Further Characterization of Human Salivary Anticandidal Activities in a Human Immunodeficiency Virus-Positive Cohort by Use of Microassays

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Saliva contains many antifungal proteins, e.g., histatins (16), lysozyme (6, 13, 24), lactoferrin (8, 11), and secretory immunoglobulin A. Several studies have demonstrated associations between oral candidal status and concentrations of salivary histatins (1, 5) or lysozyme (25). Methods to directly evaluate anticandidal activities of saliva have been reported previously (21). These assays are based on the ability of saliva to inhibit blastocandial viability of Candida albicans or to inhibit the formation of germ tubes by C. albicans. Typically, C. albicans organisms grow as single ellipsoidal cells called blastocandia. In the presence of inducing environmental signals, e.g., alterations of pH, temperature, and nutrients, C. albicans can assume a hyphal and/or pseudohyphal form (3). Germ tube formation is the first step in the conversion of blastocandia to hyphal form. Human saliva from healthy individuals will inhibit C. albicans blastocandial viability and will inhibit the formation of germ tubes by C. albicans (21). Previous reports show that salivary anticandidal activities are severely compromised in AIDS patients (18).

Assays of salivary antifungal capacities are useful for investigation of the pathogenesis of oral candidal infections. However, such investigations are limited, perhaps because the current methods require a minimum of 1 ml of saliva for a single assay. In cases of hyposalivation or xerostomia, a common occurrence for human immunodeficiency virus (HIV)-AIDS patients (21, 25), the ability to obtain 1 ml of saliva can be difficult. In the present study, we have modified existing anticandidal assays for use with smaller quantities of saliva and we have optimized the assay conditions for these modified methods. We have used these assays to characterize salivary anticandidal activities against several strains of Candida isolated from different body sites. We also used these assays against Candida strains which were either resistant or susceptible to the antifungal drug fluconazole. Finally, we used these assays to characterize the anticandidal activities of stimulated whole saliva obtained from a cohort of HIV-AIDS patients.

MATERIALS AND METHODS

Subjects. To develop and characterize the microcantidial assays, multiple stimulated whole- and glandular saliva samples were collected from four medically healthy male volunteers. The mean age was 41 years with a range from 26 to 52 years. These volunteers took no medications.

These anticandidal microassays were used to characterize the anticandidal activity of the saliva of HIV-AIDS patients (n = 12 males; 39 ± 6.7 years) who were recruited from participants in the Fluconazole Efficacy Study (T. F. Patterson, Department of Medicine, University of Texas Health Science Center at San Antonio). The mean CD4+ lymphocyte count was 162.4 ± 165.0 cells/μl (mean ± standard deviation [SD]). All patients were taking anti-HIV and/or anti-AIDS medications including lamivudine, stavudine, zidovudine, didanosine (ddI), delavirdine, nevirapine, indinavir, ritonavir, or saquinavir. No patient took fluconazole or other anticandidal medications at the time of saliva collection. Stimulated whole-saliva samples collected from 17 male healthy volunteers (23 to 53 years old with a mean of 31 years) were used as controls for the studies with the HIV-AIDS patients.

This study was approved by the Institutional Review Boards of the University of Texas Health Science Center at San Antonio and the Audie L. Murphy Division, South Texas Veterans Health Care System. Informed consent was obtained from all participants.

Saliva collection and treatment. Stimulated whole saliva was obtained by having subjects chew paradin wax. Stimulated parotid saliva was obtained using a modified Carlson-Crittenden cup placed over Stenson’s duct and held in place with gentle suction (23). Submandibular-sublingual saliva was collected...
with gentle suction by using a plastic micropipette (4) held at the orifices of Wharton’s and Bartholin’s ducts in the floor of the mouth (26). For stimulation of glandular saliva, the dorsolateral surfaces of the tongue were swabbed with a 2% citric acid solution at intervals of 30 s. The saliva collection time was 5 min. The collected saliva was immediately placed on ice. All saliva samples were adjusted to pH 4.5 with glacial acetic acid. For whole-saliva samples, 2 mM phenylmethylsulfonyl fluoride (PMSF) was added to prevent proteolysis. The acidified saliva was boiled for either 2.5 (parotid and submandibular-sublingual saliva) or 10 (whole saliva) min. After cooling on ice for 20 min, the samples were centrifuged (16,000 × g, 10 min) at 4°C. When patient and healthy control samples were evaluated, a sample of this pool was included on each day to ensure assay reproducibility. If the percent inhibition for this assay control pool was less than 90%, the saliva samples were reanalyzed on another day.

