Phenotypic characterization of *Candida* spp. isolates from chronic periodontitis patients

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Abstract

Aim: Several typing methods for *Candida* spp. have been suggested in the literature in order to distinguish isolates for studies about the virulence or infection routes of these microorganisms and, in particular, for epidemiological purposes. The aim of this study was to establish a comparison between the phenotypic profile of oral *Candida* isolates from periodontitis patients and control individuals. **Methods**: The morphotyping and biotyping of 35 *C. albicans* isolates obtained from chronic periodontitis patients and 48 isolates from control individuals were performed. For morphotyping, the isolates were plated on malt extract agar and incubated for 10 days. Sixteen different morphotypes were observed for *C. albicans*, the most frequently observed being 0000 and 0001. **Results**: Biotype 0000 (complete absence of fringe) was most prevalent among the isolates obtained from periodontitis patients compared to those from control individuals, with statistical significance. Biotyping revealed 5 different biotypes with higher prevalence of the biotype 357 among the isolates from control and periodontitis groups. **Conclusions**: The results obtained by biotyping of the isolates did not permit to differentiate a characteristic model related to periodontal disease, whilst the morphotype 0000 was most frequently isolated from periodontitis patients.

Key words:
periodontitis, *Candida albicans,* biotyping, morphotyping

Introduction

Candida spp. have been correlated to cases of severe and refractory periodontal infections, particularly in immunocompromised patients or individuals under antimicrobial therapy for long periods[1-5]. Several *Candida* spp. typing methods have been suggested in the literature in order to distinguish isolates for studies about the virulence or infection routes of these microorganisms and, in particular, for epidemiological purposes[6-10]. Among the methods based on phenotypic characteristics, serotyping, biotyping, morphotyping and sensitivity to *killer* toxins are included[7]. There are also methods based on genotypic characteristics, such as immunobloting[11] and DNA fingerprinting techniques[12].

Morphotyping is considered an efficient, reproducible and low cost method for *C. albicans* characterization[7,13]. Moreover this method reveals a good discriminatory power. The variation in the morphology of *Candida* colonies was firstly observed by Negroni[14]. Later, based on Brown-Thomsen's[16] observations about the morphological variations of colonies due to alterations in the incubation temperature or medium composition, Phongpaichit *et al.*[15] proposed a typing method with codes. This coding system was based on the characteristics of the colonies as well as on their surfaces.

Hunter *et al.*[17], studying the morphotype distribution among 446 *C. albicans* strains, suggested a typing method based in the characteristics of colony surfaces, represented by 4 digits.

The phenotypic switching and *Candida* morphotypes have been associated to their virulence. Different phenotypic expressions in relation to colony growth are related to variations that are considered responsible for the several degrees of virulence[7,18]. Phenotypic switching occurs frequently and causes changes in colony morphology and cell surface properties, such as alteration in the adherence to epithelial cells[19].

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The biotyping technique proposed by Odds and Abbott[20] has been reported as a method with an adequate discriminatory power mainly when associated with the morphotyping method[21]. Considering the adequate discriminatory power of the combination of morphotyping and biotyping methods, the purpose of this study was to establish a comparison between the phenotypic profile of oral *Candida* isolates from periodontitis patients and control individuals, aiming to correlate specific phenotypic features to the occurrence of periodontal disease.

Material and Methods

This research project has been independently reviewed and approved by the Bioethics Research Committee of the Dental School of São José dos Campos, São Paulo State University, Brazil (Protocol #72/99-PH/CEP).

Candida albicans isolates from chronic periodontitis (n=35) and control individuals (n=48) were included in this study. These strains were previously isolated and belonged to the strain collection of the Microbiology Laboratory at the Dental School of São José dos Campos, São Paulo State University.

Isolates from chronic periodontitis were obtained from 88 individuals aged 25 and 62 years (mean age of 41.33 ± 5.54 years), with at least two 5-mm deep periodontal sites and diagnosed clinically as chronic periodontitis patients. Control group isolates were obtained from 68 individuals aged 25 to 55 years (mean age of 34.45 ± 7.93 years) diagnosed as periodontally healthy patients.

