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A Simple Saliva-Based Test for Detecting Antibodies to Human Immunodeficiency Virus*

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This study was performed to determine the feasibility of using saliva as a diagnostic medium for the detection of antibodies to human immunodeficiency virus type 1 (HIV-1) and HIV-2 under nonlaboratory conditions and to evaluate the performance characteristics of such a test. We developed for this purpose a self-contained kit (Saliva · Strip [ST]), which combines the collection and processing, as well as the analysis, of the specimen. The kit's performance was evaluated in a blinded study. Saliva collection was facilitated with a specially designed device that contains a sample adequacy indicator, and immunochromatography test strips were used for the analysis. A total of 1,336 matched serum and saliva specimens (684 reactive and 652 nonreactive specimens) were tested. We tested sera using an enzyme immunoassay (EIA) and a rapid strip test. Sera reactive in one of the assays were also analyzed by Western blotting. Sensitivity and specificity were 99.4 and 99.4%, respectively, for ST, 100 and 99.1%, respectively, for EIA, and 99.7 and 100%, respectively, for the serum strip test. The saliva test performed well when HIV-2-positive sera or a low-titer performance panel (HIV-1) of serum or plasma specimens were diluted (1:2,000) in nonreactive saliva. Because the methodology we present here uses a noninvasively obtained medium, the methodology may be suitable for use in the field where laboratory support and personnel are limited, such as community outreach programs, doctors' offices, surveillance studies, and community hospitals.

Many of the problems related to human immunodeficiency virus (HIV) infection and AIDS, particularly those affecting public health policies, have not yet been fully realized, because the total number of infected individuals in many parts of the world is still rising. Among the unresolved issues surrounding HIV infection are those related to diagnosis: costs for the majority of countries most heavily affected, logistic problems associated with traditional methods, and use of specimens obtained by invasive procedures. Others are the often heated debates concerning social implications associated with appropriate counseling and rapid testing (2), the "right to know" (30), home collection of a specimen (4, 24, 29), or outright self-testing (13). We present a particular methodology which reduces some of the limitations of the contemporary means of diagnosis of HIV infection. Our report focuses solely on the presentation of a new approach to such means of diagnosis, an approach which combines noninvasive specimen collection and rapid testing.

Modern immunodiagnosis is characterized by at least some of the following: convenient access of the patient to primary health care professionals, collection of a specimen with disposable instruments, rapid specimen transport in cooled containers, specimen processing by automated analyzers in highly specialized, centralized laboratories, well-researched reference and control protocols, and establishment of feedback loops between test results and treatment regimens. Unfortunately, such factors do not apply to the world's population. There is an urgent need for methods which facilitate specimen collection and analysis while the patient is present at the testing site.

Several requirements must be satisfied in order to perform on-site analysis. One is a preference for less invasive specimen collection techniques (e.g., by use of saliva). It has now been shown that antibodies to HIV from the oral cavity can be detected with a sensitivity and specificity that are essentially identical to those of tests with serum (5, 11, 14–17, 19, 21, 22). The use of saliva in reference methods has now become equally feasible, when such protocols are appropriately modified (14, 16).

Despite advances in the use of saliva for HIV detection, the immunochemical methods have been traditional laboratory assays. The advantages of using saliva can be fully realized if it is used in simple but reliable nonlaboratory assays. The work that we present here describes the evaluation of a method for the collection, processing, and analysis of saliva which can be performed by nonspecialized personnel under nonlaboratory conditions. The kit includes the collection and processing device and test strips.

MATERIALS AND METHODS

Study population. The participants in this study were attending the Clinical Laboratory of the Hospital de Infectologia "Dr. Daniel Mendez Hernandez," Centro Medico Nacional la Raza I.M.S.S., Mexico City, Mexico. The patients were classified according to the AIDS Surveillance Case Definition for Adolescents and Adults (9), where applicable; otherwise, they were classified as clinically healthy persons, patients with other infectious diseases (e.g., hepatitis A, B, or C, herpes, cytomegalovirus infection, rubella, brucellosis, or leprosy), and patients with other clinical conditions (e.g., diabetes mellitus, aplastic anemia, or leukemia). All participants gave informed consent, and epidemiological and demographic data were collected. Counseling was offered pre- and posttesting.

