Usefulness of a commercial enzyme-linked immunosorbent assay (ELISA) kit for *Candida* mannan antigen for detecting *Candida* in oral rinse solutions

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Key words: Oral, *Candida*, Mannan, Candidiasis, Antigen, Mouth

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Abstract

Objective: To test the performance of a commercial enzyme-linked immunosorbent assay (ELISA) kit for *Candida* mannan antigen for detecting *Candida* in oral rinse solutions.

Study design: Forty-eight oral rinse solutions (38 from patients and 10 from healthy volunteers) were available. Mannan antigen was measured using a commercial sandwich ELISA kit, Unimedi *Candida*. The result of the mannan assay was compared with the result of conventional detection and identification by culture.

Results: The result of the mannan assay revealed that 31 of 38 clinical and 3 of 10 healthy volunteer samples were positive for *Candida*. Using the culture as a gold standard, the overall sensitivity and specificity of the mannan antigen detection were 90.9% and 46.2%, respectively.

Conclusions: The results of this study suggested that mannan antigen detection might be a possible and sensitive technique for the detection of oral *Candida*. The conditions of the ELISA-based assay should be optimized for oral rinse solutions.

Yeast species of the genus *Candida* are the most prevalent fungi recovered from the oral cavity. The reported rate of oral *Candida* among healthy individuals varies from 35 to 80%, depending on the population studied and the detection methods.[1,2] Debilitation of an individual can resulted in the occurrence of oral *Candida* infection (oral candidiasis). It is an opportunistic disease and the incidence of oral candidiasis has increased as a result of the escalation of human immunodeficiency virus infection and the more widespread use of immunosuppressive chemotherapy.[3] In addition, higher incidences of *Candida* isolation from oral conditions not previously associated with *Candida* infection have been reported.[3] These include high incidences of *Candida* in the mouth of patients with lichen planus, burning mouth syndrome, dry mouth, and leukoplakia.[3,4,5] The significance of *Candida* in these patients is uncertain, but it has been attracting increasing interest.

The conventional detection method for oral *Candida* is a culture of oral samples (i.e., smear, swab, or imprint specimen; whole saliva; and oral rinse solution). However, many studies have pointed out the low positivity of cultures.[3,6,7] Clinicians and researchers require a reliable and sensitive method to detect oral *Candida*. In recent years, numerous DNA-based methods have been reported to detect oral *Candida* species.[3,7] However, they are still laborious and costly, and have technical problems such as the risk of contamination. The methods for DNA extraction and detection should be both standardized and simplified to introduce a molecular diagnostic method for the routine examination of oral samples.

Various laboratory tests based on the detection of *Candida*-specific antibodies, antigens, or metabolites have been developed for serological diagnosis of candidemia. *Candida* mannan antigen detection is a reliable and sensitive method for serological diagnosis of systemic candidiasis, and an assay kit is commercially available.[8-10] The Unimedi *Candida* is a microplate enzyme immunoassay employing affinity-purified polyclonal antibodies against *Candida albicans* mannan. The detection method is generally prepared with *C. albicans* antigen, but it allows the detection of antigens against other *Candida* species. It was reported that the Unimedi *Candida* test was more sensitive and specific than other commercially available mannan antigen detection kits using a monoclonal antibody against *Candida albicans* mannan.[9, 11]

Studies have yet to be performed that employed mannan antigen detection as a detection tool for presence of *Candida* in the oral cavity. Therefore, the purpose of this study was to test the hypothesis that a mannan antigen detection enzyme-linked immunosorbent assay (ELISA) kit can be used in the detection of oral *Candida*. In this study, the performance of a commercially available mannan antigen detection kit (Unimedi *Candida*) for the detection of *Candida* in oral rinse solutions was assessed.

Material and Methods

This study was independently reviewed and approved by the University ethical board. The experiments were undertaken with the understanding and written consent of each subject and according to ethical principles, including the World Medical Association Declaration of Helsinki (version, 2002).

