Isolation of *Candida dubliniensis* from denture wearers

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Candida albicans is considered the most important *Candida* species able to cause oral infections in denture wearers. In recent years, *Candida dubliniensis* has emerged as a pathogenic yeast in humans. The close phenotypic similarities of *C. albicans* and *C. dubliniensis* have led to the misidentification of these species. In this work, our aim was to verify through PCR the presence of *C. dubliniensis* in palate and maxillary denture samples from 112 denture wearers presenting with or without denture-related stomatitis (DRS). *C. dubliniensis* was isolated at low rates from both palate (5.3 % and 10.7 %) and maxillary denture (5.3 % and 8.9 %) samples from wearers regardless of the presence of the disease. However, when *C. dubliniensis* was detected in individuals with DRS, it was always associated with *C. albicans*. In addition, our results showed that *C. albicans* was the most commonly identified candidal species in maxillary denture and hard palate samples from DRS patients (78.5 % and 89.2 %, respectively) as well as from controls (31.2 % and 28.5 %, respectively). In conclusion, *C. dubliniensis* was detected in the oral environment of denture wearers. The association of *C. dubliniensis* with *C. albicans* occurred in approximately 10 % of the DRS cases.

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INTRODUCTION

Denture-related stomatitis (DRS) is a local recurring disease (Budtz-Jorgensen *et al.*, 1975; Dar-Odeh & Shehabi, 2003) that attacks the mucosal area subjacent to dental prostheses, beneath the fitting surface of the denture, especially maxillary dentures. Many studies have discussed the involvement of *Candida albicans* in the establishment and persistence of such disease since the acrylic denture fitting surfaces seem to facilitate the adherence of this micro-organism (Webb *et al.*, 1998; Makihiro *et al.*, 2002; Ramage *et al.*, 2004; Moura *et al.*, 2006). Non-*albicans* *Candida* species can also be obtained from DRS lesions (Figueiral *et al.*, 2007).

Recently, a newly identified *Candida* species has emerged, *Candida dubliniensis*, which exhibits numerous characteristics typical of *C. albicans* species, including the formation

of green colonies on CHROMagar medium. Both micro-organisms share several phenotypic traits such as the ability to form true germ tubes, adhere to epithelial surfaces, secrete a range of aspartic proteinases and form chlamydospores (Martinez *et al.*, 2002; Sullivan & Coleman, 1998; Sahand *et al.*, 2005; Gilfillan *et al.*, 1998). *C. dubliniensis* was first obtained from oropharyngeal lesions of human immunodeficiency virus (HIV)-positive patients (Sullivan & Coleman, 1998). Diabetic HIV-negative individuals also exhibited a high prevalence of *C. dubliniensis* in oral carriage and disease states (Willis *et al.*, 2000). Recent work has documented the presence of *C. dubliniensis* in just one case of DRS lesion (Mosca *et al.*, 2005). The association between *C. dubliniensis* and DRS suggests that this micro-organism may play important roles in the establishment and persistence of DRS.

Therefore, in this work, we examined the presence of *C. dubliniensis* in samples from palate and maxillary dentures from 112 denture wearers presenting with or without DRS. In order to address this, CHROMagar culture and PCR

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Abbreviation: DRS, denture-related stomatitis.

were utilized. Our data demonstrated that *Candida* species were more frequently isolated from the palate as well as the internal surface of maxillary dentures from individuals with DRS than from control denture wearer individuals. PCR analysis indicated that *C. dubliniensis* was recovered from the palate and/or maxillary denture from both groups studied (5.3–10.7%) and confirmed that *C. albicans* was the species most associated with the disease (89.2% on palate and 78.5% on maxillary denture).

METHODS

Subject population. One hundred and twelve denture wearers were selected from the population referred to the Clinics of Prosthodontics at the Bauru School of Dentistry, University of São Paulo (Bauru, SP, Brazil). A complete clinical examination was performed, including medical and dental histories and an intra-oral examination. Fifty-six patients were clinically diagnosed as having DRS and 56 were designated healthy controls. The age of the participants ranged from 44 to 94 years. The protocol for all the procedures was approved by the Institutional Ethics Committee. All the subjects signed an informed consent.

