokers	(7)
Torque teno virus	Saliva	Healthy subjects	(113)

*Sample description as indicated in the publication.
†Including herpes simplex viruses 1 and 2, varicella zoster virus, Epstein–Barr virus, cytomegalovirus and human herpesviruses 6, 7 and 8.
‡Influenza A and B viruses; the study also included detection of two other respiratory viruses – parainfluenza and respiratory syncytial virus.
§Including human polyomaviruses BKV, JCV, WUV and KIV.
¶Tobacco mosaic virus is a plant pathogen.

virion) often utilizes the simpler technique of detection of unbound/free soluble protein rather than viral nucleic acid amplification. After seroconversion, effective testing for the presence of viral antigens is best achieved by including an immune-complex dissociation step. In HIV infection, p24 testing was proposed as a method to detect acute infections, within the seroconversion window. In August 2013, the first rapid test detecting HIV-1 antigen and HIV-1/2 antibodies was approved by the US Food and Drug Administration (FDA) for over-the-counter sales. The Determine HIV-1/2 antigen/antibody Combo test (Alere Inc., Orlando, FL, USA) is used by trained professionals and is well suited for outreach settings where access to health care is limited. However, the antigen/antibody Combo test does not replace viral nucleic acid confirmatory testing and is not approved for blood-donor screening. Multiple-test algorithms designed for validation of antibody-only tests still apply to the Combo test, even when the test indicates the presence of both anti-HIV immunoglobulins and HIV p24 antigen. The use of lower-complexity and less-expensive p24 tests as alternatives to viral nucleic acid-based viral load testing to validate serological assays is thought to be limited (97) as these antigen tests show poor clinical sensitivity once the viral load drops below 30,000 virions/ml; note that a good correlation between p24 and viral RNA is only seen within the seroconversion window.

Nucleic acid amplification is compatible with the simultaneous screening or detection of multiple pathogens in a single sample using multiple pairs of DNA primers specific for the targeted microorganisms. The development of fully integrated rapid test devices, capable of analyzing clinical samples for a panel of specific pathogen targets, would be of great value for point-of-care use. Such devices are feasible with the currently available viral nucleic acid-amplification technology; however, cost and sample-preparation issues still need to be addressed (45, 71). Recent technological advances have allowed analysis by deep sequencing of the human salivary microbiome to create a catalog of all the microorganisms present in saliva and oral mucosa. Analysis of the salivary virome (94), the virus subset of the salivary microbiome, demonstrated that the majority of oral viruses are bacteriophages. It is postulated that because these phages require bacterial cells for replication they probably play an important role in the microbial diversity and health of the oral cavity and may also influence the susceptibility to human viral infections. Optimization of oral fluid viral nucleic acid-isolation methods, combined with the availability of high-throughput sequencing and analysis technologies, will provide useful tools to identify known, as well as unknown, viruses (24).

Although not widely utilized, immunologic detection of active infection has been reported for infections other than HIV. For example, dengue virus-specific IgA in serum and saliva was detected soon after infection in dengue-endemic regions (133). Moreover, dengue-specific IgM/IgG levels may be used to distinguish between primary and secondary infections (8, 122). However, antibody testing (Table 3) does not always detect active infection, but is valid for documenting exposure to uncommon viruses such as West Nile virus. Antibody testing is also used for blood-donor screening to identify individuals exposed to human T-lymphotropic virus I and II, hepatitis viruses B and C, HIV-1 and HIV-2.

Rapid diagnosis: the need to identify the pathogen

Clinicians would like to determine if an acute infection is of bacterial or viral origin. In contrast to most systemic diseases that display multiple biomarkers, the detection of a single pathogen-specific target can diagnose a disease/infection. Currently, there is no definitive test that distinguishes between a viral and a bacterial infection, other than by testing all of the possible pathogens and/or biomarkers using microarray technology with blood or saliva (20).

Bacterial infections are ideally treated with narrow-range antibiotics if the bacterial pathogen is identified; however, broad-range antibiotics are typically used when no identification is available. Owing to the emergence of antibiotic-resistant strains of pathogens, antibiograms to determine the susceptibility of pathogens to a variety of antibiotics are increasingly required. These tests are carried out in a clinical microbiology laboratory and it may take hours to days before the appropriate information is available to select the best therapy for treatment of a specific infection.

Acute viral infections are not easily resolved with drugs and require a different approach. The misdiagnosis of a bacterial infection as a viral infection can have fatal consequences, as in the case of bacterial meningitis vs. viral meningitis. In acute infections, when the type of pathogen cannot be established, antibiotics are often prescribed as a precautionary measure. However, high-dose antibiotics in acute bacterial meningitis are toxic and can lead to allergic reactions. Laboratory-based testing to identify the