Forty-eight oral rinse solutions were available for the study. Ten samples were obtained from 10 healthy volunteers (4 women and 6 men, with a mean age of 27.9 years). Thirty-eight samples were obtained from 25 patients (20 women and 5 men, with a mean age of 71.1 years) who attended our clinic complaining of burning mouth and were clinically suspected of being colonized/infected with *Candida*. In 13 patients, repeated

samplings were carried out before and after treatment with antifungal agents.
The oral rinse solution was collected by rinsing the mouth with 10 ml of sterile phosphate-buffered saline which was held in the mouth for 1 minute prior to collection in a sterile container. A portion (10 µl) of the oral rinse solution was cultured on plates of CHROMagar *Candida*, which were incubated for 48 hours at 37˚C for isolation of multiple yeast species. Yeast identity was confirmed with the API 20C AUX system (bioMérieux, Marcy l'Etoile, France). The number of *Candida* colonies was calculated and reported in five categories: 0, 1–9, 10–99, 100–999, and more than 1,000 cfu/culture.

Mannan antigen was measured using a commercial sandwich ELISA, Unimedi *Candida* (Unitika, Amagasaki, Japan). The test was performed according to the manufacturer's instructions. Briefly, the oral rinse solution (150 µl) was mixed with 150 µl of treatment solution (including 20 nmol/L of sodium dihydrogen phosphate), boiled for 4 min, and then centrifuged at 10,000 G for 10 minutes. Supernatant (100 µl) was added to antibody-coated microtiter plate wells. After incubation at room temperature for 2 hours, the plates were washed thoroughly and 100 µl alkaline phosphatase-conjugated antibodies were added to the wells. After further incubation at room temperature for 1 hour, the plates were washed. The reaction was revealed by incubation with 100 µl substrate solution for 20 min at room temperature. After the addition of 100 µl stopping solution to the wells, optical density was read at 490–500 nm on a microplate reader. Each experiment included positive and negative controls as well as a calibration curve, which was made with a pool of normal human serum supplemented with known concentrations of mannan. Mannan concentrations greater than 0.05 U/ml (serological cut-off value) were considered a positive result, according to the manufacturer's recommendations.

Results

Candida was detected by culture in 20 (52.6%) of 38 oral rinse solutions from the clinical patients and 2 (20%) of the 10 oral rinse solutions from healthy volunteers. Of the 22 culture-positive samples, *C. albicans* was evident in 19 (86.4%) samples, *C. glabrata* in 6 (27.3%) samples, and *C. parapsilosis* in one (4.5%) sample (Table 1). Although *Candida albicans* was most frequently encountered, only non-*albicans Candida* was detected in 3 (13.6%) samples. More than one *Candida* species was detected in 4 (18.2%) samples (Table 1).

The result of the mannan assay revealed that 31 (81.6%) of 38 clinical samples and 3 (30%) of 10 healthy volunteer samples were positive for *Candida*. The agreement between *Candida* culture and mannan antigen detection of *Candida* is shown in Tables 1, 2 and 3. Using culture as a gold standard, the overall sensitivity and specificity of the mannan antigen detection was 90.9% and 46.2%, respectively. Fourteen out of 26 culture-negative samples (53.8%) were judged as positive by mannan antigen detection, while only two out of 22 culture-positive samples (9.1%) were judged as negative by *Candida* antigen detection. Of these two samples, one was culture positive for only *C. glabrata* (Table 1). The other was culture positive for *C. albicans* (Table 1) and showed a borderline result in the mannan assay (mannan concentration of 0.04 U/ml, Table 3).

The relationship between mannan concentration in the immunoassay and the number of *Candida* colonies in culture are also shown in Table 3 and there was a statistically significant correlation between them (R^2=0.488; Spearman rank correlation test, $p<0.01$).

The results of assessment of mannan concentrations before and after treatment with antifungal agent are shown in Figure 1. Eleven of 13 patients showed a significant

reduction in mannnan concentration between before and after antifungal treatment (median of 0.4 U/ml before treatment vs. median of 0.11 U/ml after treatment, Wilcoxon signed-ranks test, $p<0.05$). In the mycological assessment by culture, eight of 13 patients had *Candida*-positive cultures before the treatment. Of these eight patients, six showed negative culture after the antifungal treatment.

