TITLE

Oral *Candida* carriage of patients attending a dental clinic in Braga, Portugal

RUNNING TITLE

Candida oral carriage

TÍTULO

Colonización oral por *Candida* en pacientes que asisten a una clinica dental in Braga, Portugal

TÍTULO CORTO

Colonización oral por *Candida*

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SUMMARY

Background: The ability of the *Candida* species to colonize surfaces can be considered a risk factor for oral infection.

Aims: The aim of this work was to establish oral *Candida* carriage in patients attending a dental clinic in Braga, Portugal.

Methods: A total of 97 patients were analysed. Swab samples were collected, and directly cultured in CHROMagar Candida. Representative yeasts were identified by polymerase chain reaction.

Results: From the samples analysed 54.6% (*n*=53) were *Candida* positive, and *Candida albicans* was the most frequently isolated species, accounting for 79% of all the species identified. Non-*C. albicans Candida* (NCAC) species recovered included *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, and *Candida guilliermondii*. There was a lack of association between the presence of *C. albicans*, and NCAC species, and age, gender or prostheses wearing in this population. In 17% of the cases (*n*=9) polymicrobial cultures, with two different *Candida* species, were identified.

Conclusions: This study shows a high *Candida* carriage rate among this population, thus pointing to the relevance of an accurate diagnostic approach in *Candida* species identification.

RESUMEN

Antecedentes: La capacidad que poseen las diferentes especies de *Candida* de colonizar las superficies, puede ser considerada como un factor de riesgo para la infección oral.

Objetivos: El objetivo de este estudio fue establecer la colonización oral por *Candida* en pacientes que asisten a una clinica dental en Braga, Portugal.

Métodos: Un total de 97 pacientes fueron estudiados. Se colectaron muestras bucales con hisopo y fueron cultivadas directamente en CHROMagar Candida. Las levaduras seleccionadas se identificaron mediante reacción en cadena de la polimerasa.

Resultados: De las muestras analizadas 54.6% (*n*=53) fueron positivas para *Candida*. *Candida albicans* fue la especie más frecuentemente aislada, representado el 79% de todas las especies identificadas. Las especies de *Candida* no-*Candida* albicans (CNCA) aisladas fueron *C. parapsilosis, C. glabrata, C. tropicalis* y *C. guilliermondii*. En la población estudiada no se observó asociación entre la presencia de *C. albicans* y CNCA con la edad, el sexo o el uso de prótesis. En el 17% de los casos (*n*=9) se identificó colonización mixta con dos especies de *Candida*.

Conclusiones: Este estudio muestra una alta incidencia de colonización por *Candida* en esta población; por lo tanto, se sugiere la necesidad de un diagnóstico preciso para la identificación de las especies de *Candida*.

KEY WORDS

Candida, polymicrobial cultures, oral carriage, Braga-Portugal

PALABRAS CLAVE

Candida, Braga-Portugal, colonización oral, colonización mixta

INTRODUCTION

Colonization of the oral cavity by *Candida* species was defined as the acquisition, and maintenance of yeast cells without clinical signs. This process entails *Candida* species acquisition, growth, and removal [3].

Within the yeast oral community *Candida albicans* is the most frequently found (47–75% of the yeasts isolated) [4]. However, other yeast species have been increasingly identified, such as non-*C. albicans Candida* (NCAC) species(*Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis*, *Candida dubliniensis*, and *Candida guilliermondii*) *Saccharomyces cerevisiae*, *Trichosporon* species, and *Yarrowia lipolytica* [2,15,18,29].

