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

ALAN L. LIN,1 QINGHONG SHI,1 DORTHEA A. JOHNSON,2 THOMAS F. PATTERSON,3,4 MICHAEL G. RINALDI,1,5 AND CHIH-KO YEH1,2,4*

Departments of Dental Diagnostic Science,1 Community Dentistry,2 Medicine,3 and Pathology,5 University of Texas Health Science Center at San Antonio, and Geriatric Research, Education and Clinical Center, Audie L. Murphy Division, South Texas Veterans Health Care System,4 San Antonio, Texas

Received 16 February 1999/Returned for modification 20 May 1999/Accepted 30 July 1999

Salivary anticandidal activities play an important role in oral candidal infection. R. P. Santarpia et al. (Oral Microbiol. Immunol. 7:38–43, 1992) developed in vitro anticandidal assays to measure the ability of saliva to inhibit the viability of Candida albicans blastoconidia and the formation of germ tubes by C. albicans. In this report, we describe modifications of these assays for use with small volumes of saliva (50 to 100 µl). For healthy subjects, there is strong inhibition of blastocandial viability in stimulated parotid (75%), submandibular-sublingual (74%), and whole (97%) saliva, as well as strong inhibition of germ tube formation (>80%) for all three saliva types. The susceptibility of several Candida isolates to inhibition of viability by saliva collected from healthy subjects is independent of body source of Candida isolation (blood, oral cavity, or vagina) or the susceptibility of the isolate to the antifungal drug fluconazole. Salivary anticandidal activities in human immunodeficiency virus (HIV)-infected patients were significantly lower than those in healthy controls for inhibition of blastocandial viability (P < 0.05) and germ tube formation (P < 0.001). Stimulated whole-saliva flow rates were also significantly lower (P < 0.05) for HIV-infected patients. These results show that saliva of healthy individuals has anticandidal activity and that this activity is reduced in the saliva of HIV-infected patients. These findings may help explain the greater incidence of oral candidal infections for individuals with AIDS.

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, which is 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 microanticandidal 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 antifungal 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 paraffin wax. Stimulated parotid saliva was obtained by 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, 15 min) at 4°C. The supernatant was saved for further analysis or stored at −70°C.

For saliva treatment, stimulated whole saliva was collected from several healthy donors, and the salivas were combined. This saliva pool was processed as described above, and the supernatant was stored in small aliquots at −70°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 reprocessed as described above, and the supernatant was stored in small aliquots.

**Assay of salivary inhibition of blastoconidial viability.** The *C. albicans* isolates used in this project were obtained from HIV-AIDS patients. These isolates came from different body sites and had different susceptibilities to the antifungal drug fluconazole. An isolate was considered fluconazole sensitive when the MIC of fluconazole was ≤2 μg/mL. The MICs for the two fluconazole-resistant isolates (2520 and 566) were ≥8 μg/mL.

The microassay of salivary inhibition of blastoconidial viability was performed by modifications of the method of Pollock et al. (18). *C. albicans* organisms were grown to late log phase (optical density of 1.4 to 1.6 at 600 nm). After centrifugation (16,000 × g, for 5 min at 4°C) and washing with sterilized water, the *Candida* suspension was adjusted to 3 × 10⁶ CFU/mL with 25 mM sodium acetate buffer (pH 4.5). The incubation mixture contained 5 μl of *Candida* suspension and either 95 μl of saliva or 95 μl of 27.2 mg/mL filter-sterilized *Candida* filtrate (control). The incubation time for this mixture was 1 h at 37°C for stimulated parotid or submandibular-sublingual saliva and 4 h for whole saliva. After this incubation, the mixture was diluted 100-fold with 25 mM MES (2-[N-morpholino]ethanesulfonic acid) buffer (pH 6). The diluted mixture was then further incubated at 37°C for 4 h (glandular saliva samples) or 3 h (whole-saliva samples). After the second incubation, 100 μl was inoculated onto a Sabouraud dextrose agar plate containing chloramphenicol (0.01%). The CFU were counted after 24 h of incubation at 37°C. The percent inhibition of blastoconidial viability for each sample was calculated in comparison with the control (containing no saliva) according to the following formula: [(1 − (CFU in saliva/CFU in buffer control))] × 100. Duplicate determinations were done for each sample. All patient and healthy control saliva assays included a pooled saliva sample (described above) as an assay control.

**Assay of salivary inhibition of germ tube formation.** The salivary germ tube inhibition microassay was performed by modifications of the method described by Santarpia et al. (21). Late-log-phase *C. albicans* (optical density of 1.4 to 1.6 at 600 nm) or a static colony (diluted to 3 × 10⁷ CFU/mL in water) was used. The assay mixture for parotid and submandibular-sublingual saliva contained 6.5 μl of 27.2 mg/mL filter-sterilized N-acetylglucosamine per ml, 15 μl of fetal bovine serum (FBS), 10 μl of the freshly prepared Camida suspension, and either 95 μl of saliva or 95 μl of 20 mM sodium acetate buffer (control). In order to obtain optimal conditions for germ tube formation in the whole-saliva assay system, 32.5 μl of FBS was required. After incubation for 3 h at 37°C, the mixture was sonicated for 15 min. An aliquot of the mixture was examined under an inverted microscope (magnification, ×400). A total of 300 blastoconidia and germ tubes were counted, and the percent inhibition of blastoconidial germination was calculated according to the following formula: [(1 − (% germ tubes in saliva/µg germ tubes in buffer control))] × 100.

**Statistical analysis.** The data in the text is given as the mean ± 1 SD or as the median and the 25th to 75th percentile. Analysis of variance was used to study the differences in susceptibility of different *Candida* isolates to saliva inhibition.

---

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

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

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

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

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

* Single determination.

* 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 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).

![Graph showing salivary inhibition of germ tube formation with different Candida isolates.](http://cvi.asm.org/)

**TABLE 3.** Comparison of anticandidal activities for whole saliva from HIV-positive patients and healthy controls

<table>
<thead>
<tr>
<th>Subject group (n)</th>
<th>Salivary flow rate (ml/min)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blastoconidial viability</td>
</tr>
<tr>
<td>HIV positive (12)</td>
<td>1.21 (0.64–1.35)*</td>
<td>88.8 (70.4–95.2)*</td>
</tr>
<tr>
<td>Control (17)</td>
<td>2.00 (1.48–2.40)</td>
<td>100 (97.9–100)</td>
</tr>
</tbody>
</table>

* Each value is the median with the 25th and 75th percentiles indicated in parentheses.
* Significantly different from the control (P < 0.001).
* 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 isolates 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 blastoconidial 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 blastoconidial 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 blastoconidial 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 blastoconidia 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 concentration 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 acetate buffer 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.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE-12188 (to C.-K.Y.) and DE-11381 (to T.F.P.) from the National Institute of Dental and Craniofacial Research. We also acknowledge the contributions of Jose L. Lopez-Ribot and Marta Caceres.

REFERENCES