Discussion

The Unimedi *Candida* assay uses affinity-purified polyclonal antibodies against *Candida albicans* mannan. The detection method is generally prepared with *C. albicans* antigen, but allows for the detection of antigens against other *Candida* species. The method employs enzymatic cycling that improves the sensitivity of the immunoassay. In the detection of serum mannan antigens in patients with candidemia, a high sensitivity of 69% and specificity of 89% was reported.[9] It was also reported that the Unimedi *Candida* showed high sensitivity for the detection of mannanaemia with *C. albicans*, *C. tropicalis*, C. *guilliermondii*, and *C. tropicalis*, while low sensitivity for that with *C. glabrata*, *C. parapsilosis*, and *C. krusei*.[9,11] The Unimedi *Candida* is generally prepared for serum samples and, to the best of our knowledge, this is the first application of manna antigen detection in an oral rinse solution.

In this study, the result of the mannan assay revealed that 31 (81.6%) of 38 clinical samples and 3 (30%) of 10 healthy volunteer samples were positive for *Candida*. White et al. used real-time polymerase chain reaction (PCR) to detect *Candida* in concentrated oral rinse cultures and reported that 97 (67%) of the 145 clinical samples and 17 (38%) of the 48 healthy control samples were positive for *Candida*.[3] Liguori et al. used a multiplex PCR assay directly for oral rinse solutions and reported that 64 (82.1%) of 78 samples from symptomatic patients were positive for *Candida*.[7] Our results are compatible with their results. These results suggest that the mannan antigen detection in oral rinse solution might be a sensitive assay for the detection of oral *Candida*, possibly being as sensitive as DNA-based methods. In addition, the results of assessment of mannan concentrations before and after treatment with antifungal agent showed a significant decrease of mannan concentrations following treatment. This result also strongly supports the sensitivity of the mannan antigen detection for oral *Candida*.

In this study, the results of mannan detection achieved 90.9% agreement with those of the culture-positive samples. The mannan detection assay showed a low false-negative rate (2 samples, 9.1%). Of the two false-negative results, one showed only *C. glabrata*, with a mannan concentration of 0.02 U/ml, that was under the serological cut-off value of > 0.05 U/ml. In the results of this study, the Unimedi *Candida* showed high sensitivity for *C. albicans* (93.8%, 15/16), while lower specificity for *C. glabrata*. The difference in *Candida* species probably related to the false-negative result. The other false-negative result showed *C. albicans*, with a mannan concentration of 0.04 U/ml, which was the lowest concentration in the culture-positive samples for *C. albicans* and was a borderline concentration. As shown in this study, there was a positive correlation between mannan concentrations in the immunoassay and the number of *Candida* colonies. If all samples with a mannan concentration equal to or more than 0.02 U/ml were judged as positive for *Candida*, no false-negative results would have been reported. In the mannan assay of this study, an ELISA standard curve using pooled serum provided by the manufacturer was used. Thus, it is possible that the significance of the standard ELISA curve and the cut-off value should be reconsidered in the detection of *Candida* in oral rinse solutions with

respect to the cut-offs used for serum.

On the other hand, mannan immunoassay detected 14 mannan-positive samples among the 26 culture-negative samples, possibly highlighting the higher sensitivity of mannan antigen detection than the culture of oral rinse solutions. Considering the mechanism of detecting *Candida* by manman immunoassay and the possibility of detecting nonviable cells, the higher sensitivity of the mannan antigen detection compared to culture is understandable. This is also consistent with other studies where PCRs have been found to be more sensitive than conventional culture methods.[3,6,7,8]

In conclusion, the results of this study suggest that mannan antigen detection might be a possible and sensitive technique for the detection of oral *Candida*. Further studies which discuss the agreement between *Candida* detection by mannan antigen and that by DNA-based methods are mandatory. In addition, because the mannan antigen detection kit (the Unimedi *Candida*) employed in the study is generally prepared for serum samples, studies that evaluate optimal conditions of the ELISA-based assay for oral rinse solutions and determine the detection limit of mannan antigen detection for oral *Candida* in oral rinse solution are important to be performed.