Although the presence of *Candida* species is not an indication of disease, the ability of the yeasts to overcome the host clearance mechanisms and to colonize surfaces can be considered a risk factor for oral infection. The balance between *Candida* colonization, and candidiasis rely on the balance between pathogen characteristics (e.g. production of adhesins, secreted aspartyl proteinases), and host factors [12]. Host local predisposing conditions comprise: (i) reduced saliva secretion, (ii) epithelial changes, and local mucosal diseases, (iii) changes in commensal flora, (iv) high carbohydrate diet, and (v) denture wearing. Additionally, host systemic factors have also been associated with *Candida* oral colonization, and include: (i) age, (ii) tobacco smoking (iii) endocrine disorders, including diabetes, hypothyroidism, hyperparathyroidism, (iv) rheumatic diseases, (v) nutritional deficiencies (iron or folate deficiencies), (vi) immunosuppressive conditions, such as chemotherapy, deficiencies of humoral or cell-mediated immunity, human immunodeficiency virus infection, and acquired immunodeficiency syndrome, and (vii) drugs: broad-spectrum antibiotics, and corticosteroids [1,4,10,18].

To the authors' knowledge oral *Candida* carriage prevalence, and aetiology studies were not yet performed in the Portuguese population. Thus, the main objective of this study was to evaluate

oral *Candida* carriage in patients attending a dental clinic in Braga, Portugal.

PATIENTS AND METHODS

Candida type strains

For quality control purposes, the following *Candida* type strains were used in the identification procedures of *Candida* isolates: *C. albicans* CECT 1472, *C. dubliniensis*, strain provided by Biognostica from United Kingdom National External Quality Assessment Service, *C. glabrata* ATCC 2001, *C. guilliermondii* ATCC 6260, *C. kefyr* ATCC 204093, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750. These strains were maintained on Sabouraud dextrose agar.

Patients

Samples were collected from a total of 97 asymptomatic individuals (77 females, and 20 males) attending a dental clinic in Braga, Portugal over a 12-month period (May 2005 to 2006). Each of the potential subjects was informed of the aims, and methods of the study, and anticipated benefits, and potential risks, according to the World Medical Association Declaration of Helsinki. Data on patient age, oral hygiene habits, health status, medications, and prosthesis wearing were collected.

Sample collection

Samples were collected by passing a sterile swab (UNI-TER, MEUS, Padua, Italy) across the oral mucosa: tongue, hard palate, and gums, and replaced in its sterile container tube. Samples were kept at 4°C, and analysed within 24 h.

Candida species identification

Medium for the primary isolation

CHROMagar™ Candida medium (CHROMagar, Paris, France) was prepared according to the

manufacturer's instructions. The swab was inoculated into CHROMagar Candida medium rotating the swab head on the surface of the medium. The plates were incubated at 37°C for 48 h. Colony morphology, and colour description were assigned in a standard manner by a single investigator. Presumptive species identification was performed according to Odds and Bernaerts [20]. At least one colony exhibiting each colour was streaked into a new CHROMagar Candida plate, and then cryopreserved.

Molecular identification

Yeast DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Lisbon, Portugal) according to the manufacturer's instructions. Genomic DNA content was determined by spectrophotometry readings at 260 nm. Aliquots of 10 µl were analysed by electrophoresis in a 0.8% agarose (Bio-Rad, Lisbon, Portugal) gel in 1 × TBE buffer (Bio-Rad, Lisbon, Portugal), and visualized with a UV transilluminator, after ethidium bromide (Bio-Rad, Lisbon, Portugal) staining (0.5 mg/ml).

To assess the *Candida* speciation, a polymerase chain reaction method (PCR) previously described [14] was followed. This method uses primer mixtures of the *Candida* DNA topoisomerase II genes (Table 1). In a multiplex PCR strategy it allows the identification of *C. albicans*, *C. guilliermondii*, and *C. parapsilosis* using primer set A (Table 1) and *C. dubliniensis*, *C. krusei*, *C. kefyr*, and *C. glabrata* using primer set B (Table 1). *C. tropicalis* is identified in a third PCR reaction using a single pair of primers (Table 1).

