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Genetic relatedness of subgingival and buccal *Candida dubliniensis* isolates in immunocompetent subjects assessed by RAPD-PCR

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Background: It is recognized that *Candida dubliniensis* commonly colonizes oral and subgingival sites in immunocompetent subjects with periodontal disease.
Objective: Since there are few data available on genetic characterization of *C. dubliniensis* in periodontal pockets and other oral sites, the aim of this study was to characterize subgingival and mucosal *C. dubliniensis* isolates recovered from immunocompetent subjects and to assay the genetic similarity of such isolates from both niches in the same patient by random amplified polymorphic DNA (RAPD).
Design: *C. dubliniensis* recovered from subgingival plaque and from buccal cavity samples were studied in 240 immunocompetent non-smoking individuals. Arbitrary amplification was carried out by RAPD-polymerase chain reaction (PCR).
Results: RAPD analysis showed identical genotypes of *C. dubliniensis* in different sampling sites (buccal cavity and subgingival areas) in eight of 10 patients except for those derived from two participants who presented presumably unrelated isolates.
Conclusions: On the basis of the findings presented, the origin of the colonization of *C. dubliniensis* in subgingival biofilm seems to be the buccal cavity in a single patient. Consequently, it may be assumed that most of *C. dubliniensis* in these sites arise from the endogenous commensal strains.

Keywords: *C. dubliniensis*; *RAPD-PCR*; *subgingival biofilm*; *periodontal disease*; *genetic relatedness of C. dubliniensis*

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Yeasts of the *Candida* genus are opportunistic commensals in the human mouth, representing potential sources of oropharyngeal candidiasis, as well as other more serious forms of the disease, such as esophageal and systemic diseases (1, 2). *Candida* species have also been recovered from periodontal pockets in a large number (7.1–19.6%) of patients with periodontal disease (3–8).

Although the yeast most frequently associated to this type of infection is *Candida albicans*, other less prevalent emerging species have been isolated (4, 6, 7, 9–12).

Mucosal surfaces are the primary body reservoirs for these microorganisms (13). This reflects the ability of the yeast to colonize different oral surfaces and the variety of factors which predispose the host to *Candida* colonization and subsequent infection. Colonization of the oral cavity appears to be facilitated by several specific adherence interactions between *Candida* species and oral surfaces which enable the yeast to resist host clearance mechanisms. Mucosal and subgingival *Candida dubliniensis* colonization has been reported in immunocompetent subjects with periodontal disease (6, 7, 14) and both oral sites are appropriate niches for multiplication of this species.

It would seem relevant to know about epidemiological aspects of subgingival *C. dubliniensis*, given its capacity to adhere to bacteria in the oral microbiota, and to coaggregate, that enables it to colonize the depth of subgingival biofilm (4, 11, 15, 16).

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In recent years, several molecular typing methods have been used to characterize *Candida* species isolates and to delineate strain relatedness, although polymerase chain reaction (PCR) based methods remain the most widely used. Among these, the random amplified polymorphic DNA (RAPD) method of DNA fingerprinting has become quite popular for all infectious fungi and has been successfully applied to assess the genetic relatedness of *Candida* species (17–24). These methods have greatly enhanced knowledge about the epidemiology of oral and subgingival *Candida* species, and they can provide valuable information by their capacity to distinguish distinct isolates of the same species. Some studies have demonstrated that commensal yeasts dominate in oral candidiasis, whereas controversial evidence shows that genetically homogeneous, hypervirulent strains of *C. albicans* are involved in the disease (25–28).

Since there are few data available on genetic characterization of *C. dubliniensis* in periodontal pockets and other oral sites, the aim of this study was to characterize subgingival and mucosal *C. dubliniensis* isolates recovered from immunocompetent subjects and to assay the genetic similarity of *C. dubliniensis* isolates from both niches in the same patient by RAPD.

Material and methods

Sources of isolates

A total of 21 isolates of *C. dubliniensis*, 10 from the buccal cavity and 11 from subgingival biofilm, were collected from 240 non-smoking individuals who attended the dental clinic of the University of Buenos Aires, Argentina. Periodontal evaluations included clinical examination and radiographs with clinical measurements: clinical attachment level (CAL), pocket depth (PD), plaque index (29), gingival index (30), and bleeding on probing (BOP). Location of the gingival margin was determined and tooth mobility was assessed. Measurements were made at four sites per tooth (mesial, buccal, distal, and lingual positions) at 15 teeth excluding the third molar.