**Assay of salivary inhibition of blastoconidial viability.** The *C. albicans* isolates used in this project were obtained from patients with HIV-AIDS (1215 and 566) were used during the characterization of salivary antifungal assays. Since the results with the two strains were similar, only the results for saliva against isolate 1215 are presented in Table 1. The data shown in Table 1 is the result of multiple saliva samples taken at different times from a single healthy volunteer. All saliva samples, i.e., parotid, submandibular-sublingual, and whole saliva, had strong inhibition of both *C. albicans* blastocoidal viability and germ tube formation. Similar antifungal activities were observed for three other healthy subjects (data not shown).

The inhibition of blastocoidal viability against a variety of *C. albicans* isolates was examined in stimulated whole, parotid, and submandibular-sublingual saliva. The data is shown in Table 2. In these assays, whole and submandibular-sublingual saliva were from a single donor while the parotid saliva studies used saliva from two individuals. Since the methods to analyze antifungal activities in individual glandular and whole saliva were slightly different, a comparison of antifungal activities among whole saliva, parotid saliva, and submandibular-sublingual saliva was not performed. There were significant differences among the saliva types in the susceptibilities of different

### Table 1. Antifungal activities in whole and glandular saliva collected from a single healthy subject

| Type of saliva | Blastoconidial viability | Germ tube formation |
|---------------|--------------------------|---------------------|
|               | n | Buffer control (CFU/plate) | Saliva treatment (CFU/plate) | % Inhibition | n | Buffer control (% germination) | Saliva treatment (% germination) | % Inhibition |
| WS | 5 | 185.3 ± 25.9 | 5.5 ± 2.8 | 97.0 ± 1.6 | 3 | 94.4 ± 6.7 | 14.2 ± 1.1 | 85.3 ± 10.7 |
| PS | 5 | 175.3 ± 50.8 | 47.0 ± 19.7 | 74.7 ± 7.4 | 3 | 58.3 ± 16.7 | 0 | 100 |
| SS | 4 | 166.6 ± 44.9 | 47.5 ± 22.8 | 74.1 ± 10.6 | 5 | 63.6 ± 8.2 | 0 | 100 |

*a* Candida isolate 1215 was used. Values, except for n (number of independent measurements), are shown as means ± SDs. WS, whole saliva; PS, parotid saliva; SS, submandibular-sublingual saliva.

*b* Calculated as follows: [1 − (CFU in saliva/CFU in buffer control)] × 100.

*c* Calculated as follows: [1 − (% germ tubes in saliva/% germ tubes in buffer control)] × 100.

### Table 2. Salivary inhibition of blastocoidal viability with different *Candida* isolates

| Isolate no. and description | WS (n = 5) | PS (n = 4) | SS (n = 4) |
|-----------------------------|------------|------------|------------|
| 2520, VR                    | 91.4 ± 1.1 | 76.1 ± 1.2 | 53.1 ± 2.6 |
| 456, VS                     | 94.6 ± 1.1 | 95.6 ± 1.4 | 91.3 ± 1.6 |
| 540, OS                     | 65.5 ± 1.8 | 68.3 ± 1.6 | 21.3 ± 2.4 |
| 566, OR                     | 84.6 ± 1.6 | 97.9 ± 0.6 | 88.8 ± 1.8 |
| 546, BS                     | 61.2 ± 1.6 | 68.7 ± 1.4 | 49.9 ± 1.6 |
| 539, AS                     | 90.0 ± 1.1 | 77.0 ± 1.0 | 47.9 ± 1.8 |
| 1215, OS                    | 97.4 ± 0.9 | 75.0 ± 0.8 | 62.2 ± 0.8 |
| 996, OS                     | 98.5 ± 0.9 | 74.5 ± 2.4 | 95.7± |
| 3741, VS ND                 | 85.4 ± 1.0 | 77.7± |

*a* The saliva used in these studies was obtained from healthy donors. Each value is mean ± SD.

*b* V, vagina; O, oral; B, blood; A, aorta; R, fluconazole resistant (MIC > 64 µg/ml); S, fluconazole sensitive (MIC < 8 µg/ml).