PCR amplification was performed in 25 µl volume consisting of: 1 × PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20, and 25 mM $MgCl_2$) (Bioron, Porto, Portugal); dNTP mixture (200 µM each) (Bioron, Porto, Portugal); primer mixture (300 nM each); 1.25 U Taq DNA polymerase (Bioron, Porto, Portugal); 10–100 ng genomic DNA template; the remaining volume consisted of sterilized ultrapure water. PCR was carried out in a

MyCycler thermal cycler (Bio-Rad, Lisbon, Portugal) under the following cycling conditions: 35 cycles of 30 s at 94°C, 15 s at 57°C, and 45 s at 65°C, after a 10-min initial period of DNA denaturation, and enzyme activation at 94°C. One blank reaction was performed simultaneously per every 10 tests run by replacing the template DNA by sterilized ultrapure water. DNA from type strains was also included in each reaction as positive and negative controls.

An aliquot of 15 µl of each PCR product was analysed by electrophoresis in a 1.2% agarose gel in 1 × TBE buffer. Fragments were visualized by ethidium bromide staining (0.5 mg/ml) with a UV transilluminator. The size of the amplified DNA fragments was determined by comparison with a 100-bp DNA marker (Bioron, Porto, Portugal).

All the isolates whose presumptive identification in CHROMagar Candida did not correspond to the molecular identification were re-tested by two independent researchers in a blind assay.

Statistical analysis

CHROMagar Candida medium sensitivity was calculated as: [# true positives × 100 / (# true positives + # false negatives)], and specificity as: [# true negatives × 100 / (# true negatives + # false positives)]. Statistical analysis was performed using GraphPad Prism, version 5.00 software for Windows. Data was analysed using two-tailed chi-square test or Fischer test to measure association between *Candida* species distribution within groups. A statistical confidence interval of 95% was established.

RESULTS

***Candida* species identification**

Presumptive identification of yeasts clinical isolates was based on their colour on CHROMagar Candida medium. Samples were processed in parallel with *C. albicans*, *C. krusei*, and *C. tropicalis* type strains that presented the expected colours on CHROMagar Candida medium:

green, pink, and blue, respectively.

For each culture, representative isolates were identified by PCR using *Candida* specific primers pairs for the genomic sequences of DNA topoisomerase II gene (Table 1) [14]. Reference strains DNA was included in each assay as control. DNA of *C. albicans* (Fig. 1A, lane 1), *C. guilliermondii* (Fig. 1A, lane 3), and *C. parapsilosis* (Fig. 1A, lane 4) were amplified using primer set A (Table 1). Primer set B (Table 1) allowed the identification of *C. glabrata* (Fig. 1B, lane 2), *C. dubliniensis*, *C. krusei*, and *C. kefyr* reference strains (data not shown). Finally, the set of primers C (Table 1) allowed the identification of *C. tropicalis* reference strain (Fig. 1C, lane 5). For all *Candida* species the amplicon size obtained (Fig. 1) was as expected (Table 1). Clinical isolates identification was based on the comparison of the size of the amplified DNA products (assessed by the DNA ladder), with the respective type strain PCR product. Fig. 1 shows an example of the identification of an isolate of *C. albicans* (lane 6), *C. glabrata* (lane 7), *C. guilliermondii* (lane 8), *C. parapsilosis* (lane 9) and *C. tropicalis* (lane 10).

CHROMagar Candida phenotypic characteristics of the *Candida* species identified by PCR are specified in Table 2. As expected, *C. glabrata*, and *C. parapsilosis* did not present a distinguishable colour in this medium. Using PCR as a standard method, and considering the CHROMagar Candida identifying colours claimed by the manufacturer, CHROMagar Candida sensitivity, and specificity for *C. albicans* were found to be 97.9%, and 83.3%, respectively. For *C. tropicalis* CHROMagar Candida sensitivity was 66.7%, and specificity 100%.

Candida **species carriage**

From the 97 patients evaluated, 53 were identified as oral *Candida* carriers: 81.1% were females (*n*=43), and 18.9% males (*n*=10) with ages ranging from 28 to 91 years old (mean=61, and median=62 years old). Prosthesis wearers accounted for 84.9% of the individuals (*n*=45).