References

1. Abu-Elteen KH, Abu-Alteen RM. The prevalence of Candida albicans populations in the mouths of complete denture wearers. New Microbiol. 1998;21:41-8.
2. Ben-Aryeh H, Blumfield E, Szargel R, Laufer D, Berdicevsky I. Oral Candida carriage and blood group antigen secretor status. Mycoses 1995;38:355-8.
3. White PL, Williams DW, Kuriyama T, Samad SA, Lewis MAO, Barnes RA. Detection of Candida in concentrated oral rinse cultures by real-time PCR. J Clin Microbiol 2004;42:2101-7.
4. Krogh P, Holmstrup P, Thorn JJ, Vedtofte P, Pindborg JJ. Yeast species and biotypes associated with oral leukoplakia and lichen planus. Oral Surg Oral Med Oral Pathol 1987;63:48-54.
5. Kurnatowska AJ. Search for correlation between symptoms and signs of changes in the oral mucosa and presence of fungi. Mycoses 2001;49:379-82.
6. Ahmad S, Khan Z, Mustafa AS, Khan ZU. Seminested PCR for diagnosis of candidemia: Comparison with culture, antigen detection, and biochemical methods for species identification. J Clin Microbiol 2002;40:2483-9.
7. Liguori G, Lucariella A, Colella G, De Luca A, Marinelli P. Rapid identification of Candida species in oral rinse solutions by PCR. J Clin Pathol 2007;60:1035-9.
8. Alam FF, Mustafa AS, Khan ZU. Comparative evaluation of (1, 3)-ß-D-glucan, mannan and anti-mannan antibodies, and Candida species-specific snPCR in patients with candidemia. BMC Infectious Diseases 2007;7:103.
9. Fujita S, Takamura T, Nagahara M, Hashimoto T. Evaluation of a newly developed down-flow immunoassay for detection of serum mannan antigens in patients with candidaemia. J Med Microbiol 2006;55:537-43.
10. Persat F, Topenot R, Piens MA, Thiebaut A, Dannaoui E, Picot S. Evaluation of different commercial ELISA methods for the serodiagnosis of systemic cnadidosis. Mycoses 2002;45:455-60.
11. Yoshida K, Niki Y, Miyashita N, Matsushima T. Clinical utility of serum Candida mannan antigen detection kit using ELISA. Kansenshogakuzashi 2002;76:536-41. (Written in Japanese with English abstract)

Figure Legend

Figure 1. Mannan concentrations before and after treatment with antifungal agent (n=13).

Table 1. Result of culture identification of *Candida* species and result of mannan detection in oral rinse solutions (n=48)

	No. of culture-positive samples	No. of mannan-positive / no. of culture-positive samples (%)
C. albicans	16	15/16 (93.8)
C. glabrata	2	1/2 (50.0)
C. albicans + *C. glabrata*	3	3/3 (100)
C. glabrata + *C. parapsilosis*	1	1/1 (100)
Negative	26	14/26 (53.8)

Table 2. Comparison of *Candida* detection by *Candida* mannan antigen vs. culture

