GENETIC-RELATEDNESS OF PERI-IMPLANTS AND BUCCAL *CANDIDA ALBICANS* ISOLATES DETERMINED BY RAPD-PCR

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ABSTRACT

Molecular techniques have been used in recent studies to identify a wide range of potential bacterial pathogens in peri-implant pockets of the oral cavity. However, the prevalence and molecular epidemiology of yeasts and species distribution related to peri-implantitis are as yet unknown. The aim of this study was to determine the prevalence and distribution of yeasts in peri-implant biofilm and to study genetic relatedness of Candida albicans.

Yeasts recovered from peri-implant biofilm samples (n=89) and buccal samples (n=120) were studied in 40 immunocompetent non-smoking patients who visited the dental clinic of the Asociación Implantodontológica Argentina, Buenos Aires, Argentina, and had received oral rehabilitation with implants for more than five years. Yeasts recovered from samples were studied by typing assays using RAPD-PCR. The prevalence of yeasts in the peri-implant sulcus was 73% (n=29). C. albicans was the most prevalent species identified in this study population. The RAPD analysis showed identical genotypes in most C. albicans spp. from the two different sampling sites: buccal and peri-implant. These findings suggest that peri-implant biofilm is an ecological niche that favors the growth of yeast species. Most C. albicans found in peri-implant biofilm originate from the endogenous infection caused by commensal strains.

Key words: *Implants; biofilm; Candida albicans; RAPD-PCR; peri-implantitis.*

RELACIÓN GENÉTICA DE AISLAMIENTOS DE *CANDIDA ALBICANS* POR RAPD-PCR EN SURCOS PERI-IMPLANTARIOS DE CAVIDAD BUCAL

RESUMEN

Las técnicas moleculares se han utilizado en estudios recientes para identificar una gran diversidad de patógenos bacterianos de surcos periimplantarios de cavidad bucal. Sin embargo, la prevalencia y epidemiología molecular de especies de levaduras en relación con la periimplantitis son aún desconocidas. El objetivo de este estudio fue determinar la prevalencia y distribución de las levaduras en la biopelícula periimplantaria y estudiar la relación genética de Candida albicans. Se estudiaron 40 pacientes inmunocompetentes no fumadores que se asistieron en la clínica dental de la Asociación Implantodontológica Argentina, Buenos Aires, Argentina, y que habían recibido rehabilitación oral con implantes durante más de cinco años. Las levaduras aisladas de las muestras de biopelícula periimplantaria (n = 89) y bucales (n = 120), fueron identificadas por métodos micológicos tradicionales y moleculares. Se obtuvo el ADN de C. albicans y se realizaron estudios moleculares por RAPD -PCR. La prevalencia de levaduras en el surco alrededor del implante fue de 73 % (n = 29). C. albicans fue la especie más frecuente identificada en esta población de estudio. El análisis RAPD permitió identificar idénticos genotipos de C. albicans en ambos nichos ecológicos estudiados, periimplantar y bucal.

Según los resultados obtenidos, el surco periiplantario es un nicho ecológico que favorece el crecimiento de especies de levaduras del género Candida. La mayoría de los aislamientos de C. albicans periimplantarios se originan a partir de la infección endógena causada por cepas comensales.

Palabras clave: *Implantes; biopelícula; Candida albicans; RAPD-PCR; periimplantitis.*

INTRODUCTION

The use of osseointegrated implants, as well as their complications and problems, have increased in recent decades. Successfully osseointegrated titanium implants usually harbor low quantities of plaque and present little marginal inflammation. Supra- and sub-gingival microbiota at well maintained implant sites seem to resemble the microbiota associated with healthy gingiva. An increased proportion of putative periodontal pathogens has been documented at implant sites, suggesting that the periodontal pocket may serve as

a reservoir for colonization of titanium implants. Peri-implantitis is a chronic progressive marginal infection, defined as an inflammatory reaction that affects the tissue surrounding osseointegrated dental implants, resulting in the loss of the supporting bone. Microbiota resembling that of adult peiodontitis has been found in peri-implantitis [1-4].