C. albicans was identified in 79% of the samples being the predominant *Candida* species. Additionally, *C. parapsilosis* comprised 6.5% of the isolates, followed by *C. glabrata* (4.8%), *C. tropicalis* (3.2%), and *C. guilliermondii* (1.6%). Five percent of the CHROMagar Candida positive samples (Table 2) were not identified. The distribution of *Candida* isolates within gender, and age groups is presented in Table 3. There was no association between *C. albicans*, and NCAC species carriage within (i) gender (P=0.7), (ii) the age groups defined (P=0.83) or (iii) prosthesis wearing (P=1).

Seventeen percent of the individuals (*n*=9) presented more than one *Candida* species per sample (Table 4). These individuals (seven females, two males) had a mean age of 58 years old (range=38-88 years old). The percentage of patients wearing prostheses in this sub-population (88.9%) was similar to the observed in the population studied.

DISCUSSION

Motivation for microbiological tests in the field of oral medicine includes diagnosis, choice of therapy, treatment control, and risk evaluation [6]. This study focused on diagnostic, and risk evaluation.

The diagnosis approach used herein included a primary isolation on a chromogenic medium (CHROMagar Candida) followed by *Candida* species identification using a previously described PCR method [14]. The CHROMagar Candida phenotypic characteristics of the *Candida* species identified by PCR are specified in Table 2. In comparison with PCR, there was a lower sensitivity, and specificity of CHROMagar Candida in the identification of *C. tropicalis*, and of sensitivity regarding *C. albicans*, also described by other authors [27]. In fact, the low sensitivity shown by *C. tropicalis* to CHROMagar Candida (66.7%) is due to the fact that one isolate further identified as *C. tropicalis* presented green colour (Table 2), instead of the characteristic

blue one. *C. tropicalis* isolates developing dark pink [28], lavender [19,27], and white colour [27] on CHROMagar Candida have also been reported. Regarding *C. albicans*, two isolates that developed green colour on CHROMagar Candida were not identified as *C. albicans* (Table 1). In addition, one of the isolates, further identified as *C. albicans*, developed pink colour in CHROMagar Candida. In accordance, some literature reports show that *C. albicans* isolates can develop atypical colours in CHROMagar Candida which include pink [25], white [27], blue or lavender [19]. It should be noted that in the same plates containing these atypical strains, isolates of *C. albicans*, and *C. tropicalis* with the expected colour phenotype were identified.

Additionally, three yeast isolates were not identified by the PCR method using specific primers for the most common species (Table 2). A comparison between literature reporting yeast species frequently isolated from the oral cavity [18], and colours developed by Candida species in CHROMagar Candida [20], suggests that the unidentified isolate developing green colour might be a *Trichosporon* species, and the pink isolates might be: *C. famata*, *C. inconspicua*, *C. lusitaniae*, *C. norvegensis*, *C. pelliculosa* or *S. cerevisiae*.

Results presented herein evidence that CHROMagar Candida medium failed to identify some yeast isolates, and that species identification should be supported by other methods, such as the molecular ones. Nevertheless, this medium facilitates the recognition of polymicrobial species in cultures, as exemplified in Table 4.

The analysis of the epidemiological literature on the recovery of *Candida* species from the oral cavity is not clear concerning factors determining colonization. The reasons of such variability may include different patient selection criteria, collection data period, geographic region in which the patients live, sampling collection methods, and methodology used for sample analysis. In the present study, the prevalence of yeasts isolated was 54.6%, and between 41% to 67% in previous studies [2,7,9,17,29]. However, due to the irregular distribution of *Candida* in the oral