Table 3. Antiviral antibodies detected in saliva

Virus	Sample*	Patient group	Reference
Cytomegalovirus	Saliva	Healthy children and adolescents	(130)
Dengue virus	Oral swab	Past exposure	(2)
Ebola virus	Oral swab	Suspected Ebola patients	(44)
Epstein–Barr virus	Saliva	Intensively training athletes	(132)
Hepatitis A virus	Oral fluid	Volunteers; vaccination screening	(119)
Hepatitis B virus	Oral fluid	Schoolchildren; transmission study	(9)
Hepatitis C virus	Saliva	Seropositive hepatitis C virus and/or HIV patients	(124)
Human herpesvirus 6	Saliva	Nurses working shifts; stress marker	(48)
Human herpesvirus 8†	Whole saliva	Kaposi sarcoma patients	(77)
HIV	Oral fluid	HIV testing program	(92)
Human papillomavirus	Saliva	Oral and genital human papillomavirus infection	(51)
Herpes simplex virus 1	Saliva	Adolescents (age group, 9–14 years)	(104)
Influenza virus	Saliva	HIV-infected children	(129)
Measles virus‡	Oral fluid	Children (age group, 12–16 years)	(59)
Mumps virus‡	Oral fluid	MMR‡ surveillance samples	(126)
Norovirus	Saliva	Volunteers	(55)
Parvovirus	Oral fluid	Vaccinated children	(125)
Poliovirus	Saliva	Elderly (salivary IgA response study)	(18)
Rotavirus	Saliva	Children (age group, 6 months to 3 years)	(91)
Rubella virus	Oral swab	Surveillance testing	(95)
Varicella zoster virus	Oral fluid	Routine MMR‡ testing samples	(116)

*Sample description as indicated in the publication.
†Human herpesvirus-8 is the Kaposi sarcoma-associated herpesvirus.
‡Routine testing for presence of antibodies against measles, mumps and rotavirus (MMR).

pathogen may require several hours or days to obtain a result. A rapid point-of-care test providing immediate results is desirable and could immediately determine the appropriate treatment. In the case of emergencies, the major issue is the 'sample to assay result time' and saliva or a finger-stick blood test can be utilized for a point-of-care test. In less-acute situations, saliva as a biological matrix for point-of-care diagnosis is well accepted by patients and is preferred when they can choose between finger-stick blood or an oral sample (54). Moreover, the use of oral samples is ideal for nonhospital settings, such as a local physician's office, a small community health facility, a dental office, first responders and home care.

Studies have reported using oral samples as an alternative to blood or urine samples for the detection of pathogen-specific antigens, antibodies and nucleic acids. In general, the concentration of the target molecules in saliva is lower, but amplification technologies facilitate identification of the viral markers. The use of a saliva sample to replace a lumbar puncture to obtain a sample that unequivocally identifies the pathogen causing acute meningitis would be ideal, but is not yet available. Specific biomolecules derived from any virus circulating in the bloodstream or present in mucosa are likely to be present also in an oral sample. The detection of human antibodies against measles and mumps in oral fluid was first described more than three decades ago (47) and since then several simple platforms for antibody detection in saliva or oral mucosal transudate have been published (33).

Antibody testing is not widely used for diagnosis of infectious diseases because the presence of antibodies cannot discriminate between a past or current infection as residual antibodies can be present in the

circulation as a result of a previous immunization. However, a four-fold increase in antibody titer during convalescence may aid in the identification of a particular pathogen but requires an accurate quantitative antibody assay, whereas the existing rapid antibody assays are qualitative, providing a yes/no answer. In contrast, an HIV antibody test can be used to establish HIV-positive status because the infection is not self-cleared and there is no cure or vaccination currently available; therefore, the presence of anti-HIV immunoglobulins can be used as a screening test. However, antibody presence does not correlate with viral load or the ability to transmit the disease; HIV-infected patients on antiviral therapy are antibody positive but may have a viral load below the level of detection. An FDA-approved saliva antibody test is available over-the-counter for home testing (63). A concern regarding this test is that individuals with suspected exposure to HIV may use it too early postexposure and, as a result, receive a false-negative test result because it takes 6 weeks or longer to mount an antibody response, a time period referred to as the seroconversion window. This situation poses a potential risk as newly infected individuals in the period before seroconversion have extremely high viral loads and are thus highly contagious. There are reports indicating that saliva-based testing has a lower clinical sensitivity than blood-based testing, which may be the case if including HIV-infected individuals receiving highly active antiretroviral therapy (HAART) for an extended period (84).

In this review, we summarize our unpublished results from two studies demonstrating that the level of antibodies in saliva differ from the level in blood. Although the antibody levels in blood and saliva in HIV-infected individuals differ significantly, all seropositive results were identified when using either blood or saliva as the test fluid. More elaborate laboratory-based testing (e.g. full analysis of blood) is necessary to provide quantitative results because a rapid antibody test only provides a qualitative result. Saliva is preferred as a sample over blood because of higher patient acceptance, issues of safety (no chance of injury caused by needles) and cost (e.g. requirement for a phlebotomist), and is compatible with self-testing. Eventually, oral samples may provide an ideal matrix for diagnosing many other viral infections. Currently, only two oral-based commercial tests for infectious disease are FDA approved for use in the USA: the OraQuick oral HIV test; and the OraRisk human papillomavirus salivary diagnostic test. The OraQuick test (OraSure Technologies, Inc., Bethlehem, PA, USA) is an FDA-approved CLIA waved test using an oral swab specimen. The OraRisk human papillomavirus test (OralDNA labs; Access Genetics, LLC), also FDA approved, utilizes an oral fluid sample sent to a central laboratory for nucleic acid analysis. OraQuick was recently approved as an over-the-counter home-use test, taking 20 min from sample collection to result (a visual line on a test strip). The same platform has been used for development of a commercial oral hepatitis C virus test (66).