Candida isolates were morphotyped according to the methodology proposed by Hunter *et al.*[7] and Pongpaichit *et al* [15]. Briefly, the isolates were plated on Sabouraud dextrose agar and incubated at 25ºC for 48 h. Then, yeast suspensions were prepared in sterile distilled water adjusted to the turbidity of tube #3 of McFarland scale. Using sterile swabs, the suspensions were plated on the surface of malt extract agar (Difco, Detroit, MI, USA). Plates were maintained at room temperature in a light-proof environment for 10 days. After this period, the macromorphological aspects of the fringes and surface of the colonies were evaluated. The results of morphotyping were recorded using 4-digit codes. Biotyping methods were performed according to Odds and Abbot[22], with a combination of tests of tolerance (to pH 1.4 and NaCl), resistance (5-fluorocytosine, safranine and boric acid), enzymatic activity (proteinase) and growth in presence of urea, sorbose and citrate. The isolates were plated on Sabouraud dextrose agar and incubated at 37ºC for 24 h. Then, saline suspensions adjusted to the turbidity of tube #3 of McFarland scale were obtained. Aliquots of 100 µL of these saline suspensions were deposited in the wells of a Steers' inoculator and plated in the test culture media and the positive control (Sabouraud agar). All tests were performed in triplicate. Plates were incubated at 37ºC for different periods of time (3 to 4 days for control, safranine, urea, citrate, boric acid and sodium chloride; 6 to 7 days to resistance to pH 1.4 and tests of sorbose, proteinase and 5-fluorocytosine). Positive tests were considered when the strains grew at pH 1.4, 5-fluorocytosine, sodium chloride, boric acid, urea, sorbose and sodium citrate, as well as for the strains that formed colonies with diameter grater than 2 mm in presence of safranine.

The results of biotyping were recorded using 3-digit codes, according to Odds and Abbott[22]. Values of 1, 2, 3 and 4 were attributed to the positive tests and 0 to the negative tests.

Statistical Analysis

The data regarding the occurrence of the morphotypes and biotypes were compared between the periodontitis and control groups by two proportion Z test at 5% significance level.

Results

The morphotype 0000 was the most frequently observed (50%) among the oral isolates from control individuals. The morphotype 0001 was observed in 12.5% of the isolates. The morphotypes 1232, 7240, 0004, 2331, 1230 and 7520 were also observed (Table 1).

Among the isolates from chronic periodontitis patients, the morphotype 0000 was also the most frequently found (77.14%) differing significantly from the control group (p=0.007) (Figure 1). Other 7 morphotypes were observed among the isolates of this group (Table 1).

Biotypes 357 and 757 were the most frequently observed in both groups without statistically significant differences (p=0.522) (Table 2).

Discussion

Candida genus yeasts are considered opportunistic microorganisms[23,24] and may lead to severe periodontal infections in immunocompromised patients or individuals under antimicrobial therapy for long periods[1]. Under specific situations, such as in immunodepressed patients[25], superinfection by *Candida* can be refractory to conventional periodontal treatments. In fact, *C. albicans* presents virulence factors that can have an important role in the pathogenesis of periodontal disease such as the ability of penetrating the epithelium, inhibiting polymorphonuclear cells and causing lysis of monocytes[8,26]. Also, Lu *et al.*[27] have described the hyphal invasion capacity of *C. albicans* to inhibit the production of antimicrobial peptides by oral epithelium. *Candida* species have been isolated from the subgingival microbiota of the gingival tissues of patients with periodontal abscesses[1], periodontitis[28,29], and patients with chronic periodontitis under therapy with antibiotics[17].

Morphotyping has been employed in epidemiological and virulence studies. Ribeiro *et al.*[30] studying children with Down's syndrome showed that the *C. albicans* isolates from these patients induced more formation of fringes compared to the isolates obtained from control individuals, and the authors suggested a correlation with increased virulence. In fact, Hunter *et al.*[17] had previously suggested a correlation

Table 1 - Distribution of the morphotypes observed among *Candida albicans* isolates from control individuals and periodontitis patients.