Participation in our survey was voluntary and all the patients provided a written informed consent.

Sampling, culture conditions, and identification of isolates

All the volunteers were requested to thoroughly rinse their mouths with sterile distilled water. The dental professional then isolated the area to be sampled by means of cotton rolls and a high-speed suction device. Following removal of the supragingival plaque, in order to avoid contamination, four subgingival plaque samples were taken from each patient: the upper right and lower central incisor, the first upper right molar and the first lower left molar, to mimic the more common periodontal pockets infected in periodontitis, by using a 7/8 sterile Gracey curette. Samples were pooled and cultured on a differential chromogenic medium (CHROMagar *Candida*, Paris, France). Buccal cavity samples, including palatal, buccal mucosa, and tongue, were collected by sterile cotton-tipped swabs and were streaked directly onto the chromogenic medium. Isolated yeasts were identified by conventional mycological methods: colony color on the chromogenic medium, micromorphology in agar milk with 1% Tween 80 (31), and carbohydrate assimilation tests using the commercially available kit API ID 32C (®BioMérieux, France). Further studies were conducted for characterization of *C. dubliniensis*, including chlamydospore formation on Staib agar after incubation for 72 h at 28°C (32) and specific PCR with primers from actin gene (ACT1)-associated intron sequences of *C. dubliniensis*, DUBF (GTATTTGTCCTTCCCCTTTTC) and DUBR (GTGT TGTGTGCACTAACGTC) (7, 33).

Random amplified polymorphic DNA (RAPD) analysis

Yeast DNA was isolated according to a previously described technique (7, 34, 35). The DNA was quantified and its purity was evaluated at 260 nm (SmartSpec™ 3000 Spectrophotometer BIO-RAD).

Five different primers were included in the typing assays. Primer sequences were as follows: OPA 02 (TGCCGAGCTG), OPA 09 (GGGTAACGCC), M13F (CGACGTTGTAAAACGACGCCCAGT), M13R (CAGGAAACAGCTATGAC), and OCP 5 (GATGACCGCC). All were used in RAPD-PCR following the method of Williams et al. (36).

Arbitrary amplification was performed in a total volume of 50 µl containing: 1 × buffer 2.5 mM $MgCl_2$, 0.2 mM each of the dNTP, 0.5 µM of the primer, 1.25 U Taq DNA polymerase (Invitrogen), and 75 ng of template DNA. The cycling program was made up of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 25°C, 2 min at 72°C followed by a final extension of 5 min at 72°C. These steps were carried out in a Minicycler DNA thermal cycler (TM MJ Research Inc., NY, USA). Products were separated by electrophoresis in 2% agarose gel and stained with ethidium bromide. They were visualized under UV light and digitalized by the image analyzer software (EPI-Chemi Darkroom. UVP Laboratory Products, California, USA). Band profiles were analyzed and compared visually. Each band was scored as positive or negative for all isolates, and for each isolate, the presence or absence of each band was registered. The resulting matrix was interpreted using the Treecon program, where isolates were grouped according to their pattern resemblance. Based on matrix of similarity coefficients (SC), a dendrogram was generated by the unweighted pair group method using arithmetic averages (UPGMA). The criterion used for genotyping was as

follows: arbitrary threshold at an SC of 90% for high relatedness isolates (20).

Statistical analysis

Statistical analysis was performed by using Statistix 7.0 and SPSS 11.0 versions. Confidence intervals (CI) were calculated at 95% employing the Epi-Info 6.04 program (Atlanta University, USA).

Results

Clinical features

The 240 subjects included in the study ranged in age from 18 to 75 years (mean age 37), 55% were females (132/240) and had not received any antibacterial or antifungal agents before sampling. Table 1 shows clinical periodontal parameters (PD and CAL Mean ± SD) of subjects at the time of sampling. Patients were classified into groups according to the periodontal health status as shown in Table 1: healthy ($n = 53$), gingivitis ($n = 58$), and chronic periodontitis ($n = 129$). Gingivitis and chronic periodontitis patients were BOP positive.