HIV antibody levels in blood vs. saliva

Although antibody assays generally cannot unequivocally diagnose active viral infections, they are valuable tools to track exposure to a pathogen. Antibody screening is useful for epidemiological studies and for monitoring a large population for immunity to common viral infections, whereas the screening of young children for the presence of antibodies against infectious agents thought to be eliminated can be useful to monitor potential re-emergence of a pathogen. Using saliva as a clinical sample for this (large-scale) approach is cost-effective because samples can be collected noninvasively by the participants themselves at home and mailed to a national test laboratory (81, 85). It is widely accepted that any antibody detectable in blood can also be detected in saliva. However, there are fluctuations and differences in concentration because salivary flow rates vary with circadian rhythm and saliva composition is more variable than that of blood/plasma. Moreover, the antibody isotype differs; salivary glands produce and secrete salivary IgA, whereas IgG and IgM enter the oral cavity through leakage from the bloodstream and gingival crevicular fluid and are present at lower concentrations. Another issue in studies utilizing oral samples is the method of collection; for example, with respect to antibody type, the composition of whole saliva is quite different from the composition of gingival crevicular fluid, which is a transudate that more closely resembles blood. For antibody detection, as a consequence of the high abundancy after seroconversion, the collection method is less critical than is the case when targeting pathogen-derived analytes, such as nucleic acids. In previous studies (22, 67) exploring the development of a modular microfluidic test device for multiplex analysis of saliva samples, a versatile generic antibody assay [Fig. 1 (22, 29, 31, 32)] was evaluated using two sets of saliva samples collected using different protocols. Set 1 [WIHS, obtained from the Woman's Interagency HIV Study (10)] comprised samples, which, upon collection, were immediately stored in a preservative, whereas

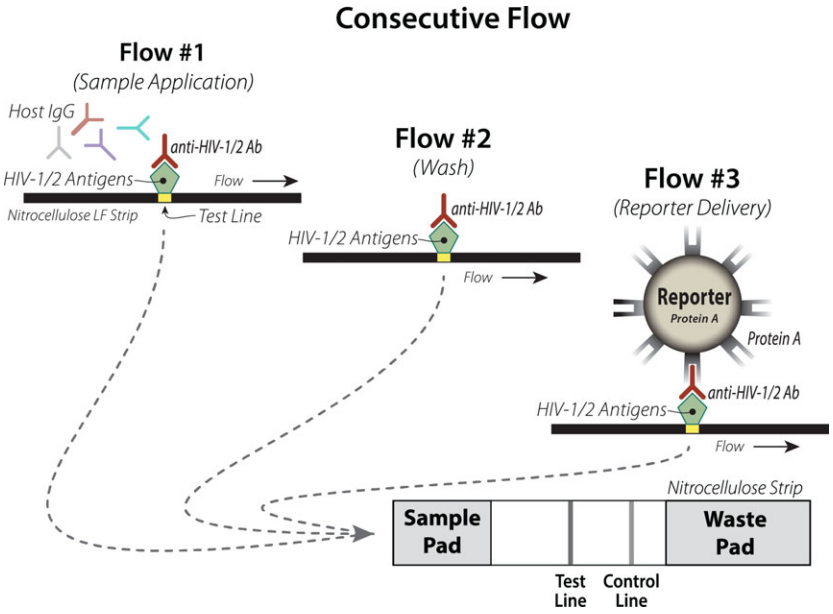

Fig. 1. Consecutive flow assay format for detection of human anti-HIV1/2 immunoglobulins. Antibodies indicated in red represent human anti-HIV immunoglobulins in saliva that can bind to the HIV-1/2 antigen Test line. Other IgG antibodies will bind to the flow Control line comprised of anti-human IgG antibodies, located downstream of the Test line. Following a wash flow, the fluorescent reporter (30) that can bind to IgG on the Test and Control lines is flowed. The lateral flow strip is then scanned to record the presence of the reporter.

set 2 (UO1, samples collected during a study funded by National Institute of Dental and Craniofacial Research, NIH grant UO1DE017855) comprised samples, which, upon collection, were clarified using the saliva-collection sponge from the UPlink collector (73). From both sets, paired serum and saliva samples were tested in parallel to validate saliva results.

Figure 2 shows the results obtained with the WIHS (Fig. 2A) and UO1 samples (Fig. 2B). The assay results are presented as a normalized value, the ratio of the signal measured at the Test line divided by the signal measured at the Flow Control line (27). Assay cut-off thresholds (the value above which a sample is designated reactive) are determined from the average value obtained with the HIV-negative control samples. The HIV-positive samples uniformly generated a higher assay value than the HIV-negative samples, apparently independently of the saliva-collection protocol. Similar patterns comparing saliva and blood-antibody levels are also observed for other infectious diseases (34).

Viral infection of the oral cavity

Saliva plays a key role in protecting the body from a wide variety of viral infections in addition to its role