CONTROL GROUP (n=48)		
Morphotype	n	%
0000	24	50.0
7340	1	2.1
7540	1	2.1
5340	2	4.1
0001	6	12.5
1040	1	2.1
1240	2	4.2
7520	1	2.1
7320	1	2.1
7330	1	2.1
7531	1	2.1
0002	2	4.1
0006	2	4.1
1240	1	2.1
7321	1	2.1
3340	1	2.1
PERIODONTITIS GROUP (n=35)		
0000	27	77.14
1232	1	2.86
7240	2	5.70
0004	1	2.86
2331	1	2.86
1230	1	2.86
7520	1	2.86
7540	1	2.86

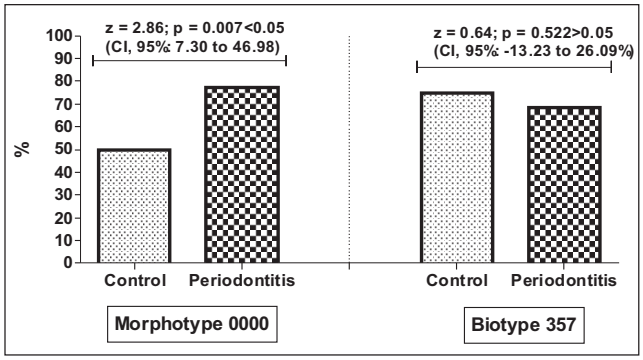

Fig. 1 - *Statistical comparison of the morphotype 0000 and biotype 357 occurrence in the studied groups.*

between invasive infections and isolates with discontinued fringes. The increased virulence of isolates with fringes was also related by Spadari *et al.*[31]. These authors observed a higher degree of adherence in these isolates compared to strains without fringes. In the present study, this tendency was not observed, as similar proportion of isolates in the groups showed discontinued fringes. Four isolates from the control group (8.33%) and 3 from the periodontitis group (8.57%) presented discontinued fringes. These results suggest that no correlation between colonies with discontinued fringes and periodontitis occurrence could be done.

Table 2 - Distribution of biotypes observed among *Candida albicans* isolates from control individuals and periodontitis patients

CONTROL GROUP (n=48)		
Biotype	n	%
357	36	75.0
757	9	18.7
157	1	2.1
313	1	2.1
353	1	2.1
PERIODONTITIS GROUP (n=35)		
357	24	68.6
757	5	14.2
777	3	8.6
377	2	5.8
577	1	2.8

The morphotype 0000 (complete absence of fringes) was the most frequently isolated from the oral cavity of control and periodontitis patients, and this result corroborates the absence of correlation between the presence of discontinuous fringes, which is associated to filamentation, and periodontitis occurrence. Previous studies[6,17,32] also observed the higher incidence of this morphotype among isolates from the oral cavity. This result may suggest that the mycelial form formation might not be an essential feature of *Candida* in the periodontal milieu, and other factors (i.e. proteolytic enzymes and toxins production) might be more important contributors to the pathogenesis of this disease. For more detailed data, further studies on these features could be useful. Also, studies on genotyping could provide important results. In fact, several authors have pointed out the importance of combining phenotyping and genotyping methods[6,9,33,34].

Hunter *et al.*[7] have demonstrated the occurrence of the morphotypes 7540 and 7340 among isolates from several sites of the body. These morphotypes were also observed among the isolates of the present study. The great variability of morphotypes in the control group (16 models) has been reported elsewhere[17]. Ribeiro *et al.*[29] did not observe the occurrence of morphotype 0000 and found a high prevalence of morphotype 5530 in Down's syndrome patients.

The biotyping method proposed by Odds and Abbott[20] has been considered as a method with adequate discriminatory power. However, in the present study, it was not able to distinguish between periodontitis and control isolates. Biotyping permitted the identification of 5 different biotypes among the isolates from the oral cavity of control individuals. The biotypes 357 and 757 were the most frequently observed (75% e 18.8%, respectively). Among

the isolates from periodontitis patients, the biotypes 357, 757, 777, 377 and 577, and the model 357 (68.6%) were the most frequently observed. In the present study, most isolates assimilated urea and citrate and did not assimilate sorbose. Also, these isolates were tolerant to salt and developed in presence of boric acid and safranine. Neely *et al.*[35] also verified a high prevalence of isolates with these features among *Candida* isolates from children.

In conclusion, the results obtained by biotyping of the isolates did not allow distinguishing a characteristic model related to periodontal disease, whilst the morphotype 0000 was most frequently isolated from periodontitis patients.

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