Extensive antibiotic treatment and irrigation with chlorhexidine may cause etiological changes. Microorganisms not primarily associated with periodontitis, such as *Staphylococcus spp*., enterics and *Candida spp*., have also been isolated [2-5]. Molecular techniques have been used in recent studies to identify a wide range of potential bacterial pathogens in peri-implant pockets [6,7]. However, the prevalence of yeasts and species distribution related to peri-implantitis are as yet unknown.

The same has been found to be true for dental biofilm [2,8] . Dahlen *et al.* [9], and Reynaud *et al.*[10] claim that there was colonization by the genus *Candida spp*. in periodontal pockets, refractory periodontitis[3,10,11], and implant failure. Other studies report presence of *Candida albicans* in the subgingival plaque microbiota of human immunodeficiency virus (HIV) positive individuals [12].

In recent years, several molecular typing methods have been used to characterize *Candida spp*. isolates and to delineate strain relatedness, the most widely used being polymerase chain reaction (PCR) based methods. 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 spp*. [13-18]. These methods have greatly enhanced knowledge on the epidemiology of oral and subgingival *Candida spp*., and can provide valuable information through their ability 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[19].

Since there is no available data on the epidemiology of yeasts and genetic characterization of peri-implant *C. albicans*, the aim of this study was to characterize peri-implant biofilm and mucosal *C. albicans* isolates recovered from immunocompetent subjects with more than 5 years of implant treatment, and to assay the genetic similarity of *C. albicans* isolates from the two niches in the same patient by RAPD.

MATERIAL AND METHODS

Study population

This study was approved by the Ethics Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires (Res. 41, File 727.071/10). Yeasts recovered from peri-implant plaque (n=89) and buccal samples (n=120) were studied in 40 immunocompetent non-smoking patients with more than five years of implant treatment on oral prosthesis who attended the dental clinic of the Asociación Implantodontológica Argentina, Buenos Aires, Argentina.

Evaluations included clinical examination and radiographs with clinical measurements: pocket depth (PD), considered regular up to 3 mm around implants, plaque index, gingival index [11,20] and bleeding on probing. Measurements were taken at four sites per tooth (mesial, buccal, distal and lingual positions) on 15 teeth, excluding third molars.

Bone resorption was assessed by comparing the radiographic examination in the patients' medical records taken at the time of implant placement to those taken at the appointment for this study. In order to analyze bone resorption, implants were classified into two groups according to time of implant placement: "immediately loaded implants" if they were placed during the same session as tooth extraction or "delayed loaded implants" if they were placed on healed bone, months or years after extraction.

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

The volunteers were requested to rinse their mouths thoroughly with sterile distilled water, after which sterile swabs were used to take samples from tongue, palate and cheek.

The dental professional then isolated the area using cotton rolls and a high-speed suction device. Following removal of the supragingival plaque using a Teflon curette to avoid salivary contamination, peri-implant biofilm was collected from the interdental plate by inserting 3-4 sterile paper points number 30-35-40 for 15-30 minutes in the four sites: mesial, buccal, distal and lingual positions. Samples were cultured in a differential chromogenic medium

(CHROMagar Candida, Paris, France). Yeast isolates were identified using conventional mycological methods: colony color on the chromogenic medium, micromorphology in agar milk with 1% Tween-80 [21], carbohydrate assimilation tests using a commercially available kit API ID 32D (BioMérieux, Lyon, France), and specific PCR[22].

Random amplified polymorphic DNA (RAPD) analysis

Yeast DNA was isolated using a technique described previously [22-24]. 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). They were all used in RAPD-PCR, following the method developed by Williams *et al*.[23]. 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 mM of the primer, 1.25 U Taq DNA polymerase (Invitrogen), and 75 ng of template DNA. The cycling program consisted of 4 min at 94ºC, 35 1-minute cycles at 94ºC, 1 min at 25ºC, 2 min at 72ºC followed by a final extension of 5 min at 72ºC.