cavity [26], it cannot be discarded that swab samples can yield false-negative results, and thus a misclassification of true carriers as non–carriers. The *Candida* carriage frequency observed herein was: *C. albicans* > *C. parapsilosis* > *C. glabrata* > *C. tropicalis* > *C. guilliermondii* (Table 3), with NCAC species standing for 21% of the total *Candida* species. The increased prevalence of *C. parapsilosis* within NCAC species was also observed in Portuguese patients with fungaemia [5], suggesting that *C. parapsilosis* might be an important fungal pathogen in Portugal. Nevertheless prevalence of *C. tropicalis* [9], *C. parapsilosis* [11], *C. famata* [15] or *C. glabrata* [17,29] over other NCAC species in the oral cavity has been shown, and such variation may be due to patient age or underlying disease.

The distribution of *Candida* isolates within gender, and age groups are presented in Table 3. In the current study there was no association between *C. albicans* versus NCAC species carriage within gender or age, likewise the observed by other authors [13]. Nevertheless, NCAC species recovered from samples of patients with more than 80 years old were exclusively *C. parapsilosis*, and *C. glabrata* (Table 3), suggesting an association between this age group, and these *Candida* species. Even so, as only two NCAC species have been isolated in this age group, an increase in sample size would be necessary to establish a conclusive association.

Earlier studies scarcely report the identification of mixed *Candida* cultures. However, in recent years, researchers became aware of it, and the refinement of identification procedures allowed the discrimination of multi-*Candida* species in culture. As observed in other studies [2,8,16,17,24,29], the most common association found herein was *C. albicans* plus *C. glabrata* (Table 4). Nevertheless, the epidemiological data available report the association between other *Candida* species [2,8,15-17,24,29]. It is interesting to note that in the current study *C. parapsilosis* was the only NCAC species identified that was not exclusively co–isolated with other *Candida* species (Table 3, and 4). In fact, other authors have reported that the colonization

with NCAC species, as the sole species, is lower when compared with its co–colonization with other species [17]. This suggests that multi–species colonization may support the maintenance of the oral NCAC population contributing to increased interactions with molecules, and surfaces in the oral cavity. In fact, it was shown that the intensity of colonization by more than one *Candida* species was higher than the observed with single species [17]. However, when host natural defences decay, the benign colonization can develop into oral candidiasis, and antifungal therapy may support NCAC species emergence as the sole detectable species from oral lesions. In fact, results from the ARTEMIS DISK Global Antifungal Surveillance Program [21-23] show that *Candida* species resistance to fluconazole can be ranked as follow: *C. glabrata*, 16%, *C. guilliermondii*, 13%, *C. tropicalis*, 2.6%, *C. parapsilosis*, 2.4%, and *C. albicans*, 1.2%. These findings suggest that multi–species carriers might be at higher risk, than the mono–species carriers, of developing oral candidiasis, and of being resistant to antifungal therapy.

According to the World Medical Association Declaration of Helsinki, purposes of research involving human individuals might be the advance in prophylactic, diagnostic, and therapeutic procedures as well as a better understanding of the aetiology of the disease. The present study fulfilled some of these issues. The diagnosis of oral *Candida* carriage before the presentation of clinical symptoms allowed the possibility of (i) dental hygiene education for routine oral care, (ii) control of the spread of the colonization through the monitoring of colonization, and (iii) use of therapeutic approaches when appropriated. Finally, the main observation that may contribute to a better understanding of *Candida* oral carriage arose from the high frequency of polymicrobial cultures, which may represent an increased risk of infection to patients, requiring careful surveillance.

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AUTHOR'S DECLARATION

Authors have nothing to declare.

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Figure 1 *Candida* species-specific amplification of DNA topoisomerase II fragments. Genomic DNA was amplified using different sets of species-specific primers (from A to C), in accordance to Table 1. Lanes: M, 100-bp DNA marker with their molecular size in bp is indicated in the left margin; 1- *C. albicans* CECT 1472; 2- *C. glabrata* ATCC 2001; 3- *C. guilliermondii* ATCC 6260; 4- *C. parapsilosis* ATCC 22019; 5- *C. tropicalis* ATCC 750; 6–10- example of clinical isolates of each species; 11- blank. *C. dubliniensis*, *C. krusei* ATCC6258, and *C. kefyr* ATCC 204093 identification was omitted for simplicity. Arrows on the right indicate the molecular weight of the amplified products.