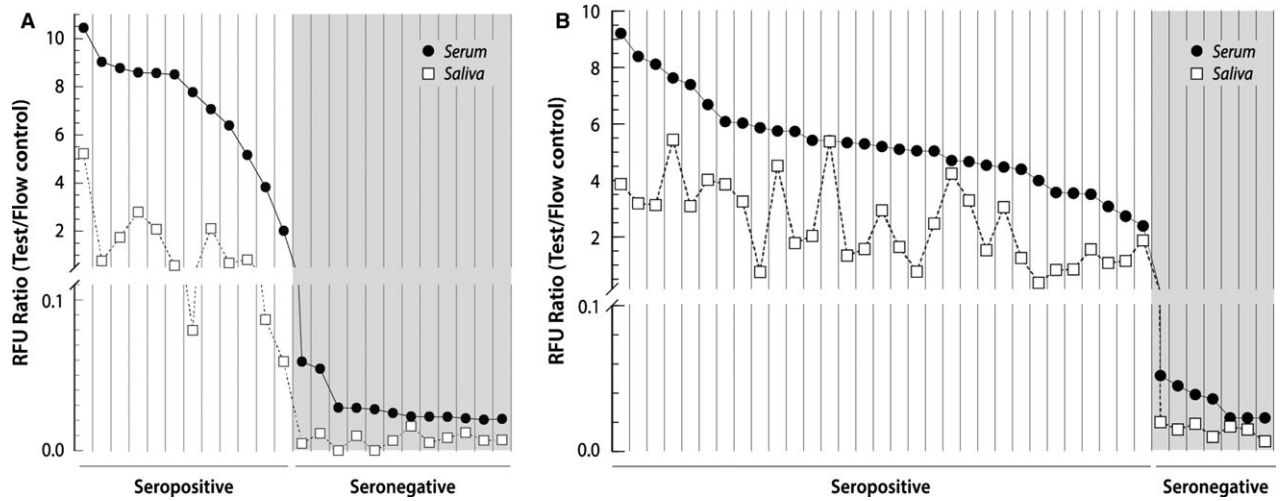

Fig. 2. Detection of HIV antibody in serum and saliva. Relative amounts of HIV antibodies detected in paired serum and saliva samples. (A) Results obtained with the Woman's Interagency HIV Study sample set (n = 24); (B) results obtained with the National Institute of Dental and Craniofacial Research UO1 grant sample set (n = 38). The amounts of sample used in the test were 0.5 µl and 5 µl for serum and saliva, respectively. Samples were ranked by the Test/Flow Control ratio value determined with serum, with the HIV-negative controls grouping together on the right side of the graph. RFU, Relative Fluorescent Units, is a measure the signal strength measured at the Test and Flow Control lines.

in controlling the colonization of bacteria in the oral cavity. Many biomolecules in saliva have antiviral activities for specific viruses (74). Most viral infections occur across mucosal membranes. The mouth and the eye are common sites for viral entry, although viral infections of both the eye and the oral cavity are relatively rare. The mechanism for viral infection control includes many biomolecules, including mucins, antibodies and antiviral proteins that are present in a continuous flow of fluid. It is of interest that many of the same antiviral molecules are present in both saliva and tears and that the hypotonicity of saliva (12) is capable of lysing enveloped viruses.

Responses to an infection involve complex, highly regulated processes. The innate immune system is the first nonspecific defense against both bacteria and viruses. Specific responses include cell-mediated immunity and the adaptive immune system with increased cytokine levels from T-helper cells and the production of anti-pathogen-specific antibodies against pathogen antigens. The secondary response involves the reservoir of specific memory cells, permitting a faster response to a recurring infection by the same pathogen. The presence of specific antibodies and a specific cytokine response from mononuclear blood cells to pathogen-specific antigens can be used as a diagnostic tool (28, 32), but it does not distinguish between active and latent infections. Confirmation and monitoring of active infections generally requires the detection of pathogen-specific antigens, nucleic acids or cultivation of the pathogen.

As the gateway to the gastrointestinal tract, antiviral activity present in the oral cavity may also prevent viruses from reaching the lower portions of the gastrointestinal tract. Mucins present in saliva, and their production along the entire gastrointestinal tract, serve to trap both bacteria and viruses and prevent colonization. Epithelial surfaces are coated with a group of molecules derived from the innate immune system [e.g. lysozyme, lactoferrin, lactoperoxidase, secretory leukocyte protease inhibitor, gp340 (DMBT1) and defensins], and these are also present in tears and saliva. Considering the vast array of pathogens to which humans are exposed on a daily basis, the level of infection is surprisingly low, indicating that the host-derived protective mechanisms do a remarkable job of bacterial and viral control. Whilst there are many host factors, each pathogen presents a unique mode of infecting and a unique means of host control of infection. Typically, a virus enters a cell by attaching to a specific receptor on the cell surface. In the case of HIV, the virus binds to CD4 and a co-receptor (CCR5 or CXCR4), rabies virus attaches to the neural cell adhesion molecule receptor, whilst herpes simplex virus utilizes a number of cellular receptors, including toll-like receptors. Epstein–Barr virus, responsible for infectious mononucleosis and oral hairy leukoplakia, appears to enter cells by utilizing multiple viral surface proteins and multiple cell receptors, which may increase its oral transmission efficiency. Norovirus, a major cause of gastroenteritis, binds to blood group antigens present on red blood cells and in saliva of secretor-positive individuals. Note that 80% of the population secrete these blood type antigens into saliva.

In the next sections we focus on a number of viral infections detectable in oral fluids, the protective role of saliva and the targets and mechanism involved in the infection pathway (Table 4).