These steps were carried out in a Minicycler DNA thermal cycler (TM MJ Research Inc., NY, USA). Products were separated by electrophoresis in 1.4% agarose gel and stained with ethidium bromide. They were visualized under UV light and digitalized by 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 the presence or absence of each band was recorded for each isolate. The resulting matrix was interpreted using the Treecon program, where isolates were grouped according to the resemblance of their patterns. 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 closely related isolates.

Statistical analysis

Statistical analysis was performed using Statistix 7.0 and the SPSS 11.0 software. Confidence interval was 95% (CI 95%). Fisher and ANOVA were calculated at 95% using the Epi-Info 6.04 program (Atlanta University, GA).

RESULTS

Clinical features

The 40 subjects included in the study ranged in age from 33 to 76 years (mean age 56 years), 50% were female (20/40). None of them had received antibacterial or antifungal agents before this treatment. Of the total population, 68% were non-smokers. This population had an average of 12.80 teeth and 2.58 implants; 1.85 loaded implants and 0.38 non-loaded implants.

Of the total number of original implants (n=103) in the study population, we found that only 89 were present. The percentage of bone resorption in immediately loaded implants (n=13), was significantly higher ($p<0.001$) than in delayed loaded implants (n=76) (Fig.1).

Comparison of bone resorption in relation to the kind of prosthesis placed on the implants (n=89) showed significantly higher resorption rates ($p<0.001$) in the group with removable prostheses (36/48) than in the group with fixed prostheses (6/26) and without load (7/15) (Table 1).

Pocket depth (PD) was more than 3mm in 18/40 patients and less than 3 mm in 22/40 patients (Table 2).

Carriage of *C. albicans* and other yeast species

The prevalence of yeasts in the peri-implant sulcus was 73% (n=29, CI 95%:55.9- 84.9). In buccal mucosa, the distribution of yeasts was: 73% in palate and cheek (n=29), CI 95%; 0.559 0.859), and

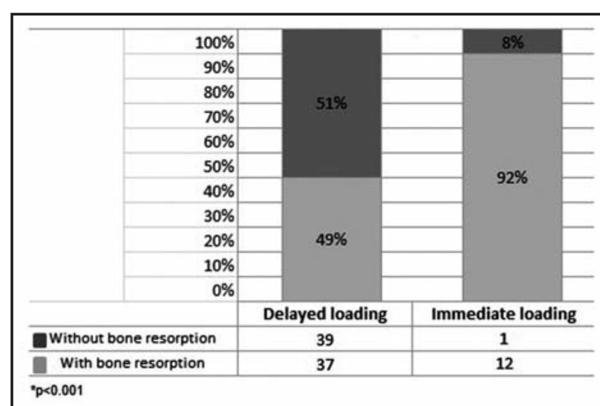

Fig. 1: Percentage of bone resorption in immediately-loaded and delayed-loaded implants. (N=89).

Table 1: Study of bone resorption in 89 implants.

Prosthetic load		With bone resorption	Without bone resorption
Totals	**89**	**49**	**40**
Fixed prosthesis	26	6	20
Removable prosthesis	48	36	12
Without prosthesis	15	7	8

Table 2: Pocket depth greater and smaller than 3mm.

Cultures	PD>3mm.		PD≤3mm.		Total
Positive	15	83%	13	59%	28
Negative	3	17%	9	41%	12
Total patients	18	100%	22	100%	40

Table 3: Prevalence of yeasts in the peri-implant sulcus and mucosa.

Cultures	Cheek	IC95%	Tongue*	IC95%	Palate	IC95%	Sulcus	IC95%
Positive	29 (73%)	55.9 84.9	34 (85%)	70.2 94.3	29 (73%)	55.9 84.9	29 (73%)	55.9 84.9
Negative	11(27%)	15.1 44.1	6 (15%)	05.7 29.8	11 (27%)	15.1 44.1	11 (27%)	15.1 44.1

*p<0.001

85% in lingual mucosa (n=34, CI 95%; 70.2- 94.3), representing a high statistically significant prevalence (*p< 0.001*) (Table 3).