*c* WS, whole saliva; PS, parotid saliva; SS, submandibular-sublingual saliva; n, number of determinations.

*d* Single determination.

*e* ND, not determined.
Candida isolates to inhibition of blastoconidial viability ($P < 0.001$ for each saliva type). In order to see if salivary inhibition of blastoconidial viability was related to fluconazole sensitivity, strains of fluconazole-sensitive and -resistant Candida were included. Strong salivary inhibition of blastoconidial viability was detected for all Candida isolates, regardless of fluconazole sensitivity or resistance. The range of salivary inhibition of blastoconidial viability was 98.5 to 55.3% for whole saliva, 97.9 to 68.3% for parotid saliva, and 91.3 to 21.3% for submandibular-sublingual saliva. This data suggests that the susceptibilities of different candidal isolates to inhibition of blastoconidial viability by saliva might be independent of body site of isolation and the susceptibility of the Candida strain to fluconazole.

Germ tube formation of all tested Candida isolates was also inhibited by whole, parotid, and submandibular-sublingual saliva. The susceptibilities of six candidal isolates to inhibition of germ tube formation by parotid saliva ($>86.3\%$) and submandibular-sublingual saliva (73 to 95\%) are shown in Fig. 1. Again, this data suggests that there were no differences specifically related to isolation site or to fluconazole susceptibility.

Anticandidal activities in paraffin-chewing-stimulated whole saliva of 12 HIV-infected patients and 17 healthy controls were evaluated by these anticandidal assay methods. The HIV-infected individuals had significantly lower (~40\%) median salivary flow rates than those of healthy controls ($P < 0.05$, Table 3). Isolates 1215 and 540 were used to study salivary inhibition of blastoconidial viability, whereas isolates 1215 and 566 were used to study salivary inhibition of germ tube formation. Almost all saliva samples from the healthy controls had 100\% inhibition of both blastoconidial viability and germ tube formation. The median inhibition of blastoconidial viability by saliva from HIV-positive patients was significantly lower than that of the controls ($P < 0.05$, Table 3). The median salivary inhibition of Candida germ tube formation was also significantly reduced in HIV-positive patients compared to that for controls ($P < 0.001$, Table 3).

![FIG. 1. Salivary inhibition of germ tube formation with different Candida isolates. The saliva of a healthy person was used. The height of the column indicates the mean percentage of inhibition with the SD being indicated by the error bars. The mean is based on three separate determinations. Closed columns indicate stimulated parotid saliva (SP), while open columns indicate stimulated submandibular-sublingual saliva (SS).](image)

| TABLE 3. Comparison of anticandidal activities for whole saliva from HIV-positive patients and healthy controls$^a$ |
|---|---|---|---|
| Subject group ($n$) | Salivary flow rate (ml/min) | % Inhibition |
| | | Blastoconidial viability | Germ tube formation |
| HIV positive (12) | 1.21 (0.64–1.35)$^b$ | 88.8 (70.4–95.2)$^c$ | 80.3 (11.1–87.7)$^d$ |
| Control (17) | 2.00 (1.48–2.40) | 100 (97.9–100) | 100 (100–100) |

$^a$ Each value is the median with the 25th and 75th percentiles indicated in parentheses.

$^b$ Significantly different from the control ($P < 0.001$).

$^c$ Significantly different from the control ($P < 0.05$).
DISCUSSION

In this study, we have modified published salivary antican didal assays (21) for use with smaller quantities of saliva, and we have further characterized these assays. Using these in vitro bioassays, we have demonstrated that stimulated whole, parotid, and submandibular-sublingual saliva have strong antican didal activities, i.e., inhibition of blastoconidial viability and inhibition of germ tube formation. Saliva appears to have a very broad spectrum of antican didal activities. Although the susceptibilities of Candida isolates to saliva may be varied, the relative susceptibility or resistance of different C. albicans iso lates to saliva was not related to the body site of isolation or to fluconazole resistance or susceptibility (Table 2 and Fig. 1).