As expected, periodontitis sites showed significantly more signs of disease, including higher mean PD ($p < 0.001$) and mean CAL ($p < 0.001$) than healthy patients.

The carriage of C. dubliniensis

Table 2 summarizes species distribution of yeast isolates in the buccal cavity and the subgingival biofilm according to the periodontal health status in 240 immunocompetent individuals. The yeast microflora had similar species in both studied sites (Table 2).

Out of the 203 recovered yeasts, *C. albicans* was the most frequent species corresponding to 26.7% (64/240) in the buccal cavity and 21.7% (52/240) in the subgingival biofilm, as shown in Table 2.

C. dubliniensis was isolated in 4.2% (10/240) and 4.6% (11/240) of patients in both niches, respectively. Distribution of *C. dubliniensis* isolates from the subingival biofilm according to periodontal health status (Table 2), was as follows: 2.5% from subjects with chronic periodontitis, 1.7% with gingivitis, and 0.4% from healthy individuals.

RAPD-PCR assay

We selected five RAPD primers, based on their reproducibility, after pre-screening to analyze 21 isolates of *C. dubliniensis*. The number of bands ranged from three splitters (M13r) to 12 (M13f). Two of the five primers were the most informative (M13f and OPC5) and they generated the highest number of band patterns (between 10 and 12).

Fig. 1 shows the dendrogram of RAPD fingerprints of *C. dubliniensis* isolates, the SC ranged from 50 to 100%. Eight genetic clusters and five main genotypes were obtained at an SC of 90%, genotypes I, II, III, IV, and V, as shown in Fig. 1.

All isolates unclustered or belonging to different clusters by RAPD analysis were observed to differ by three or more bands.

RAPD analysis showed identical genotypes of *C. dubliniensis* in different sampling sites in eight of the 10 patients (buccal cavity and subgingival areas), except for those observed in two participants. One case was patient 1, who presented presumably unrelated isolates (Fig. 1, green arrows). The other patient, number 3 presented two strains in subgingival biofilm (Cd3b and Cd3bb), which represented genotype I and IV, respectively (Fig. 1). Moreover, participant 3 presented a mouth *C. dubliniensis* strain, Cd3a, which corresponded with genotype III (Fig. 1, red arrows). Those yeasts were presumably unrelated isolates (20).

Discussion

C. dubliniensis is a yeast species that is characterized by its *in vitro* resistance to the antifungal azole group in HIV patients, for their capacity to adhere *in vitro* to human buccal epithelial cells and other microorganisms in the oral microbiota, as well as the high proteinase activity (9, 15, 37, 38). Originally, this species was isolated from oropharyngeal candidiasis in AIDS patients. Later, numerous papers have been published indicating its

Table 1. Clinical periodontal parameters (Mean ± SD and 95% Confidence interval) of subjects at the time of sampling according to periodontal health status*

Clinical parameters	Healthy	Gingivitis	Chronic periodontitis
Pocket depth** (mm)	1.5 ± 0.80 (1.2–1.8)	4.0 ± 1.2 (3.6–4.3)	6.2 ± 1.9 (5.8–6.5)
Clinical attachment level (mm)	0	0	6.4 ± 3.6 (5.7–7.1)
Plaque index[a]	0.70 ± 0.18	0.90 ± 0.85	1.70 ± 0.18
Gingival index[b]	0.10 ± 0.24	0.95 ± 0.35	2.10 ± 0.14

*Healthy, $n = 53$; Gingivitis, $n = 58$; Chronic periodontitis, $n = 129$.

**Kruskal–Wallis test: (Differences in the median values among the groups*) H = 902,675 with two degrees of freedom ($p < 0.001$).

[a]Silness and Loe (1964).

[b]Loe and Silness (1963).