Norovirus

Norovirus refers to a family of RNA nonenveloped viruses that are the major cause of nonbacterial gastroenteritis, transmitted by the fecal–oral route (52, 76) following exposure to fecal-contaminated fomites, food or water and possibly also direct person–person contact. Shellfish grown in contaminated waters are

Table 4. Examples of viral infections detectable in oral fluid

Virus	Disease	Main transmission route	Main cell-type infected
Epstein–Barr virus	Infectious mononucleosis, oral hairy leucoplakia	Saliva	B-cells and epithelial cells
HIV	AIDS	Sexually transmitted infection	Macrophages and $CD4^+$ T-cells
Human papillomavirus	Genital warts, cancer	Sexually transmitted infection	Keratinocytes
Herpes simplex virus	Oral and genital herpes	Body fluid or lesion (blister)	Neurons
Hepatitis C virus	Liver cirrhosis	Blood exposure/contact	Hepatocyte
Norovirus	Acute gastroenteritis	Contaminated food	Macrophages and dendritic cells
Rabies virus	Acute encephalitis	Animal bite (saliva)	Muscle and nerve cells

among the most common source of outbreaks of norovirus infection. The viral receptor utilizes blood types O and B, Le^a and Le^b (121) that are all present in saliva, but to date there is no definitive evidence that the virus is directly transmitted by saliva. Typically the pathogen is transferred amongst individuals in close contact, including locations such as hospitals, cruise ships and airlines, as well as by food handlers. The virus can survive for days in water and only extremely small doses need to be transferred to the hands/mouth from contaminated surfaces (e.g. doorknobs, bed sheets, ceramic surfaces) to transmit the infection. The virus is relatively resistant to common disinfectants, thus increasing the risk of transmission. The most common human strain of norovirus is the GII.4 genetic cluster (90), and several laboratories are working to develop compounds to block the norovirus receptors based on the blood group carbohydrate structures that bind the virus.

Rabies virus

In contrast to norovirus, rabies virus is usually transmitted via saliva following a bite from an infected animal, which results in encephalitis. It is suggested that spelunkers may become infected after inhalation of aerosols from bat colonies hanging upside down in caves. The disease is most prevalent in areas with endemic dog rabies or infected bats. The virus binds to nicotinic receptors on muscle fibers of a newly infected animal and then migrates via nerves to the brain, where it replicates and spreads via neuronal routes to the salivary gland and ultimately appears in saliva. When the rabies-infected animal bites a human, the spread once again is to the central nervous system and subsequently virus is found in the saliva of the human host. Rabies antigens and antibodies can be detected in saliva of both infected animals and humans, but this is typically late in the infection, making serological detection of little practical value. PCR detection of rabies virus in saliva has been reported (36). Appropriate pre-exposure (vaccine) and postexposure prophylaxis (rabies immune globulin and vaccine) can be effective with early treatment along with antiviral drugs, otherwise the disease is typically fatal (127). Massive animal control and pre-exposure vaccines has made rabies-related mortality in the USA relatively rare, with fewer than two deaths per year.

Human papillomavirus

Human papillomaviruses are a family of DNA viruses that infect epithelial cells of the skin and mucosa. There are over 100 different strains of human papillomavirus and these are able to infect the genital and reproductive tracts, the oral cavity and cause warts predominantly on the hands and feet. In the USA, up to 80% will have a human papillomavirus infection in their lifetime, but most will clear without causing disease. Antibody tests to screen for a potentially active human papillomavirus infection are therefore not considered relevant (38).

Twelve strains of human papillomavirus are considered 'high risk', meaning that they can lead to cancers, primarily cervical and oral cancer. Human papillomavirus strains 16 and 18 are two high-risk strains most frequently associated with cancers or premalignant conditions;~70% of cervical cancers are attributed to these two strains. However, Beachler et al. (16), in a study of 404 individuals, found that the most prevalent oral strains were human papillomaviruses 55, 83 and 72. Human papillomavirus strains 16 and 18 are included in national prevention programs, including administration of anti-human papillomavirus vaccines. Oral human papillomavirus infection has a higher prevalence in HIV-infected individuals than uninfected HIV individuals and as a result have a higher risk of developing human papillomavirus-associated cancers, particularly head and neck cancers (15). Of interest, in a case study of two couples with human papillomavirus-associated tonsillar carcinoma, the human papillomavirus strain 16 was identical in both partners, suggesting the possibility of the infectious nature of oropharyngeal cancer (3). At present, OralDNA Labs (Access Genetics, LLC) has the only commercially available salivary diagnostic test for human papillomavirus. Oral samples are collected with a swab, placed in transport media and sent to a central laboratory for nucleic acid-based human papillomavirus detection and strain identification. The salivary test, OraRisk human papillomavirus, identifies several human papillomavirus types, with a focus on human papillomavirus-16 and human papillomavirus-18, the types most commonly linked to oral cancers.

Epstein–Barr virus

Epstein–Barr virus is a member of the *Herpesviridae* family (DNA viruses) and known to cause disease in humans. It is a lymphocryptovirus targeting B-cells and epithelial cells and is also referred to as human herpesvirus 4. This orally transmitted virus is one of the most common viruses, with a worldwide infection rate of over 90% of the adult population. Although exposure to the virus is high, most primary infections

are asymptomatic. However, long-term carriage is associated with epithelial-cell malignancies, including nasopharyngeal carcinoma and oral hairy leukoplakia, and may play a role in many other benign and malignant diseases (56). Oral hairy leukoplakia is often a consequence of an opportunistic infection of Epstein–Barr virus in HIV-infected individuals.

