Original Article

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Detection of single and mixed colonization of *Candida* species in patients with denture stomatitis

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Abstract

Aim: To evaluate the profile of the colonization by *Candida spp*. using presumptive identification to classify the patients with denture stomatitis as having single or mixed colonization, correlating with oral and systemic status. **Methods:** The CHROMagar Candida™ Medium (CC) for yeast culture and exfoliative cytology was used to identify colonization by *Candida* spp. and distinguish the different species of the *Candida* genus from patients with denture stomatitis (DS) and denture wearers without DS (control group). In addition, colonization was correlated with specific habits, such as tobacco and alcohol use, as well as with the use of systemic drugs. **Results:** Direct swabbing of whole unstimulated saliva (WUS) and palatal mucosa revealed colonization in 97.3% of the patients with DS. In the control group, 55.0% patients presented colonization. The presumptive identification found *C. albicans* as the most prevalent between both groups, respectively in 89.4% of the DS group and 40.0% from the control group. Regarding the non-*albicans* species in the DS group, the most frequent were *C. krusei* (31.5%), *C. glabrata* (21.0%) *C. tropicalis* (15.7%) and *Candida spp* (2.6%). Smokers presented 90% of mixed isolates, and no *C.albicans* single colonization in the DS group, with statistically significant difference between smokers and non-smokers (p=0.0051). In the control group, the non-*albicans* species were *C.glabrata* (23.0%) and *C.tropicalis* (23.0%). The results of cytology from the DS group showed positive results in 22.2% of the cases. **Conclusions:** The use of CC was effective as a complementary method for the diagnosis of colonization by *Candida spp*. and DS, with the additional advantage of enabling a rapid presumptive identification of the specie. Smoking seemed to play a role in the colonization of oral mucosa by mixed *albicans* and non-*albicans* species. Mixed colonization seems to be more prevalent between patients with DS.

Keywords: culture media, *Candida albicans,* candidiasis, denture stomatitis.

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Introduction

Candida infections are common and often recurrent, and represent a significant clinical problem[1]. Host factors play a more important role than organism virulence in the pathogenesis of oral candidosis, and intraoral environment conditions, such as the presence of dentures, also play a crucial role in the disease[2].

Candidosis is most commonly caused by *Candida albicans*[3]. Other species including *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei* have also been isolated from denture wearers[4]. In these patients, an important infection described as *Candida*-associated denture stomatitis (DS) occurs in about 50-60% of them[5].

The role of non-*albicans* species has become increasingly important, especially in high-risk subjects, such as HIV-positive patients[6]. Another recent study with patients with hematological malignancies and head and neck solid tumors revealed that the majority of cases of oral candidiasis were caused by *C. albicans,* but almost one third of patients harbored non-*albicans* strains, such as *C. glabrata,* which were often more resistant to anti-fungal agents[7]. These facts underline the importance of an appropriate sampling method, for a more accurate diagnosis. Cytology is the most frequently used technique in dental clinics because it is easy to perform and les time consuming when compared with other diagnostic techniques. For the culture techniques, the usual collecting methods to obtain yeasts are whole unstimulated saliva (WUS) collection for swabbing, swabbing the oral mucosa, scraping it with a spatula or rinsing the mouth[8].

The identification of yeasts is crucial to ensure an effective antifungal treatment. Several chromogenic media have been developed to aid the rapid identification of *Candida* species. These media contains chromogenic substrates that react with yeast enzymes, resulting in colored colonies[9]. CHROMagar Candida™ medium (CC) is a differential culture medium that allows the presumptive identification of yeasts species, with sensitivity and specificity higher than 99% to C. *albicans*[10].

The aim of this study was to evaluate CC and cytology methods to identify colonization and candidiasis as well as to distinguish the presumptive species on *Candida* genus from denture wearers with and without DS, correlating single and mixed colonization with some specific oral conditions namely denture wearing and predisposing factors to the installation of infection, such as tobacco and alcohol habits and the use of systemic drugs.

Material and methods

Fifty-eight patients undergoing dental treatment at the School of Dentistry of University of São Paulo were selected for this study. Informed consent was obtained from all subjected and the study protocol was approved by the university's Ethics Committee (FR 268407 – PR 95/2009).