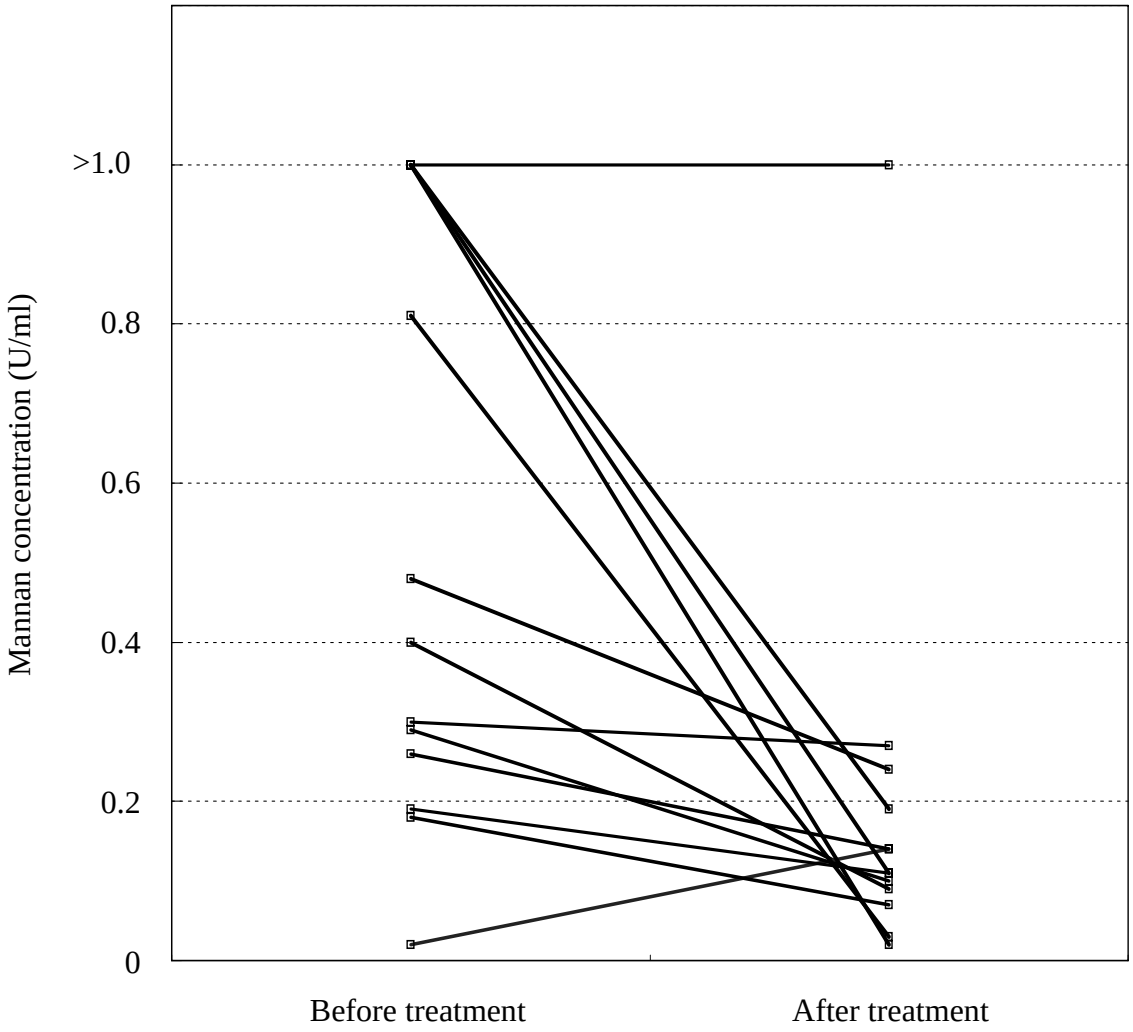
	Culture	
	Positive	Negative
Mannan antigen		
Positive	20	14
Negative	2	12

Table 3. Comparison between mannan concentration and result of culture identification of *Candida*

Mannan concentration (U/ml)	Result of culture			Total (n=48)
	Negative (n=26)	Positive (n=22)		
		1-9 cfu*	10-99 cfu*	
0.00	8			8
0.02	3	1		4
0.03	1			1
0.04		1		1
0.07	1			1
0.08	1			1
0.09	1			1
0.10	1			1
0.11	2			2
0.14	2			2
0.18		1		1
0.19	1	1		2
0.24		1		1
0.26	1	1		2
0.27		1		1
0.29		1		1
0.30	1			1
0.40	1			1
0.42			1	1
0.47	1			1
0.48		1		1
0.63		1		1
0.66		1		1
0.81	1			1
0.87		1		1
>1.00		5	4	9

Dashed line; Borderline between positive and negative result in the mannan assay, *; Number of colonies per culture

Figure 1


Mannan concentration (U/ml)
>1.0
0.8
0.6
0.4
0.2
0
Before treatment
After treatment