Table 2. Species distribution of yeast isolates in buccal cavity (BC) and subgingival biofilm (SB) according to periodontal health status* in 240 immunocompetent individuals

Yeast species	Healthy *n* (%)		Gingivitis *n* (%)		Chronic periodontitis *n* (%)		Total *n* (%)	
	BC	SB	BC	SB**	BC	SB**	BC	SB
C. albicans	11 (4.6)	9 (3.8)	15 (6.3)	13 (5.4)	38 (15.8)	30 (125)	64 (26.7)	52 (21.7)
C. parapsilosis	0	1 (0.4)	2 (0.8)	2 (0.8)	7 (2.9)	10 (4.2)	9 (3.8)	13 (5.4)
C. dubliniensis	2 (0.8)	1 (0.4)	4 (1.7)	4 (1.7)	4 (1.7)	6 (2.5)	10 (4.2)	11 (4.6)
C. tropicalis	1 (0.4)	0	2 (0.8)	3 (1.2)	1 (0.4)	4(1.7)	4 (1.7)	7 (2.9)
C. guilliermondii	0	0	1 (0.4)	2 (0.8)	2 (0.8)	5 (2.1)	3 (1.3)	7 (2.9)
C. krusei	0	0	0	0	4 (1.7)	1 (0.4)	4 (1.7)	1 (0.4)
C. glabrata	0	0	1 (0.4)	1 (0.4)	3 (1.3)	4 (1.7)	4 (1.7)	5 (2.1)
Rhodotorula spp	0	2 (0.8)	1 (0.4)	2 (0.8)	3 (1.3)	1 (0.4)	4 (1.7)	5 (2.1)
Total (*n*)	14	13	26	27**	62	61**	102	101

*Healthy, $n = 53$; Gingivitis, $n = 58$; Chronic periodontitis, $n = 129$.
**Kruskal–Wallis test, $p < 0.01$.

presence in all types of clinical samples as well as in healthy individuals (7, 10, 14, 33, 39–50).

In this study, *C. dubliniensis* showed a prevalence of 4.2% in the buccal mucosa from 240 patients. These results do not match those found by other authors, who found low prevalence of this species in healthy HIV-negative patients (14, 44, 51–53). This disagreement could be due to the different study populations evaluated (healthy subjects and periodontitis patients).

In this study, the prevalence of *C. dubliniensis* was 4.6% (95% CI: 2.4–8.3) in subgingival biofilm in patients from Buenos Aires, Argentina. This species was isolated both in healthy patients and in patients with periodontal disease. Table 2 shows an increased prevalence of this species in