Epstein–Barr virus can spread through sexual contact, blood transfusions and organ transplantation. However, most frequently, transmission is thought to occur through saliva. The virus can survive on objects and surfaces as long as they stay moist and thus can be easily transmitted (e.g. by sharing drinks). There is no vaccine available to prevent infection. Epstein–Barr virus is the cause of the majority of infectious mononucleosis cases (also known as Pfeiffer or the Kissing Disease), common among young adults and teenagers. Infection may cause fatigue and malaise, from which it may take several months to recover (35). When clinical symptoms indicate mononucleosis-like symptoms, infectious mononucleosis can be confirmed by the presence of atypical lymphocytes and serological tests. In the past, serological diagnostic tests detected the presence of heterophile antibodies (weak antibodies with multispecific activity against poorly defined antigens). Epidemiological studies, using the detection of specific antibodies in saliva to determine population immunity, have been reported (37). Currently, more sensitive IgG (reflecting past infection) and IgM (reflecting current infection) diagnostics have been developed that reduce the false-negative rate and thus rule out infectious mononucleosis. Previously, the presence of infectious Epstein–Barr virus in saliva was demonstrated by the ability of saliva extracts to transform lymphocytes in culture. Now, nucleic acid-based detection is the most sensitive standard method for detecting infections (61, 110).

Herpes simplex viruses

Herpes simplex virus is a double-stranded DNA virus that enters cells through the interaction of viral coat glycoprotein with cellular receptors. The viral capsid is eventually transported to the cell nucleus where its DNA is released into the nucleus and replication is initiated. The linear viral DNA is not integrated in the human genome but remains as an extrachromosomal circular DNA (viral episome). Some oncogenic viruses from the *Herpesviridae* family, including the Epstein–Barr virus, have been reported to integrate at low frequency into the host chromosome (80). An association of herpes simplex virus-1 and Epstein–Barr virus with aggressive periodontal disease has been reported, suggesting the possibility that these viruses interact with bacteria linked to periodontal disease (109).

Herpes simplex virus 1 and herpes simplex virus 2 are two members of the *Herpesviridae* family. Both are highly contagious and spread easily during periods of shedding. Shedding periods are recognized when individuals have moist blisters on mucous membranes of the lips, mouth and genitals. Cold sores, most frequently on the lips, are a well-recognized sign of herpes simplex virus infection; salivary contact, as well as sharing drinks and food, should then be avoided to prevent transmission of the virus. Herpes simplex virus 2 infection is a common sexually transmitted disease, generally referred to as genital herpes, although both herpes simplex virus 1 and herpes simplex virus 2 have been detected in both oral and genital infections. Herpes simplex virus is a neurotropic virus, capable of infecting nerve cells, and thus can avoid some human immune responses. After active infection and periods of shedding, the virus becomes latent, remaining in neural ganglia. Reactivation of the virus occurs with periods of stress, fatigue and illness, after which the virus is transported through the nerve cell axon to the epithelium where virus replication and shedding occurs.

Herpes simplex virus infections do not self-cure, but remain life-long latent infections that may lead to recurrent infections, although antiviral drugs may reduce the severity of reactivation and decrease the chance of transmission. Herpes infection in neonates can become life threatening as the virus can spread to the central nervous system and cause meningitis or encephalitis. Antiviral medication may be recommended for pregnant woman infected with herpes simplex virus. Currently, no vaccine has been approved for herpes simplex virus, but several studies, mainly focused on a vaccine against genital herpes (herpes simplex virus 2), are ongoing. Most carriers of the virus do not know that they are infected. Reports linking herpes simplex virus 1 infection, and other viruses from the *Herpesviridae* family, to an increased risk of developing Alzheimer's disease, if confirmed, may change the focus of herpes simplex virus research (19, 75, 78). Although herpes simplex virus infections are widespread, routine testing for herpes simplex virus is not recommended as the benefits of testing are not clear. As the disease cannot be cured and a vaccine is not available, questions such as 'would it lead to effective reduction of transmission' and 'is testing cost efficient also in relation to false positive test results and confirmatory testing', remain unanswered. Diagnostic laboratory

testing, in fact, is only recommended for people who show symptoms of an active infection in order to confirm the diagnosis. Typically, herpes simplex virus 2 infections are considered linked to recurrent genital herpes, but half of the first-episode cases are actually caused by herpes simplex virus 1. Recommended laboratory testing includes a serological test, as well as a virological test (viral culture of the lesion). Viral culture may take up to 10 days and in the future might be replaced by the more accurate nucleic acid testing using PCR.

Herpes simplex virus and saliva

Several studies describe the detection of anti-herpes simplex virus antibodies and nucleic acid in oral samples. The antibody test is specific to the type of herpes simplex virus and, like nucleic acid testing, can distinguish between herpes simplex virus 1 and herpes simplex virus 2. One of the first reports showing the presence of herpes simplex virus 1-specific IgA antibodies in saliva was published in in 1984 (101) and demonstrated elevated herpes simplex virus 1-specific IgA levels in patients with peptic ulcer. The current dogma is that herpes simplex virus 1 is frequently found in upper gastrointestinal tract ulcers but not in normal gastric and duodenal mucosa, where ulcers are linked to infection with *Helicobacter pylori*. IgA present in saliva can block the infectivity of herpes simplex virus, as demonstrated *in vitro*, but the efficiency of viral neutralization *in vivo* is still unknown. Viable herpes simplex virus can be isolated from saliva, but current methods utilize viral nucleic acid detection, although this is not a quantitative measure of infectious virions. In 1994, Tateishi et al. (117) demonstrated that nucleic acid detection is the most sensitive method for determining herpes simplex virus 1 infection. Rapid antibody tests have been developed for use with blood samples, and modifying the existing rapid tests for detection of antibody in oral fluid would appear to be straightforward. An over-the-counter rapid test would primarily serve to reassure an individual of the absence of herpes simplex virus infection. Whether a rapid test based on the detection of viral antigen or viral nucleic acid in saliva would be useful is a matter of debate because localized sampling of a lesion for diagnosis of genital herpes is available. The situation for viral infections of hepatitis C virus and HIV is clearly different (see below).