Although saliva contains many antifungal proteins, it also contains the nutrients for Candida growth (5, 17). Therefore, salivary inhibition of blastoconidial viability in vitro can be detected only indirectly. The assay for inhibition of blasto conidial viability is based on damage of the C. albicans cell membrane by preincubation in saliva followed by incubation in a nonnutrient buffer leading to the inability of the organism to grow (21). As indicated in the previous report (21), the assay for inhibition of blasto conidial viability was sensitive to pH, saliva preincubation time, boiling time, and yeast cell concentration. We found that the saliva sample could be stored at a low temperature and successfully used for the antican didal assays if the saliva had been acidified and boiled before freezing. If the saliva samples were frozen before being acidified and boiled, about 40% of the activity for inhibition of blasto conidial viability was lost (data not shown). Studies have also suggested that germinated Candida organisms are less susceptible to killing by other salivary antican didal proteins, i.e., histatins (24). We have tested the salivary inhibitory activities toward germinated Candida and blasto conidia by using glandular saliva. There was no difference in salivary inhibition of Candida blastoconidial viability between these two forms (data not shown).

In our assay, a relatively high yeast-to-saliva ratio was used, which should increase the possible detection of mild alterations in salivary inhibition of blastoconidial viability. The protease inhibitor, PMSF, was not needed in the previous report, for which a larger quantity of saliva was used in the assay (21). However, in our study, the addition of the protease inhibitor PMSF was critical to preserve the inhibition of blastoconidial viability for treated whole saliva. However, it is possible that PMSF could have a negative effect on germ tube formation. Previous studies have shown that germ tube formation is dependent on the concentrations of serum (3a). We found that the concentration of FBS in the incubation mixture had to be almost doubled (increased from 18% in the non-PMSF-treated glandular saliva samples to 33% in the PMSF-treated whole saliva samples) for germ tube formation to occur at a low level in the saliva-treated sample. By increasing the FBS concentra tion for the whole-saliva samples so that a low level of germ tube formation occurred in saliva from healthy people, we believe we have overcome any negative effect of PMSF on germ tube formation.

It has been suggested that the hyphal form of Candida is more virulent than the blastoconidial form in vivo. Formation of hyphae appears to enhance the adhesion of Candida to host epithelial cells and also to enhance tissue invasion (7, 20). In vitro, saliva inhibition of germ tube formation is dependent on the concentrations of FBS and yeast cells as well as pH (2, 21). In our assay system, 18 and 33% FBS were found to be the optimal concentrations for glandular saliva and whole saliva, respectively. Unlike the previous report, which used water for the control in the germ tube formation assays (21), we used a sodium acetate buffer (25 mM, pH 4.5) as the control germination mixture, since saliva samples were acidified to 4.5 with acetic acid immediately after collection. We found that germ tube formation with use of acetic acidbuffer for the control was reduced (approximately 20%; data not shown) compared to that with use of water.

Oral candidal infection is a common oral manifestation in HIV-infected patients (9, 14). HIV-positive patients usually have lower salivary flow rates, as demonstrated in our study. Most of our HIV-infected cohort were taking multiple anti-HIV drugs as well as drugs for management of HIV infection. Many of these are known to cause mouth dryness. A small proportion of the HIV-positive subjects may develop Sjögren’s-like syndrome during the course of HIV infection (22). Several studies have demonstrated a relationship between the occurrence of oral candidiasis and a decreased salivary flow rate (12, 14). In our current study, we have used our antican didal microassays to demonstrate that the salivary antican didal activities of whole saliva are compromised in HIV-positive patients. These results confirm observations of a previous study (18) which demonstrated that stimulated whole, parotid, and submandibular-sublingual saliva from AIDS patients had decreased salivary antican didal activities (12). Both the inhibition of blastoconidial viability and the inhibition of germ tube formation in whole saliva were reduced in our HIV positive patients. Reductions of concentrations or activities of salivary antifungal proteins, such as histatins and secretory immunoglobulin A, may account for the loss of antican didal activities in HIV-positive patients (10, 15, 19). Current investigations in our laboratory are studying whether salivary antifungal components are altered in these HIV-positive patients and whether there is a relationship between salivary antican didal activities and the progression of HIV infection.

In conclusion, we have modified salivary antican didal assays for use with small volumes of saliva. The requirement for smaller volumes of saliva enables the study of salivary antican didal activities in subjects with very low flow rates. We have found that human saliva has strong antican didal activities and that there is a decrease in the salivary antican didal activities in severely immunocompromised patients. This loss of salivary antican didal activity in AIDS patients merits further elucidation.

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