Table 1 Primers sets and species specific primers used in this study (as described by Kanbe et al. [14])

Primers sets	Target species	Forward primer sequence (name)	Reverse primer sequence (name)	Expected PCR product size (bp)
A[a]	*C. albicans*	5'-TTGAACATCTCCAGTTTCAAAGGT-3' (CABF59)	5'-AGCTAAATTCATAGCAGAAAGC-3' (CADBR125)	665
	C. guilliermondii	5'-CCCAAAATCACAAAGCTCAAGT-3' (CGLF41)	5'-TACGACTTGAAGTTGCGAATTG-3' (CGLR61)	205
	C. parapsilosis	5'-GGACAACATGACAAAAGTCGGCA-3' (CPPIIF41)	5'-TTGTGGTGTAATTCTTGGGAG-3' (CPPIIR69)	310
B[b]	*C. dubliniensis*	5'-AAATGGGTTTGGTGCCAAATTA-3' (CDBF28)	5'-GTTGGCATTGGCAATAGCTCTA-3' (CDBR110)	816
	C. krusei	5'-GAGCCACGGTAAAGAATACACA-3' (CKSF35)	5'-TTTAAAGTGACCCGGATACC-3' (CKSR57)	227
	C. kefyr	5'-CTTCCAAAGGTCAGAAGTATGTCC-3' (CKFF35)	5'-CTTCAAACGGTCTGAAACCT-3' (CKFR85)	532
	C. glabrata	5'-CCCAAAAATGGCCGTAAGTATG-3' (CGBF35)	5'-ATAGTCGCTACTAATATCACACC-3' (CGBR103)	674
C[c]	*C. tropicalis*	5'-CTGGGAAATTATATAAGCAAGTT-3' (CTPIIF36)	5'-TCAATGTACAATTATGACCGAGTT-3' (CTPIIR121)	860

In the original report [14] primers sets had the designation of [a]PsI, [b]PsII, and [c]PsIII

Table 2 Colour of colonies on CHROMagar Candida medium for species identified by PCR

Species	**# Colony colour**		
	Green	Blue	Pink
C. albicans	48	-	1
C. parapsilosis	-	-	4
C. glabrata	-	-	3
C. tropicalis	1	2	-
C. guilliermondii	-	-	1
Unidentified	1	-	2

Table 3 Frequency of distribution of *Candida* species isolated and patients' characteristics

Patients characteristics	**Species[a] frequency, % (#)**					
		NCAC species				
	C. albicans	*C. parapsilosis*	*C. glabrata*	*C. tropicalis*	*C. guilliermondii*	Unidentified
Gender						
Female	80 (40)	6 (3)	4 (2)	4 (2)	0 (0)	6 (3)
Male	75 (9)	8.3 (1)	8.3 (1)	0 (0)	8.3 (1)	0 (0)
Age (years)						
≤ 54	82.6 (19)	4.3 (1)	4.3 (1)	4.3 (1)	0 (0)	4.3 (1)
55-79	75.9 (22)	6.9 (2)	3.4 (1)	3.4 (1)	3.4 (1)	6.9 (2)
≥ 80	80 (8)	10 (1)	10 (1)	0 (0)	0 (0)	0 (0)

[a], PCR identification

Table 4 Number of patients with more than one *Candida* species

Species[a]	**#patients**
C. albicans–C. glabrata	3
C. albicans–C. tropicalis	2
C. albicans–C. parapsilosis	1
C. albicans–unidentified	2
C. parapsilosis–C. guilliermondii	1

[a], PCR identification

Figure 1

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