Exclusion criteria were age under 18 and over 95 years, use of antibiotics and/or steroids up to 1 month before the study, use of antifungal drugs (systemic or topical use) in the 3 months before the study and complaints of dry mouth sensation. The patients were divided in two groups: DS group (patients with denture stomatitis; n = 38) and control group (denture wearers without DS; n = 20).

Clinical Examination

Clinical examination was performed according to the criteria proposed by Newton (1962) for DS classification in types I, II, III[11]. Type I showed localized inflammation or pinpoint hyperemia; type II showed a generalized erythema and type III comprised papillary hyperplasia of the palate. All patients who met these criteria were included in the test group.

Denture evaluation was done by direct examination and the patients were questioned about their age, gender, smoking and alcohol use, systemic health status, use of medicines (class, dose and frequency), oral health and denture wearing. The criteria to establish smoking and alcohol habits were the following: smoker was the individual who had the habit of daily use of tobacco at the time of enrolment in the study and alcohol habit was the use of distilled and/or fermented beverage two or more times a week at the time of enrolment in the study.

Samples and Techniques

WUS was collected during 3 min in a sterile tube, under standard conditions: between 8 AM and 11 AM, no feeding, drinking, smoking or hygienic habits allowed for 60 min before the test section.

After this step, a sterile swab was inserted in the tube and was direct sampled in half of a CC Plate (PlastLabor, Rio de Janeiro, RJ, Brazil). On the other half of the plate, an oral swab from palatal mucosa were taken by passing this sterile cotton several times across the mucosal surface and then seeding onto the CC plate.

Exfoliative cytology was performed following the swabbing. The technique consisted in passing a Cytobrush several times across the palate surface and disposed the collected material on two glass slides and fixed in absolute alcohol. These slides were sent to the Oral Pathology Department and stained by Periodic Acid Schiff (PAS) and Papanicolaou techniques. The fungal presence was searched by analyzing the presence of hyphal outgrowths (abundant, moderate, scarce and absent).

After 48 h of incubation at 37ºC, the CC plates were photographed and the digitized images were entered into the ImageJ *software* (ImageJ 1.44f, Wayne Rasband, Bethesda, MD, USA) for analysis of colonies. The presumptive identification of *Candida* species was based on the criteria proposed by Odds and Bernaerts (1994), who described the species by the color of the colony. *C. albicans* colonies are described by their green color, *C. tropicalis* colonies based on their dark-blue to blue-gray color, surrounded by a dark/pink halo, *C. glabrata* by their white/dark pink/purple range of colors and *C. krusei* colonies based on their pale pink color and downy/rough appearance with pale edges. The colonies presenting other colors were classified as *Candida ssp.*[12]. The identification of the colors of the colonies in the digitalized images was made by two calibrated dentists.

Patients who had moderate and abundant proportions of hyphal outgrowths in the cytological exams, along with positive clinical signs and positive culture tests, were considered as having candidiasis.

The statistical tests were made in the DS group only, with comparison between single and mixed colonization when considering the tobacco and alcohol habits and use of systemic drugs, by the Fisher's exact test.

Results

The patients with DS had mean age of 58.1 years (SD 11.6) being 28 females and 10 males. In the control group, the mean age was 62.1 years (SD 6.9), with 18 females and 2 males. Direct swabbing from the WUS and palatal mucosa revealed colonization in 37 (97.3%) of patients with DS. In the control group, 11 (55.0%) patients presented colonization from WUS, and swabbing of palatal mucosa was positive to reveal colonization in 6 (30.0%) patients.

In the DS group, 22 patients were using medicines and the major group of drugs taken was osmotic diuretics (10 patients). In the control group, 13 patients were using medicines and angiotensin-converting enzyme (ACE) inhibitors were the most frequently taken drugs, used by 8 patients.

Direct examination of the denture prosthesis revealed the majority of them with some inadequacy in shape, contour and surface as well as inadequate hygiene. The mean time of denture wearing was 11.6 years (SD 12.3).

The isolates showed *C. albicans* as the most prevalent between both groups, respectively in 89.4% from the patients with DS and 40.0% from the control group. About the non-*albicans* species in the DS group, the most encountered were *C. krusei* (31.5%), followed by *C. glabrata* (21.0%), *C. tropicalis* (15.7%) and *Candida spp* (2.6%). In the control group, the non-*albicans* species encountered were *C. glabrata* (23.0%) and *C. tropicalis* (23.0%). To obtain the prevalence of colonization, when a patient harbored two or more species, these were counted as separate events, i.e., if a patient had colonization by *C. albicans* and *C. krusei*, they were counted as two species.