Inclusion criteria were as follows: participants had to be complete acrylic (heat-cured resin) denture wearers, presenting with and without DRS, and who reported wearing their complete maxillary dentures for at least 12 h a day over the previous 2 years. The exclusion criteria were: smoking, alcoholism, types 1 and 2 diabetes, any diseases or medicines generating immunosuppression, autoimmune diseases and drug use (including antifungal medication).

Identification of DRS and *Candida* species. DRS associated with the palate was diagnosed as pinpoint hyperaemia, diffuse hyperaemia or granular, as reported by Newton (1962). The microbiological diagnosis was carried out by collecting material from the hard palate in a denture stomatitis-associated erythematous area, from underneath the denture and from the surface of dentures in contact with the mucosa with a sterile swab. The samples from the control group were obtained from the denture fitting surface and hard palate covered by the maxillary denture. All samples were grown on Sabouraud dextrose broth (Difco, Becton Dickinson) with 1% chloramphenicol (Sigma-Aldrich) at room temperature. Such a step was completed in order to avoid false-negativity for the presence of *Candida* and therefore c.f.u. were not counted. After 1 week, broth along with samples was inoculated onto CHROMagar *Candida* plates (Becton Dickinson) to identify *Candida* species. CHROMagar *Candida* allowed identification of *C. albicans*, *Candida tropicalis* and *Candida krusei* through the growth of yeast colonies showing different colours and morphologies (Pfaller *et al.*, 1996; Willinger *et al.*, 2001). Samples grown on CHROMagar *Candida* that displayed a dark- or light-green colour were selected.

DNA extraction. The modified technique of Romeo *et al.* (2006) to differentiate *C. albicans* and *C. dubliniensis* was used. After the yeast samples were grown on CHROMagar plates, green colonies were obtained and maintained on Sabouraud dextrose agar at room temperature until DNA purification. Yeasts were washed three times at 10 000 *g* for 5 min at 4 °C. The pellet was suspended in 0.1 ml DNase/Rnase-free water + 0.1 ml InstaGene (InstaGene Matrix; Bio-Rad Laboratories) and incubated for 30 min at 56 °C. The samples were then boiled for 10 min and centrifuged at 10 000 *g* for 5 min at 4 °C (Sakai *et al.*, 2007). Supernatants were obtained for the analysis of *C. albicans* or *C. dubliniensis* DNA.

PCR amplification of *C. albicans* and *C. dubliniensis*. Samples were amplified under standardized conditions using a species-specific primer set for the hyphal wall protein 1 gene (CRR forward primer, 5'-GTTTTTGCACCTTCTCTTTGTTA-3'; and CRR reverse primer, 5'-ACAGTTGTATCATGTTTCAGT-3'). The PCR mixture (total volume 25 µl) contained 3 µl genomic DNA template, 0.2 mM each deoxynucleoside triphosphate (IDT primer), 0.4 µM each primer, 2.5 U GoTaq polymerase (Promega) and 1.5 mM MgCl₂. Amplification was performed after denaturation at 95 °C for 5 min followed by 34 cycles of denaturation at 94 °C for 45 s, primer annealing at 50 °C for 60 s, and an extension at 72 °C for 90 s, followed by a final extension at 72 °C for 10 min in a thermocycler (Progene; Techne).

PCR products were separated on a 1.5% (w/v) agarose gel, stained with ethidium bromide (0.5 µg ml⁻¹), and compared with the DNA size marker (100 Base-Pair Ladder; Amersham Biosciences) using a transilluminator (Sigma T2202). Two different amplicons were expected: 1180 bp for *C. albicans* and 930 bp for *C. dubliniensis*.

Statistical analysis. Data were analysed with GraphPad Prism version 5.00 for Windows (www.graphpad.com), using the chi-square test (with Yates' correction) and Fisher's exact test, to compare the proportion of each variable in the groups with or without DRS and where yeasts were identified or not. Values of *P* < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

DRS is the major oral infection in elderly denture wearers, and *C. albicans* is the micro-organism mostly involved in this disease (Budtz-Jorgensen *et al.*, 1975; Dar-Odeh & Shehabi, 2003; Webb *et al.*, 1998; Figueiral *et al.*, 2007). Clinical evaluation showed that type I DRS was detected most often in our patients, followed by type II and type III (*n*=36, 17 and 3, respectively; Table 1). Our data are in accordance with those of Figueiral *et al.* (2007). Regardless of the DRS type, *Candida* species were present in almost all of the cases (Table 1). Also, a higher occurrence of *Candida* species was found in the palate and maxillary denture samples from DRS patients than in samples from the control individuals (Table 1).