Hepatitis C virus

The common hepatotropic viruses that cause viral hepatitis are hepatitis viruses A–E. Although their name suggests similarities, the viruses are not related, and they have different properties. Hepatitis D virus, for instance, can only replicate when an individual is already infected with hepatitis B virus. Vaccines are available to prevent infection with hepatitis type A, B and E, although they are not available globally. No vaccine is available for hepatitis C virus, which, according to the US Centers for Disease Control and Prevention, is the most common chronic bloodborne infection in the USA, currently resulting in more mortalities than HIV (69, 70). In Europe, the prevalence of hepatitis C virus varies from 0.1% in Northern Europe (Finland) to 6% in Eastern Europe (Romania). The core of the current hepatitis C virus epidemic (42) is attributed to injection drug use with contaminated needles. Transmission through sexual contact and from mother-to-child occurs, but at a much lower frequency. In fact, hepatitis C virus transmission follows similar routes as HIV, but the risk of transmission through blood is higher for hepatitis C virus because of its significantly higher viral load.

Like HIV, hepatitis C virus is an RNA virus, but it does not typically integrate into the host DNA. Hepatitis C virus belongs to the flavivirus group of single-stranded enveloped RNA viruses. Replication and mutation rates of hepatitis C virus are much higher than those of HIV, but hepatitis C virus infection is self-cleared in up to 40% of cases. If not cleared, the virus may become latent and activated at a later time. Treatment of acute hepatitis C reduces the risk of chronic infection, and individuals infected with hepatitis C virus are at high risk for developing liver cirrhosis upon infection with hepatitis A virus or hepatitis B virus. Vaccination against hepatitis A virus and hepatitis B virus for individuals with chronic hepatitis C virus infection is therefore advised. Liver cirrhosis caused by a chronic hepatitis C virus infection may lead to liver failure and cancer. Hepatitis C virus infections can be cured, but the existence of multiple genotypes complicates therapeutic approaches and current costs are extremely high, in the order of $100,000 (68). Currently, six genotypes (1–6), with different geographical distribution, are known; in the USA, genotypes 1–3 are the most common. HIV infection is believed to increase the rate of progression to hepatitis C disease and therefore it is recommended that individuals with a confirmed HIV infection should also be tested for hepatitis C virus. However, the impact of hepatitis C virus infection on HIV disease progression is not significant.

Screening for hepatitis C virus infection is initially performed with a rapid antibody test that delivers results within 20 min (111). Recently, the OraQuick

hepatitis C virus Rapid Antibody Test (67) was approved by the FDA for use with finger-stick blood and it is likely that a saliva test will be made available in the near future. The antibody test does not distinguish between a current and a previous infection; therefore, a confirmatory test is needed if a screening test is positive. Historically, an immunoblot assay was used to confirm infection, but testing for hepatitis C virus RNA is currently the method of choice.

Hepatitis C virus and saliva

A strong correlation between serum and salivary antibody levels against hepatitis C virus infections have been reported since 1986 (4). One of the first studies successfully demonstrating anti-hepatitis C virus antibodies in saliva was published in 1992 (118). Since then, rapid testing using saliva has evolved (131) to a rapid test that requires only 20 min from 'sample to result' (66). The latter, low-complexity, user-friendly test was built on the same platform as the OraQuick Advance HIV1/2 Rapid Test. Although the hepatitis C virus test device may become FDA approved to determine the presence of antibodies in oral samples in the future, a confirmatory test to determine an active infection will still be needed. As hepatitis C virus transmission requires blood–blood contact, it was proposed that a saliva-based diagnostic might be safer than blood for testing. Infectious hepatitis C virus in saliva has been observed, but generally only for individuals with periodontal disease or gingivitis and a serum viral load higher than 10^6 virions/ml. Infection through salivary contact is unlikely, but cannot be fully excluded.

HIV

HIV contains two separate, but identical, strands of (+) RNA, which, upon infection, are transcribed into DNA that enters the nucleus and integrates into the human genome. Once this occurs, the infected cell produces viral particles that can infect new cells. The virus targets $CD4^+$ T-helper cells and other cells from the immune system and thereby affects cell-mediated immunity, making the individual progressively more vulnerable to other opportunistic infections and AIDS-related cancers. Upon integration of the viral DNA into the human genome, the infection cannot be cleared and remains life-long in still-unknown reservoirs. For patient survival and blocking progression to full-blown AIDS, compliance with a strict drug regime needs to be followed to suppress virus production and the continuous infection of new CD4 cells.

Early HIV infection is not easily diagnosed as the symptoms resemble influenza. Individuals aware of having been at risk for HIV infection may request an HIV test; however, an antibody test can demonstrate HIV infection only after seroconversion, which can take up to 6 weeks. Recent infections require another approach. The best way to demonstrate early infection is by nucleic acid amplification.

Depending on the risk factor, postexposure prophylaxis in health-care workers or individuals suspecting exposure to HIV (e.g. unprotected sexual contact or use of a contaminated needle) can be considered. In the case of health-care workers, after post-exposure prophylaxis, individuals will have to remain alert for several weeks for potential signs of HIV infection. Pre-exposure prophylaxis with antiretroviral drugs has been demonstrated to reduce the risk of infection for individuals involved in high-risk behaviors (www.cdc.gov/hiv/prevention/research/prep/).