The WUS swabbing from DS patients revealed mixed colonization in 20 of them, *C. albicans* only in 12, non-*albicans* in 3 and no colonization in 3 patients. The swabbing of palatal mucosa revealed mixed colonization from 15 patients, 17 by *C. albicans* only, 3 of non-*albicans* and 3 with no colonization.

Cytology showed positive results only in 8 (22.2%) patients from the DS group, with 4 in the moderate range and 4 in the scarce range. In the control group, only 1 patient (7.6%) presented positivity to the test, in the scarce range. All patients with positive exfoliative cytology results were also positive for CC.

Regarding the presumptive identification, the data were distributed with all patients allocated as shown in Tables 1 and 2. The patient was considered as harboring mixed yeast population when presented mixed colonization by swabbing of WUS and/or palatal mucosa. When the patients were allocated in different groups, smokers revealed an increased proportion in mixed colonization, with a significant difference between the patients with and without the tobacco habit ($p = 0.0051$).

Four patients with positivity to the tests and clinical indication of therapeutics received treatment for candidiasis with nystatin topical and all individuals were sent to receive new dentures.

Discussion

DS is a frequent finding among denture wearers. Although its etiology is unknown, the influence of the colonization by *Candida spp.* has already been described. At the same time, not all patients with DS present colonization by *Candida spp.*, although some of them were not classified even as carriers. In the present study, only 1 patient with DS showed absence of colonization.

Abaci *et al.* (2010), demonstrated that the number of yeast cells in the saliva of patients with DS was <400 CFU/mL in 11 individuals and ≥ 400 CFU/mL in 19 individuals. In addition, all patients with DS, who were complete denture and removable partial denture users, presented colonization by *Candida spp.* from saliva samples. However, one had no colonization from swabs taken from the palatal mucosa[13].

No patient, in either the DS and or control group, complained about symptoms like continuous burning sensation or taste disorder. Although these symptoms are relatively common in patients with DS, it has been reported that there is no association between burning mouth syndrome and prevalence of *Candida* spp.[14]. In the same way, an increase

Table 1 - Distribution of primary colonization of *Candida* species in patients with Denture stomatitis.

Colonization	Smoking		Alcohol use		Use of systemic drugs	
	Yes	No	Yes	No	Yes	No
C. albicans only	0	14	3	11	8	6
C. albicans and non-*albicans species*	9	12	7	14	12	9
p value	**0.0051***		0.7041		1.0000	

*Significant difference ($p < 0.05$).

Table 2 - Distribution of primary colonization of *Candida* species in patients without denture stomatitis (Control Group).

Colonization	Smoking		Alcohol use		Use of systemic drugs	
	Yes	No	Yes	No	Yes	No
C. albicans only	0	6	0	6	4	2
C. albicans and non-*albicans species*	0	2	0	2	2	0

in oral yeasts is not necessarily associated with changes in mouth sensation alone[15].

The direct seeding without saliva dilution or any other procedure for sampling is preferable for clinical use, as performed in our study, bringing advantages such spending less time to carry out the technique and achievement of satisfactory presumptive results that can guide the choice of therapeutic support. The time elapsed for presumptive identification with CC, as used in the present study, was about 48 h. The identification of yeasts in a mycology laboratory with conventional media is time-consuming and cumbersome as it requires between 4 and 6 days[16].

Cytology showed positive results in 22.2% of the cases in the DS group, demonstrating the inefficiency of this method for the diagnosis of oral candidosis when the material is obtained from scraping and/or swabbing. Although the method is valuable to distinguish yeasts from hyphal forms, it is less sensitive than culture methods[17], as seen in the present study.

Colonization by *Candida* spp. in both groups represented the importance of the denture use on oral yeast carriage. Old age, clinical signs of oral dryness, denture wearing and reduction in whole unstimulated salivary flow increase the prevalence of oral yeasts[15]. Also, if the balance of the normal flora is disrupted or the patient's immune defenses are compromised, Candida spp. can invade mucosal surfaces and cause disease manifestations[18]. In addition, the high incidence of colonization in the patients with DS (97.3%) corroborates with the long-term period of the denture use (11 years on the average). This may have positively influenced the colonization and predisposed to the installation of DS.