Saliva specimens. Saliva was obtained from the patients with the collector portion of Saliva · Sampler or Omni · Sal (Saliva Diagnostic Systems, Inc., Vancouver, Wash.) (12, 17a) which is included as a portion of the Saliva · Strip (ST) test kit. This device consists of a cellulose pad attached to a plastic stem which contains an indicator dye which notifies the person collecting the sample that an adequate sample has been obtained. The pad is placed in the patient's

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mouth sublingually until the indicator panel turns blue. This occurs when ~1 ml of specimen has been collected. The device is deposited into a tube containing a modified phosphate buffer at physiological pH which contains sodium azide, a nonionic detergent, and avian serum (Saliva Diagnostic Systems, Inc.). The stem is removed from the cellulose pad by vigorous shaking (e.g., vortexing) or by manual twisting of the stem in the tube, and the stem is discarded. The saliva is processed (extracted from the remaining cellulose material and mixed with buffer) with a piston filter, which is manually pressed into the tube. About 1 ml of a clear solution consisting of approximately equal volumes of saliva and buffer is obtained in the filter. A test strip is dropped into the processed specimen in the filter.

Serum specimens. Blood was collected by venipuncture and placed into Vacutainers (Baxter Diagnostic, Inc., McGraw Park, Ill.) to obtain the serum. The serum was aliquoted and assigned a numerical code for subsequent blind analysis.

Sera from 1,336 patients were analyzed by an enzyme immunoassay (EIA; Abbott 3A10 EIA; Abbott Laboratories, Abbott Park, Ill.) and a rapid test, Sero · Strip HIV-1/2 (Saliva Diagnostic Systems, Inc.). The aliquots for the reference assays were coded separately, and the results from the ST (available immediately) were not known to the technicians performing the serum tests. After the three assays were completed, the code was broken by the supervisor.

All specimens reactive by any of the three assays were analyzed by Western blotting (Organon Teknika Co., Durham, N.C.) as a confirmatory method (1). If indeterminate results were obtained, an additional specimen was sought from the patient(s) at a later time (>8 weeks after the first collection).

HIV type 2 (HIV-2)-positive serum specimens were obtained from the Ivory Coast. These were analyzed by approved strategies by EIA and Western blot analysis (Cambridge Biotech Corp., Worcester, Mass.). One additional HIV-2-positive saliva specimen was collected from an individual in the United Kingdom.

The low-titer performance panel (Anti-HIV 1 Low Titer Performance Panel; PRB105) was obtained from Boston Biomedica, Inc. (West Bridgewater, Mass.).

Saliva test strips. The test strips used for this study (ST; Saliva Diagnostic Systems, Inc.) were lateral-flow immunochromatography devices. Two synthetic peptides (4a) representing highly conserved regions of viral transmembrane glycoproteins gp41 and gp36 were used to capture antibodies to HIV-1 and HIV-2, respectively. These two antigens are identical to those used in similar test strips designed for use with serum (5a) and whole blood (26a). The strips themselves consist of several layers of composite materials that accommodate all necessary reagents. When a strip is placed in the saliva filter, liquid rises vertically by capillary action and reconstitutes a dried protein A-colloidal gold conjugate, which in turn becomes immobilized on a membrane in the presence of antibodies to HIV. The liquid passes a 1-mm-wide line of immobilized HIV antigen, then passes a similar line that comprises immobilized protein A, and finally flows into a fibrous reservoir. For a reactive specimen, two lines develop in the middle of the test strip after a few minutes. For a nonreactive specimen, only one line develops. If the test is run without a saliva specimen (e.g., with water), no lines develop, indicating an invalid result. A specimen with a highly positive or a moderately positive reaction can be read after about 5 min. Some weakly reactive specimens require 10 to 20 min for the signal line to develop sufficiently. No additional equipment is required for performance of the assay, and the test strips are designed for storage at ambient temperature before use.