Table 4 summarizes species distribution of yeast isolates in peri-implant biofilm and buccal mucosa. Of the 140 yeasts recovered, *C. albicans* was the species most frequently found in all niches, peri-implant and mucosa.

The prevalence of *C. albicans* was 55% (n=22) in peri-implant biofilm. Other non-*C. albicans spp*. and other yeasts were found: *C. dubliniensis* (n=11), *C. parapsilosis* (n=5), *Saccharomyces cereviciae* (n=5), *C. krusei* (n=2), *C. tropicalis* (n=1), *C. lusitaniae* (n=1) and *Rhodotorula spp*. (n=1).

The occurrence of two or three co-isolated species was observed in 22/120 buccal mucosa samples. *C. albicans* and *C. krusei* (n=6) followed by *Saccharomyces cereviciae* and *C. dubliniensis* (n=4) were the associations most frequently observed.

The combinations in peri-implant sulcus was 16.7% (n=8). Of the associations of the species found, the most predominant were *C. dubliniensis* with *C. krusei,* and *C. albicans* with *C. glabrata* (2% each) (Table 5).

In relation to pocket depth and presence of yeasts, patients with peri-implant sulcus >3 mm exhibited an increase in positive cultures (83%, 15/18) compared to negative cultures (17%, 3/18), whereas patients with peri-implant sulcus ≤3 mm, positive cultures (59%, 13/22) and negative cultures (41%, 9/22) exhibited much lower discrepancy. This difference was not statistically significant (Table 6).

Of the 89 implants studied, 43 showed no colonization by *Candida*, of which 23 had bone resorption (53 %) and 20 did not (47%). Of the 46 implants where there was colonization by *Candida*, 26 had resorption (47%) while the other 20 did not (43%). In all four cases, the percentages were similar. According to these results, peri-implant *Candida* colonization would not be the determining cause of bone resorption around implants. (Fig. 2)

Table 4: Prevalence of Candida albicans in peri-implant sulcus.

Yeast Species	Sulcus	%	IC95%
C. albicans	22	**55.0**	38.7 70.4
C.dubliniensis	11	**27.5**	15.1 44.1
C. parapsilosis	5	12.5	4.2 26.8
C.tropicalis	1	2.5	0.1 13.2
C. guilliermondii	0		
C. krusei	2	5.0	0.6 16.9
Saccharomyces cerevisiae	5	12.5	4.2 26.8
C. glabrata	0		
C. lusitaniae	1	2.5	0.1 13.2
Rhodotorula spp.	1	2.5	0.1 13.2
Total	**48**		

Table 5: Distribution of yeasts in mucosa.

Colonization of yeasts in mucosa	CHEEK	TONGUE	PALATE	TOTAL CULTURES	Percentage of total
Negative	11	6	11	28	
C. albicans	14	11	12	**37**	40%
C. dubliniensis	5	3	4	**12**	13%
C. parapsilosis	2	5	2	9	10%
Saccharomyces cerevisiae	3	2	1	6	7%
C. tropicalis	0	1	1	2	2%
C. glabrata	0	1	0	1	1%
C. krusei	0	1	0	1	1%
C. guillermondii	1	0	0	1	1%
C. lusitaniae	1	0	0	1	1%
C. krusei and C. albicans	1	3	2	**6**	7%
Saccharomyces cerevisiae and C. dubliniensis	1	2	1	**4**	5%
C. parapsilosis and C. tropicalis	0	1	2	3	3%
C. parapsilosis and C. albicans	0	2	0	2	2%
C. parapsilosis and dubliniensis	0	0	2	2	2%
C. guillermondii and C. tropicalis	0	1	1	2	2%
Saccharomyces cerevisiae and C. glabrata	0	1	0	1	1%
C. glabrata and C. dubliniensis	1	0	0	1	1%
C. krusei and C. dubliniensis	0	0	1	1	1%
Total Positive	29	34	29	92	100%

Implants with removable prostheses exhibited significantly higher (*p<0.001*) rates of *Candida spp.* colonization (19/22) than those with fixed prostheses (9/18) (Table 7).