Regarding the mixed yeast populations from patients with DS, non-*albicans* species have been found in only 14.7% individuals in a previous investigating Candida spp. incidence in denture wearers[13]. In the present study, the frequency of colonization by non-*albicans* and *C. albicans* plus non-*albicans* was higher. These findings suggest that our patients presented more mixed fungal populations because their clinical and/or systemic conditions might have predisposed them to the Candida infection.

In addition, when the patients with DS were evaluated by groups, which means separation of the patients according to their specific conditions, smokers presented 90% of mixed colonization and no *C. albicans* colonization alone. A recent study[19] found a frequency of 54.8% of *C. albicans* and 45.2% of non-*albicans* species in healthy smokers, demonstrating a possible influence of smoking habit on colonization, although nonsmokers presented 35.1% of non-*albicans* colonization. In addition, they concluded that there was a marginally significant positive correlation between the number of cigarettes smoked *per* day and the density of candidal growth from oral rinse cultures. Figueiral et al. [20] (2007) indentified *C. albicans*, *C. glabrata* and *C. tropicalis* in a group of patients with DS, and *C. tropicalis* appeared always together with one of the other species. Those authors concluded that yeasts, particularly *C. albicans*, are associated with DS. In the present study, only 2 patients in the control group were smokers and neither of them presented colonization of *C. albicans* only.

Our results revealed a higher colonization in patients using diuretics, which could explain the reduced salivary flow, favoring the presence of yeasts. However, it is important to emphasize that there were no dryness complains. Torres et al.[21] reported a frequency of 28% of mixed colonization in patients with xerostomia, being 88.4% users of medications and 58% of these patients diagnosed with hyposalivation.

The data obtained in the present study and reported in the literature revealed the influence of some variable conditions on yeast carriage and the presence of infection. When we separated the patients into different groups, we intended to evaluate the influence of each variable in colonization, aiming at determining whether some clinical aspects and habits could result in a difference between groups in an important rate. Smoking showed a high difference when the patients were allocated as smokers and non-smokers, with rates of 90% of mixed colonization.

It is worth mentioning that our study used the presumptive identification, but our objective was focused on sampling techniques. CC can be extremely helpful in a clinical stomatology service routine, facilitating the detection of mixed cultures of yeasts and allowing direct identification of C. *albicans*, as well as rapid presumptive identification of other species like *C. glabrata*, *C. tropicalis*, C*andida krusei* and even *Sacharomyces cerevisiae*[22]. However, Tintelnot et al.[23] (2000) described CC as an insufficiently selective medium for isolation of C. *dubliniensis* as it can produce dark green in primary cultures. Our findings also reinforces the need to employ other common tests, such as germ tube observation and carbohydrate assimilation, to institute an appropriate therapy for cases of undiagnosed *Candida* spp., recurrent infections or lack of response to antifungal drugs.

It has been reported that there is increasing evidence that more than one *Candida* spp. may simultaneously colonize mucosal habitat. The need to investigate the factors that play a role in the initial attachment and subsequent colonization of denture-base materials and the oral mucosa of patients subjected to *Candida* infection should be known, controlled and may prevent the disease[24]. Differentiation between species is also important for the identification of infection. CC was used as an easy and rapid technique, reaching the target to an appropriated diagnosis and institution of adequate therapy. The findings of the present study also revealed the prevalence of infections by *C. albicans* followed by C. *krusei* and *C. glabrata.* In an extensive review, Samaranayake and Samaranayake[25] (2001) reported that *C. glabrata* emerges as the second most prevalent species, frequently isolated from acrylic denture surface and the palatal mucosa. Our results led us to encourage the use of CC in stomatology daily practice. It enables better knowledge of the most prevailing species of the genus *Candida* in the oral mucosa, resulting in a better management and control of infections, and, even for screening purposes, with faster and less expensive results.

Finally, it is important to diagnose patients with DS, recommending the adequate treatment for each case. Even asymptomatic patients should be oriented and treated if

necessary, due to the importance of the oral mucosa as a primary source of reinfection, mainly in the event of an eventual hospitalization, which would affect substantially their quality of life.

In conclusion, the use of CC was an effective subsidiary technique for the diagnosis of oral candidosis in patients with DS, with the additional advantage of enabling presumptive species identification within the genus. Smoking seemed to play a role in the colonization of oral mucosa by mixed *C. albicans* and non-*albicans* species. The results also points to a more prevalent mixed colonization in patients with DS.

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