Only one saliva specimen from an individual infected with HIV-2 was available. Eighteen serum specimens from HIV-2-infected persons were diluted (1:1,000) in freshly collected saliva from a noninfected individual, processed as described above (i.e., at a final dilution of 1:2,000), and analyzed with the ST strips.

For performance evaluation, saliva samples were prepared by spiking them with members of the PRB105 anti-HIV 1 low-titer performance panel (Boston Biomedica, Inc.). This panel contains one nonreactive specimen, which is included as a negative control. Saliva from a nonreactive individual was spiked with these sera (as described above) and was then analyzed with the ST strips.

Stability of antibodies in specimen and assay buffer. To test the integrity of saliva in the assay buffer over time at various temperatures, a large volume of saliva spiked with HIV-positive serum (saliva control) was used. The serum used for spiking (positive control serum) was a pool of eight positive serum specimens (equal volumes of each) diluted with a pool of four negative serum specimens (equal volumes of each) made by mixing one part of the positive pool with seven parts of the negative pool. Saliva specimens (positive control saliva) were prepared by collecting 30 ml of expectorated saliva from a noninfected individual and adding 60 μl of a positive control serum, and then the mixture was exhaustively vortexed in a closed container. This pool was processed in aliquots of 1 ml with the saliva collector and the saliva filter, as described above, to simulate in situ collection. The filtrates from 45 collectors of equal volumes of saliva and buffer were pooled and divided into three aliquots (15 ml each); these three aliquots were incubated at room temperature (20 to 25°C), 37°C, and 45°C, respectively (i.e., three treatment groups). The final dilution of antibodies to HIV (reactive serum pool) in saliva was 1:8,000. The use of this control provided a weak but clearly readable positive signal on the test strips at the start of this stability study.

Stability of test strips at elevated temperature and humidity (ST strip stability). The test strips were stored in screw-cap polypropylene containers at room temperature, 32°C, 37°C, and 45°C (i.e., four treatment groups). The containers were placed in tightly sealed plastic boxes, each containing a water-saturated sponge (100% relative humidity). Bottles containing the test strips were removed from these humid chambers and were allowed to equilibrate to laboratory conditions (20 to 25°C, ~50% relative humidity) before analysis. The test strips were analyzed weekly through week 10 and biweekly thereafter with a saliva specimen from an HIV-negative individual as well as this same individual's saliva spiked with an HIV-positive plasma specimen, with the final dilution of the reactive serum being 1:8,000.

Buffer stability. The buffer used in the processing step was investigated for stability after storage over time. It was kept in a polypropylene bottle at room temperature (20 to 25°C) and 45°C and was analyzed at week 30 with test strips stored at the corresponding temperatures. Also, additional lots of buffer of various ages (stored at room temperature) were tested for their ability to function with nonreactive or simulated (serum-spiked) reactive saliva specimens.

TABLE 1. Staging of HIV-infected patients

Stage	No. of patients
A1	0
A2	105
A3	22
B1	53
B2	68
B3	70
C1	47
C2	50
C3	269
Total	684

RESULTS

Diagnostic sensitivity and specificity. On the basis of the results for specimens from 684 HIV-infected and 652 noninfected individuals (total, 1,336 specimens), the sensitivity and specificity of the ST were each 99.4% (4 specimens had false-negative results and 4 specimens had false-positive results) by using EIA, Western blotting, and the clinical symptoms combined as an acceptable indicator of the true clinical state. The saliva specimens that gave false-negative results were from patients classified as having stage A2 (three individuals) and stage C3 (one individual) HIV infection. The saliva from the patient with stage C3 infection was also negative by the serum strip test. The EIA results for all of these individuals were positive. The false-positive specimens were from individuals with typhoid fever (one individual), unclassified hepatitis (one individual), and hepatitis C (two individuals). The EIA and serum strip test results for these patients were all negative. The staging of HIV-infected patients and the clinical conditions of the non-HIV-infected individuals are summarized in Tables 1 and 2, respectively.

The sensitivities and specificities of the reference assays were 100 and 97.2%, respectively, for EIA and 99.7 and 100%, respectively, for the rapid serum test. Upon reassay (after breaking the code), the sensitivities and specificities (respectively) were 100% (no reassay required) and 99.1%, respectively, for EIA and 99.9% (one sample with a false-negative result was no longer available for retesting) and 100%, respectively, for the rapid serum test.