RAPD-PCR ASSAY

We selected five RAPD primers, based on their reproducibility, after the pre-screening process in order to analyze 68 *C. albicans* isolates. The number of bands ranged from two to three splitters (M13r) to 12 (M13f). Three of five primers were the most informative (M13f, OPA 9 and OPC5) and generated the highest number of band patterns (10 to 12).

The dendrogram generated by the UPGMA clustering method, using the RAPD-PCR technique

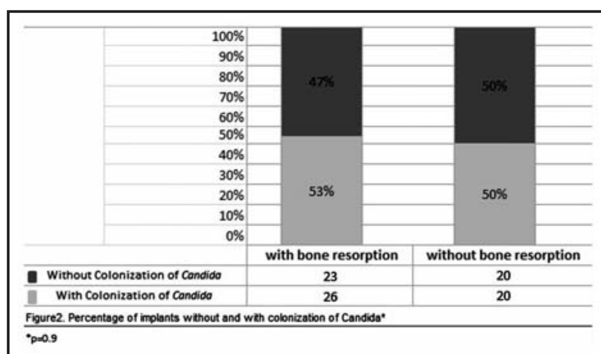

Fig. 2: Percentage of implants with and without Candida colonization.

Table 6: Presence of yeasts in relation to pocket depth.

Cultures	PD>3mm.		PD≤3mm.		Total
Positive	**15**	**83%**	**13**	**59%**	28
Negative	*3*	*17%*	*9*	*41%*	12
Total patients	**18**	**100%**	**22**	**100%**	40

Table 7: Colonization of *Candida spp.* in implants with removable and fixed prosthesis.

Culture	Fixed prosthesis		Removable prosthesis		Total
Positive	**9**	**50%**	**19**	**86%**	28
Negative	**9**	**50%**	**3**	**14%**	12
Total	**18**	**100%**	**22**	**100%**	40