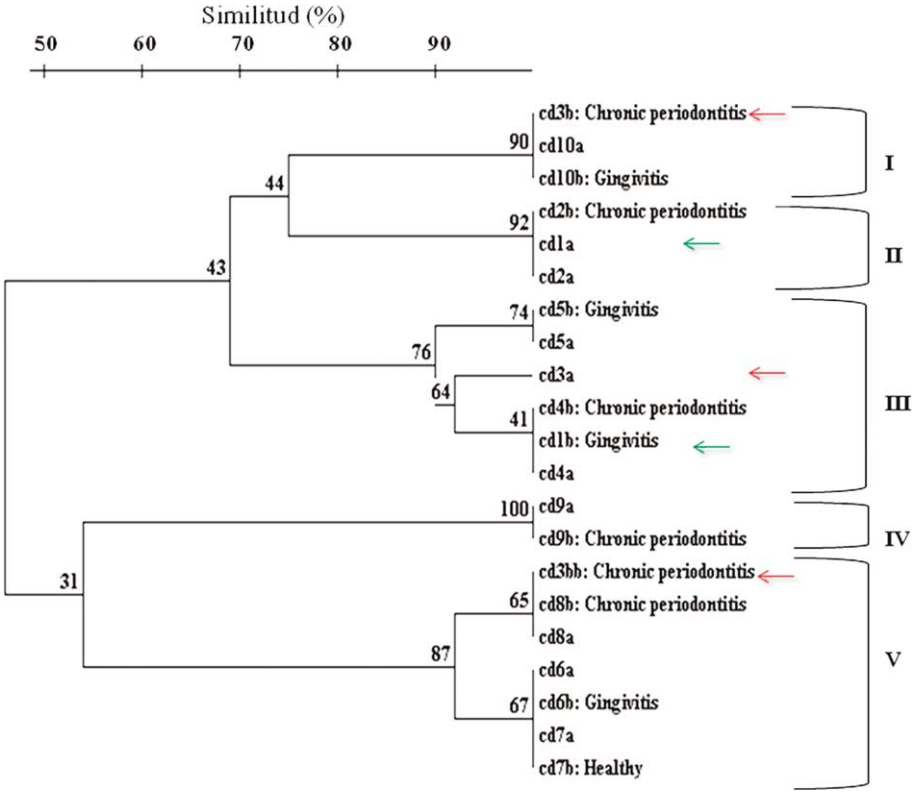

Fig. 1. Dendrogram generated by UPGMA clustering method, using the coefficient of similarity between RAPD-PCR of *C. dubliniensis* in oral cavity (a) and subgingival biofilm (b) in immunocompetent individuals.

individuals with chronic periodontitis, compared to healthy individuals and individuals with gingivitis, which is not a statistical significant difference. Based on the findings in this study, it is confirmed that *C. dubliniensis* can colonize subgingival biofilm in immunocompetent individuals with periodontal disease and healthy ones (6, 7). Similar findings have been reported by Song et al., since these authors found that *C. dubliniensis* dominated among *Candida* species other than *C. albicans*, being most prominent in periodontal pockets (54).

Others authors have not found *C. dubliniensis* in the gingival sulcus in healthy individuals (4, 10, 11).

The RAPD-based typing was used to assess the diversity of *Candida* species isolates, as it has been described as a simple, rapid, and reliable discriminatory method for clinical epidemiological studies of oral *Candida* infections (17, 23, 24, 27, 35). In spite of this, and considering the reproducibility pitfalls of the RAPD method, it was considered adequate to compare the reliability of the RAPD-based clustering with that obtained with pulsed field gel electrophoresis analysis, referred to as a more robust method (20, 23, 55).

In the present study, the RAPD analysis showed similar genotypes of *C. dubliniensis* in different sampling sites from the same patient (buccal cavity and subgingival areas), except for those observed in two patients (Fig. 1).

RAPD profiles of *C. dubliniensis* isolates from the participants were generally distinct; therefore almost every individual harbored his/her own specific isolate.

Such genetic heterogeneity within isolates was reported from other oral and non-oral sources in *C. albicans* (25, 26, 56).

These results led us to the conclusion that, the origin of the colonization of *C. dubliniensis* in subgingival biofilm seems to be the buccal cavity, consequently, it may be assumed that most of *C. dubliniensis* in these sites arise from endogenous commensal strains. As the only mode of reproduction known for *C. dubliniensis* is asexual, these results suggest a common clonal origin of isolates in both niches (57).

In our series, *C. dubliniensis* genotypes had some differences between oral and subgingival isolates in two patients. Participants 1 and 3, harbored subgingival strains genetically presumably unrelated, to the isolated ones from their buccal cavity. Pizzo et al. (25) noted the presence of a different *C. albicans* DNA type in subgingival sites. This observation suggests that the presence of *C. albicans* or *C. dubliniensis* could also occur due to the colonization with subgingivally adapted strains, possibly as a result of genetic variations such as gene conversion and/or chromosomal translations (19, 25–27).

Similar *C. dubliniensis* genotypes may be distributed among healthy and periodontal disease individuals, as shown in Fig. 1, genotypes I, II, III, and V included isolates from individuals that were healthy or had periodontal disease. Therefore, a hypervirulent strain of *C. dubliniensis* that is involved in disease patients should be excluded. Other authors also observed that genetically identical yeasts appeared in both healthy and diseased subjects when they investigated *C. albicans* isolates (22, 25, 27).

To date, no studies have been aimed at genetic characterization of subgingival *C. dubliniensis* isolates. Therefore, our yeast isolates were subjected to RAPD-PCR analysis, which has proved to be a rapid, simple, cost-effective and discriminatory technique for molecular typing of *C. dubliniensis*.

This is the first survey in Argentina to study the molecular characterization of *C. dubliniensis* by RAPD-PCR clinical isolates in different ecological niches of the oral cavity. Such findings may be useful as baseline information on subgingival *C. dubliniensis* colonization in our country.

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Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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