Processed specimen stability. Aliquots from each treatment group were analyzed weekly in triplicate with the test strips, with readings made 20 min after the start of the test. A positive signal line was observed through week 8 for all treatment groups (i.e., when the tested liquid was stored at the various temperatures). The relative signal strength at each temperature was comparable for each triplicate assay, with perhaps a slight decrease in intensity at the elevated temperatures (37 and 45°C). Also, at these temperatures a precipitate in the saliva and buffer mixture was observed at 1 week (37°C) or 2 weeks (45°C) and thereafter. The precipitate did not in any noticeable way interfere with the assay results.

TABLE 2. Clinical conditions of individuals whose specimens were nonreactive by ST[a]

Condition	No. of individuals who were ST negative
Infectious diseases, viral	
Hepatitis (viral, unclassified)	24
Hepatitis A	75
Hepatitis B (chronic or carrier state or a contact)	41
Hepatitis C (or contact with hepatitis C)	175
Herpes	18
Mononucleosis	4
Rubella (or contact)	8
Parasitic disease (toxoplasmosis)	16
Infectious diseases, bacterial	
Brucellosis	15
Tuberculosis	18
Leprosy	9
Urinary tract infection	4
Hematological disorders	22
Renal disorders	4
Diverse infections or conditions	90
Other	
Adenopathy	4
Asthma or allergy related	4
High risk for HIV infection or AIDS	55
Pregnancy (or suspected pregnancy)	5
Spontaneous abortion	44
Healthy	17

[a] The four false-positive specimens in this study were from individuals with typhoid fever ($n = 1$) and hepatitis ($n = 1$ unclassified hepatitis and $n = 2$ hepatitis C).

Buffer stability. Ten different lots of buffer solution, stored in bulk at room temperature for between 8 and 23 months, showed no evidence of deterioration or deviation from expected performance when they were tested with either freshly collected nonreactive saliva or nonreactive saliva spiked with positive serum.

Test strip stability. A clearly positive control and signal were observed through week 34 for samples stored at all storage temperatures. Strips tested with the negative control did not show a signal line (no false-positive results), and no increased background was noticeable. The time for migration of aqueous medium along the strips increased slightly after storage at 37 and 45°C but remained well within the recommended read time, i.e., 20 min.

HIV-2. The one bona fide HIV-2-positive saliva specimen used in this study was reactive by the strip test. The other 18 saliva-based specimens (prepared from confirmed HIV-2-positive sera by dilution into negative saliva) were all reactive by the ST.

Performance panel. ST performed better than the immune fluorescent assay, as well as two EIAs, but not as well as six EIAs, with saliva spiked with the serum or plasma members of the low-titer performance panel (Table 3).

DISCUSSION

The assay described here can be performed under nonlaboratory conditions and requires little training and no special skills. To our knowledge, this is the first systematic study of a simple, rapid test with saliva as a diagnostic medium that incorporates the collection, processing, and analysis of saliva for antibodies to HIV. The performance of the test is better than those of some and equal to those of many laboratory tests that use saliva. In a number of studies, diagnostic sensitivities for saliva analyzed by EIA ranged from 95 to 100% (3, 6, 8, 10, 12, 18, 20, 27, 28). Diagnostic specificity of under 90% has been reported, even by established assays (e.g., Abbott 3A11 and GACELISA [16, 22]), but ranges more likely between 98 and 100% (28). It should be noted that the diagnostic sensitivity and specificity of the ST rapid test reported here, which are comparable to those of EIAs, are achieved without reliance on an amplified signal (as in EIAs).