In many cases, HIV infection will initially go unnoticed until an individual has a medical consultation related to a secondary opportunistic infection. Usually at that point the individual is past the HIV seroconversion window and infection can be identified with a rapid antibody screening test. As HIV infection is not curable, it is highly likely that a person with antibodies against HIV is infected, but a confirmatory test to validate the antibody test result is required to exclude a false-positive result. For the patient this waiting period, up to a week or more, to get the final test result can be difficult psychologically. For this reason, point-of-care devices that can be used to diagnose and validate HIV infection within 1 or 2 h are important. If the device is also able to determine an accurate viral load, counseling could be included concomitantly with the immediate start of appropriate antiretroviral therapy. This would also reduce the number of individuals required to return to the clinic to receive their confirmatory test result and may thus decrease transmission of the virus. Fourth-generation immunoassays that detect the p24 antigen and anti-HIV immunoglobulins have been developed but are not readily available for the most point-of-care settings (57, 128). The combination of nucleic acid and antibody detection is a better option as a result of the availability of nucleic acid-amplification methods that achieve the desired sensitivity (22) and also allow detection of an early infection (before seroconversion). The ultimate (point-of-care) device allows detection of anti-HIV immunoglobulins, viral antigen (e.g. soluble p24), viral RNA, as well as pro-viral DNA (34). This type of device provides both screening and confirmation of infection within the seroconversion

window as the presence of viral RNA could be confirmed either by the presence of viral antigens or by proviral DNA. Note that detection of anti-HIV immunoglobulins is the simplest method to screen for an HIV infection. However, neonates from HIV-infected mothers carry maternal anti-HIV immunoglobulins for up to 1 year whilst not being infected (25).

HIV and saliva

The three main transmission routes of HIV infection are unprotected sexual intercourse, the use of contaminated needles and transmission from mother to child during pregnancy, birth and breastfeeding. The role of saliva in both the diagnosis and inhibition of HIV has been a widely discussed topic (for reviews see Page-Shafer et al. and Shiboski et al. (89, 103)). The presence of viable HIV particles in saliva is controversial (11, 105). Detection of HIV nucleic acids in saliva has been demonstrated, but it is not clear whether the viral RNA detected is derived from viable viral particles or instead represents free nucleic acid fragments. In general, the viral loads in saliva are lower than those in the corresponding blood sample (46). Occasionally, high secretors of HIV have been reported, perhaps indicating the presence of reservoirs for HIV in salivary glands or oropharyngeal tissue. Infected lymphocytes from various oral sources are a possible source of HIV in viral hypersecretors (106). It is important to recognize the presence of reservoirs as a potential source of transmission. Thousand-fold higher viral RNA levels have been reported in tonsil biopsies than in the corresponding blood samples (43). Even more remarkable is the detection of 10^7 HIV copies per ml in parotid cyst aspirates in individuals with undetectable blood viremia (120). Although the presence of reservoirs is acknowledged and the existence of HIV in epithelial cells of the buccal mucosa has been demonstrated (40, 79), saliva is not regarded as a relevant transmission route (88). Saliva lyses HIV particles *in vitro* because of its hypotonicity (11) and many salivary proteins have been demonstrated to inhibit HIV infectivity *in vitro*, including lysozyme, cysteine-rich defensins, lactoferrin, secretory leukocyte protease inhibitor and salivary agglutinin (gp340, DMBT1). In the oral cavity, soluble gp340 is thought to inactivate HIV by a mechanism involving binding to a HIV glycoprotein (gp120) exposed on the HIV envelope (74, 83).

Concluding remarks

The oral cavity is a major site for infection and transmission of viruses (108). The innate immune system present in the mouth can prevent some infections (e.g. HIV), whereas other viruses, including rabies, herpes simplex virus and Epstein–Barr virus, can infect and be transmitted orally. Of particular importance is that defensive antibodies to most bacterial and viral pathogens can be detected using saliva or a swab of the oral mucosa. The frequency of oral-based testing for viral and bacterial diseases is increasing as more commercial entities become involved and new products are being developed. The major advantages of oral-based testing are cost, patient acceptance, equivalence to blood testing and the possibility of home testing. Whether increased testing for viral infections should be recommended depends on several factors, of which cost is extremely important. Inexpensive and rapid saliva diagnostics can be used in a dental office to provide screening tests for many viral diseases, including HIV, hepatitis C virus and influenza – in fact, all situations where a blood-based antibody test is performed. Moreover, when oral symptoms suggest, or when requested by the patient, the dentist could conveniently initiate viral nucleic acid-based testing by collection of the appropriate oral sample to send to a central laboratory for further analysis and inform the patient's physician for follow up. Alternatively, the rapid oral antibody test could be performed in the dentist's office and, if the screening result is reactive, the patient can be referred immediately to a clinic for a confirmatory test.

Scientific literature indicates that several commercial assays may be suitable for use with oral samples. Several research laboratories are developing oral fluid-based tests for infectious diseases and several noninfectious systemic diseases. Special attention is required for assays that can be performed at home by individuals themselves when they involves diseases with potentially great social impact (e.g. HIV) as the patient may not always act appropriately upon receiving the test result. As large numbers of individuals visit a dentist annually as their only health professional (114), screening for viral infections in the dental office followed by referral to a physician could have a major impact on controlling the transmission and symptoms of infectious diseases.

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