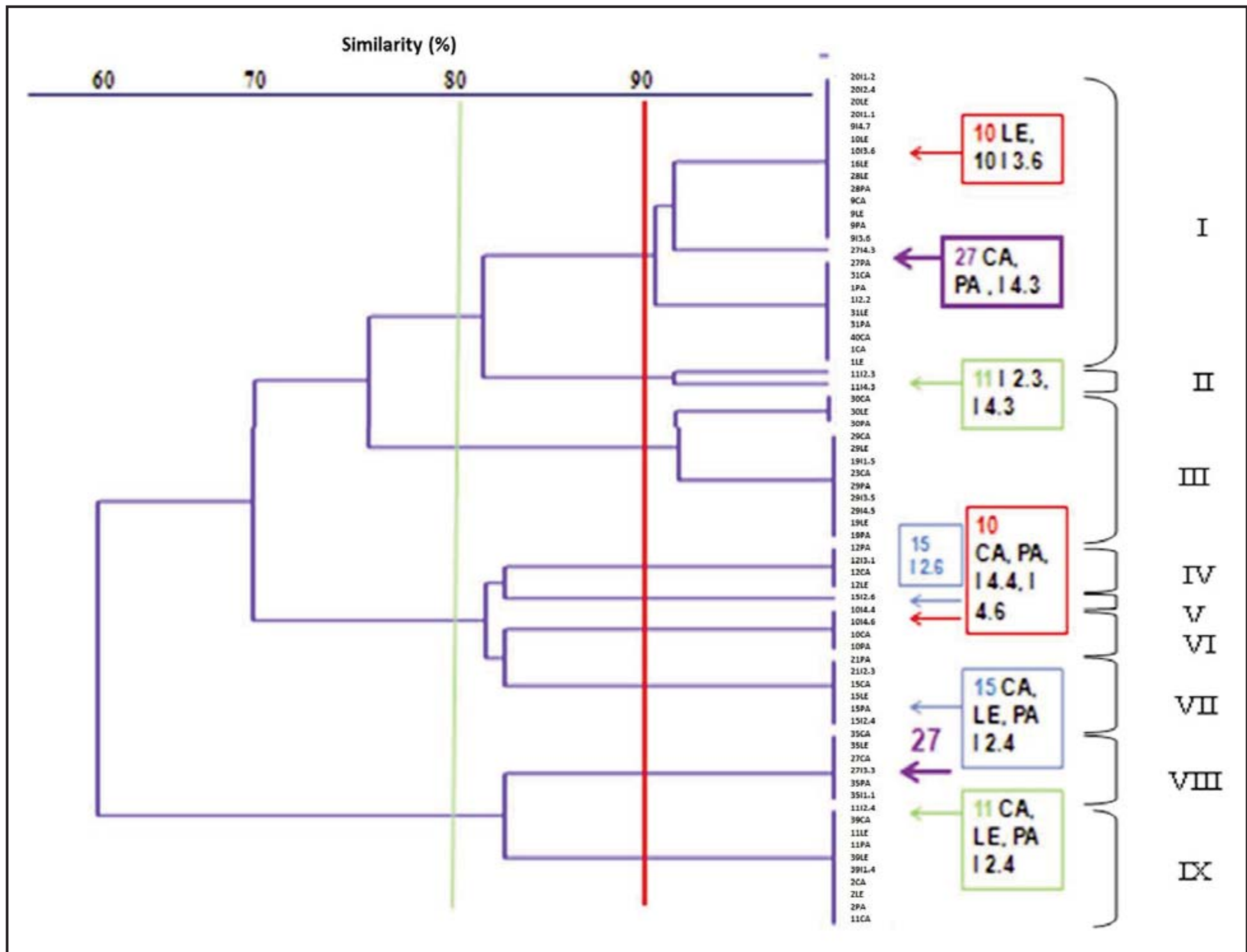

Fig. 3: The dendrogram generated by the UPGMA clustering method, using the coefficient of similarity between RAPD-PCR of C. albicans in oral cavity, tongue (LE), palate (PA), cheek (CA), and peri-implant sulcus (I) shows that the similarity coefficient (SC) ranged from 60 to 100%. Thirteen genetic clusters and nine main genotypes were obtained at a similarity coefficient (SC) of 90%, genotypes I, II, III, IV and V.

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DISCUSSION

In this study, 40 immunocompetent adult patients with more than 5 years' treatment were recruited and grouped according to their health status and pocket depth into peri-implantitis or healthy. As expected, patients with peri-implantitis presented more infectious sites, including higher rates of percentage similarity (PS) (Anova Test $p < 0.001$).
Eighty-nine peri-implant sulcus samples and 120 swabs from buccal mucosa were cultured directly in CHROMagar Candida medium to enable the presumptive identification of *C. albicans* or *C. dubliniensis, C. tropicalis* and *C. krusei*. This also enabled identification of the presence of infections caused by more than one species simultaneously. Similar findings have been reported by other authors who analyzed other populations[22,25,26-28].
The prevalence of yeasts in sulcus was 73% (n=29), showing that the surrounding ecological niche and peri-implant sulcus enabled yeast growth. Other studies have reported the presence of *Candida spp.* in peri-implant lesions[29,30], and found *Candida spp.* in 55% of peri-implant sites.
The comparison of yeast distribution in relation to clinical markers of peri-implantitis revealed no significant difference in the prevalence of yeasts at sites with PD >3 mm or at sites with bone resorption.

These findings revealed the presence of yeast species in peri-implant sulcus as well at sites with or without peri-implantitis.

Of the 120 buccal mucosa samples studied here, the tongue was the site with highest prevalence of *Candida spp*. (85, CI95%, 0.702 0.943), in contrast to cheek and palate, with a statistically significant difference ($p<0.001$).