The concentration of immunoglobulin G (IgG) in saliva varies but has been reported to be in the range of 1:1,000 (25) compared to that in serum. For the evaluation of the low-titer performance panel, we used saliva samples spiked with serum at a dilution of 1:2,000 (final dilution). At this concentration, the ST performed as well as or better than three of nine commercial laboratory assays (including an immunofluorescence assay). Three specimens for which either four or six of the conventional assays detected anti-HIV antibodies but for which the ST did not contained antibodies exclusively to either the p24 antigen or the p24 and p55 antigens (possibly some gp160 in one of three Western blots). These antigens are not present in the ST. Therefore, it is likely that analytical specificity rather than analytical sensitivity (as opposed to clinical sensitivity and clinical specificity) was the reason that HIV was not detected in these specimens. This is supported by the fact that for these specimens higher concentrations of antibodies in saliva (i.e., dilution of the respective sera 1:1,000 and 1:500) still did not promote signal line development by the strip test.

In a study that compared the utility of saliva as a diagnostic medium with seroconversion panels, Connell and Parry (7), using a 1/1,000 dilution of seroconversion panel members' sera to give an antibody concentration comparable to that found in saliva, concluded that one of five commercially available laboratory EIAs was able to detect seroconversion in saliva as soon as or within a few days of the appearance of anti-HIV antibodies in plasma following primary HIV infection.

A limitation in the present study was that only one anti-HIV-2-positive saliva specimen was available. Spiked saliva samples

TABLE 3. Comparative performance of ST with low-titer performance panel

Test[a]	No. of reactive specimens[b]
1	14
2	14
3	14
4	14
5	12
6	12
7	12 (2)
8	11
9	11
10	11
11	10
12	10 (4)
13	10 (4)

[a] The tests used were as follows: 1, Abbott HIV-1; 2, Abbott HIV-1/2; 3, CBC HIV-1; 4, Syva HIV-1; 5, CPI HIV-1; 6, Gen Sys HIV-1; 7, Ortho/Cambridge Western blot; 8, ST strip; 9, Org Tek HIV-1; 10, Gen Sys HIV-1/2; 11, Fluorognost IFA; 12, Bio-Rad Western blot; 13, Organon Teknika Western blot.

[b] See text for details concerning the panel. The data for all but the ST test strip were provided by Boston Biomedica, Inc.; the EIA data were from in-house analyses performed by Boston Biomedica, Inc. The total possible number of reactive specimens was 14. The values in parentheses are the number of specimens with indeterminate results.

were the best alternative, and field studies need to be conducted to confirm the results for the diagnosis of HIV-2 infection. Also, there was no information on saliva specimens from subjects infected with various HIV-1 subtypes. By implication, with data from the serum version of this test (24a), the authors feel that detection of many HIV-1 subtypes would certainly be possible.

Although the method described here allows immediate analysis at the site of specimen collection, it may also be suitable for analysis of specimens stored for some time (up to several weeks) after collection and processing. Given the environmental conditions one can encounter in tropical countries, we investigated the stability of specimens in assay buffer and the exposure of test strips (in bottles) at elevated temperatures (up to 45°C) and elevated external humidity. In this study, processed specimen in assay buffer was stable at this temperature for 8 weeks, which is sufficient time to transport a collected specimen from a remote location to a point of analysis (e.g., if off-site analysis is desired).

Likewise, the test strips themselves are stable for extended periods (30 weeks) at temperatures and humidities at or exceeding "ambient conditions" in tropical climates. The time limit of the functional integrity at lower temperatures is still under investigation. However, by way of projection we expect unaltered performance for at least 1 year at storage temperatures of 4 to 33°C. This estimate is derived in part from experience with test strips that use identical technologies but that use serum or plasma instead of saliva as the biological medium (unpublished results).

It has been emphasized by some that saliva enriched with gingival fluid would be advantageous as a diagnostic medium. Although it has been shown that the concentration of IgG and other proteins (23, 26) is, on average, higher in such a medium, there is no guarantee that any of the published methods for collection increase the content of gingival (and mucosal) exudate in a specific individual. The exudate component in saliva is relatively low, and large variations in IgG concentrations in exudate-enhanced saliva have been observed (23). Therefore, analytical methods that require a high sensitivity and specificity, such as those for the diagnosis of HIV infection, should prove their validity for the worst-case scenario, which is exclusion of stimulation of gingival exudate. For the method of saliva collection described here, no effort was made to stimulate exudation.

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