We further investigated which *Candida* species were present in individuals presenting with and without DRS through CHROMagar *Candida*. Our results showed that *C. krusei* and *C. tropicalis* were cultivated from patients with and without DRS. However, an association of different *Candida* species (*C. krusei*, *C. tropicalis* and *C. albicans*) on the hard palate mucosa and internal surface maxillary denture was verified only in DRS patients (data not shown). The importance of interactions between *Candida* species in biofilm formation has been extensively studied (Thein *et al.*, 2007a, b). However, further studies are necessary to understand the role of non-*albicans* *Candida* species in DRS as well as in the absence of the disease.

The colonies that were identified as *albicans* (green colonies) by CHROMagar *Candida* were submitted to molecular analysis by PCR in order to investigate the presence of *C. dubliniensis* in such samples. Our data revealed that *C. dubliniensis* was obtained in 10.7% (*n*=6)

Table 1. Demographic, microbiological and clinical data obtained from subjects with and without DRS

Variable	DRS patients (n=56)	Healthy controls (n=56)	P-value
Age (mean $\pm$ standard deviation)	69.4 $\pm$ 9.2	68.2 $\pm$ 5.5	0.9
Female/male ratio	39/17	34/22	0.99
<i>Candida</i> species on maxillary denture (n)	98.2 % (55)	44.7 % (25)	0.01*
<i>Candida</i> species on palate (n)	98.2 % (55)	42.9 % (24)	0.01*
Type of lesion (n)	64.2 %, type I (36) 30.3 %, type II (17) 5.3 %, type III (3)	Not present	

*Statistically significant difference comparing *Candida* species on maxillary denture or *Candida* species on palate in both groups.

of palate and 8.9 % ($n=5$) of denture samples from DRS patients, and in 5.3 % ($n=3$) of both palate and maxillary denture samples from controls. Although a slight difference was verified for *C. dubliniensis* detection between DRS patients and controls, it was not statistically significant ($P=0.09$). However, there was a significantly higher percentage of *C. albicans* in palate (89.2 %) and maxillary denture (78.5 %) samples from patients with DRS in comparison with controls (28.5% and 31.2%, respectively; $P<0.05$). In fact, DRS has been suggested to occur due to the outgrowth of *C. albicans* commensal strains (Mathaba *et al.*, 1995). Interestingly, all maxillary denture and palate samples from DRS patients that contained *C. dubliniensis* also contained *C. albicans*.

Another study identified *C. dubliniensis* in palate mucosa and denture samples from a patient with DRS; however, this micro-organism was not found in individuals without DRS (Marcos-Arias *et al.*, 2009). Together, our results show the presence of *C. dubliniensis* in complete maxillary denture wearers regardless of the presence of DRS. Therefore, our data could suggest that *C. dubliniensis* does not have an essential role in the pathogenesis of DRS.

The presence of *C. dubliniensis* has been more frequently detected in individuals with systemic metabolic alterations, i.e. symptomatic HIV-positive and diabetic patients (Sullivan *et al.*, 1995; Sullivan & Coleman, 1997, 1998; Kirkpatrick *et al.*, 1998; Martinez *et al.*, 2002). However, *C. dubliniensis* has been shown to present physiological and phenotypic traits similar to those of *C. albicans*, and this constitutes an important virulence factor for its pathogenicity (Moran *et al.*, 1997; Gilfillan *et al.*, 1998; Ramage *et al.*, 2001; Shin *et al.*, 2002). In addition, the treatment of patients with some antifungal drugs has generated the replacement of *C. albicans* by *C. dubliniensis* (Martinez *et al.*, 2002). Our control and DRS patients had not been given any antifungal therapy and perhaps this might explain why they maintained concomitantly *C. dubliniensis* and *C. albicans* colonizing their palate and maxillary denture.

Future studies will be necessary to understand the influence of *C. dubliniensis* in the oral environment of the systemically normal population, including denture wearers.

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