Candida spp. prevalence was higher in our study than in previously reported series[31-34] in which it ranged from 25% to 65%, suggesting that the presence of implants in our study population increases prevalence. In relation to the type of implant rehabilitation −fixed or removable− the latter yielded significantly higher ($p<0.001$) prevalence of yeasts. It is worth noting that these findings suggest that peri-implant plaque is an ecological niche that favors the growth of yeast species; especially in implants with removable rehabilitation, even though they can be removed for cleaning. Moreover, these implants are made of acrylic, which favors adhesion of *Candida spp.* These are the first data results reported in Argentina.

The use of buccal devices induces alterations within the oral cavity. Hägg *et al.*[35] observed that the presence of prosthesis or other buccal devices increases the number of *Candida spp*., not only at the site but throughout the mucosa. Dental prostheses are made of acrylic resins in which surface defects favor the development of plaque and prevent its removal[36]. The surface of the prosthesis is very porous and thus susceptible to being colonized by large numbers of microorganisms, which may give rise to different pathologies in the oral cavity.

Comparison of the two study samples showed "high" concordance, with colonization or infection by the same yeast in both ecological niches in 95% of the patients (*Kappa*= *0.8*).

In relation to the distribution of yeast species, *C. albicans spp*. was the most prevalent (55%, n=22), but it is important to highlight that non-*C. albicans spp*. were also found in peri-implant sulcus: *C. dubliniensis* 27.5% (n=11), *C. parapsilosis* 12.5% (n=5), *Saccaromyces cereviciae* 12.5% (n=5), *C. tropicalis*, *C. lusitaniae* and *Rhodotorula spp.* 2.5% (n=1), and *C. krusei* 5% (n=2), (Table 1). Many of these less prevalent species are emerging and characterized by the presence of diminished sensitivity to antifungals[37]. No data is available in the literature reviewed.

Epidemiological surveillance is very important for identifying the prevalence of yeast species in the biofilm of peri-implant sulcus since they create reservoirs for opportunistic microorganisms which, in certain clinical situations such as patients with immune deficiencies, play a significant role in diseases such as buccal candidiasis and disseminated diseases [34, 38].

In this study, *C. albicans* isolates from the buccal cavity and peri-implant sulcus of the same patient were considered to be closely related in 90% of the cases (16/20) according to RAPD-PCR. Similarity among isolates from both ecological niches suggests that the source of *C. albicans* colonization in peri-implant biofilm is the patient's buccal cavity. Thus, it can be assumed that most *C. albicans spp*. found in peri-implant biofilm originate from endogenous infection by commensal strains.

Coincidentally, other authors have found identical genetic patterns in yeasts from different anatomical sites in the same patient. However, the results obtained highlight the fact that the same patient carries different species[39]. It is important to consider that *C. albicans* colonization in peri-implant sulcus could also occur due to the presence strains adaptable to the peri-implant environment, which is likely as a result of genetic variations such as gene conversion and/or chromosomal translocations [15, 19].

To date, scientific literature has not provided any information on the genetic characterization of *C. albicans* isolates in peri-implant sulcus. Hence, yeast isolates were analyzed by RAPD-PCR, which has proved to be a rapid, simple, cost-effective technique and discriminatory for the molecular typing of *C. albicans* isolates. Other authors have used the same techniques to assay several yeasts species [13, 15-17, 22].

This is the first study conducted in Argentina on the molecular characterization of clinical *C. albicans* isolates in peri-implant sulcus by RAPD-PCR.

We confirm that the peri-implant plaque is an ecological niche that favors the growth of yeast species; especially in implants with removable rehabilitation.

C. albicans spp. were the most prevalent in peri-implant samples, but it is important to highlight that non-*C. albicans spp*. were also found in peri-implant sulcus, e.g. *C. dubliniensis*, *C. parapsilosis*, *Saccaromyces cereviciae*, *C. tropicalis*, *C. lusitaniae* and *C. krusei*.

The findings suggest that most peri-implant *C. albicans* originate from endogenous infection by commensal strains.

ACKNOWLEDGEMENTS
The authors would like to thank Prof. Ricardo Macchi for his advice on statistics (School of Dentistry, University of Buenos Aires, Argentina).
This study was supported by grants UBACYT 20020100200204 and 20020150100219BA from the University of Buenos Aires.

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