# Prevalence and antifungal susceptibility profiles of *Candida glabrata*, *Candida parapsilosis* and their close-related species in oral candidiasis

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## Abstract

### Objective

To evaluate the importance of *Candida glabrata*, *Candida parapsilosis* and their close-related species, *Candida bracarensis*, *Candida nivariensis*, *Candida metapsilosis* and *Candida orthopsilosis* in patients with oral candidiasis and, to determine the *in vitro* activities of antifungal drugs currently used for the treatment.

### Methods

One hundred fourteen isolates of *C. glabrata* and 97 of *C. parapsilosis*, previously identified by conventional mycological methods, were analysed by molecular techniques. *In vitro* antifungal susceptibility to fluconazole, itraconazole, miconazole, and nystatin was evaluated by CLSI M44-A2 disk diffusion test, and by CLSI M27-A3 microdilution for fluconazole.

### Results

All *C. glabrata* isolates were identified as *C. glabrata sensu stricto*, 93 out of 97 *C. parapsilosis* isolates as *C. parapsilosis sensu stricto*, three as *C. orthopsilosis* and one as *C. metapsilosis*. *Candida glabrata* was mainly isolated in mixed cultures but *C. parapsilosis* complex was more frequent in pure culture. *Candida metapsilosis* and *C. orthopsilosis* were isolated as pure culture and both species were susceptible to all antifungal agents tested. Most *C. glabrata* isolates were susceptible to miconazole and nystatin, but resistant to fluconazole and itraconazole. Azole cross resistance was also observed. *Candida*

*parapsilosis* isolates were susceptible to fluconazole although azole cross resistance to miconazole and itraconazole was observed.

**Conclusion**

This study highlights the importance of accurate identification and antifungal susceptibility testing of oral *Candida* isolates in order to have an in-depth understanding of the role of *C. glabrata* and *C. parapsilosis* in oral candidiasis.

**Keywords**: *Candida glabrata*, *Candida parapsilosis* complex, oral candidiasis, antifungal susceptibility.

## 1.- Introduction

Oral candidiasis is an infection caused by *Candida* which is often related to the characteristics of the patient, such as, age, immunological status, and denture wearing among other predisposing factors (Samaranayake, Keung Leung, & Jin, 2009). Oral candidiasis frequently produces discomfort, pain and dysgeusia and, manifests itself in a wide variety of chronic and acute clinical manifestations, such as pseudomembranous, erythematous or hyperplastic candidiasis. *Candida albicans* is the major aetiological agent, although other species of *Candida*, such as *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* or *Candida glabrata* can be isolated from oral lesions (Muadcheingka & Tantivitayakul, 2015; Razzaghi-Abyaneh et al., 2014; Sadeghi et al., 2018; Samaranayake, Keung Leung, & Jin, 2009). Since 1990, changes in the distribution of *Candida* species causing invasive candidemia are being increasingly reported: *C. albicans* frequency is decreasing while that of *C. glabrata* remains stable and *C. parapsilosis* incidence has risen (Guinea, 2014; Quindós, 2014; Vaezi et al., 2017). An improvement in diagnostic procedures that enables a more rapid and accurate identification has been arising during the last 20 years by molecular and proteomic technics (Alonso-Vargas et al., 2008; Aslani et al., 2018; Yazdanparast et al., 2015).

Development of molecular based identification methods has allowed the finding of new species phylogenetically close to *C. glabrata* and *C. parapsilosis*. The new species, *C. nivariensis* and *C. bracarensis*, are phylogenetically similar to *C. glabrata* (Alcoba-Flórez et al., 2005; Correia, Sampaio, James, & Páis, 2006); while *C. metapsilosis* and *C. orthopsilosis* are closely related to *C. parapsilosis* (Kurtzman & Robnett, 1997; Tavanti, Davidson, Gow, Maiden, & Odds, 2005). These new species are considered significant

74 pathogens that can be isolated from oral lesions (Borman et al., 2008; Jahanshiri et al.,
75 2018; Wahyuningsih et al., 2008).
76 Patient characteristics and prior antifungal therapy play an important role in the increasing
77 isolation of these cryptic species in candidiasis (Guinea, 2014). In our setting, patients with
78 oral candidiasis are often treated with nystatin or miconazole which are suitable topic
79 agents for the treatment of superficial infections, while other antifungal agents such as
80 fluconazole, itraconazole, or voriconazole are mainly indicated for the treatment of deep
81 seated infections or for the treatment of recalcitrant oral candidiasis when a topic treatment
82 has failed (García-Cuesta, Sarrion-Pérez, & Bagan, 2014).
83 Miconazole, fluconazole and voriconazole have shown excellent *in vitro* activities against
84 oral *Candida* isolates (Kobayashi et al., 2002; Marcos-Arias, Eraso, Madariaga, Carrillo-
85 Muñoz, & Quindós, 2012; Tscherner, Schwarzmüller, & Kuchler, 2011). However, the
86 reduced susceptibility of *C. glabrata* to azoles could be a problem for the treatment of
87 infections caused by this species (Arendrup et al., 2013; Pemán et al., 2012; Pfaller et al.,
88 2012b; Quindós, 2014; Tscherner et al., 2011).
89 *Candida bracarensis*, *C. nivariensis*, *C. metapsilosis* and *C. orthopsilosis* share many
90 phenotypic characteristics or are undistinguishable from *C. glabrata* or *C. parapsilosis*.
91 Hence, some oral clinical isolates routinely identified as *C. glabrata* or *C. parapsilosis*
92 could be actually misidentified isolates of their cryptic species. Knowledge about the
93 prevalence and distribution of these emerging species of *Candida* is still needed to elect the
94 best antifungal treatment against them. Therefore, the present study aims to evaluate the
95 importance of *C. glabrata*, *C. parapsilosis* and their phylogenetically close-related species
96 in oral candidiasis, and to assess their *in vitro* susceptibility to itraconazole, fluconazole,
97 miconazole, and nystatin.

## 2.- Materials and methods

### *2.1.- Clinical isolates*

A total of 211 *C. glabrata* and *C. parapsilosis* were isolated from oral swabs of 1126 episodes of patients suffering from clinical oral candidiasis attending at the Dental Clinic Service of the Universidad del País Vasco / Euskal Herriko Unibertsitatea (UPV/EHU), Bilbao (Spain) from 2003 to 2013. Oral isolates were identified by conventional mycological methods, such as colony morphology on *Candida* Chromogenic agar (Laboratorios Conda, Spain) and ChromID Candida (BioMérieux, France), the germ tube test, microscopic morphology on corn meal agar and carbon source assimilation kit API ID 32C system (bioMérieux) (Eraso et al., 2006). These isolates, stored in the UPV/EHU yeast stock collection at room temperature in vials containing sterile distilled water, were cultured on Sabouraud dextrose agar medium (Difco, USA) at 37 °C for 24 h for molecular identification and for *in vitro* antifungal susceptibility testing.

### *2.2.- Candida glabrata complex identification by 5.8S rRNA gene and the internal transcribed spacer (ITS1) analysis*

Identification of *C. glabrata* and its phenotypically related species, *C. bracarensis* and *C. nivariensis*, was performed by multiplex-polymerase chain reaction (multiplex-PCR) using four primers targeting the ITS1 region and the 5.8S ribosomal RNA gene (Table 1) previously described (Romeo, Scordino, Pernice, Lo Passo, & Criseo, 2009). Briefly, the master mixture was prepared from BioMix™ Red (Bioline Reagents Ltd, United Kingdom) with 0.42 µM of the primer UNI-5.8S-Reverse primer and 0.21 µM of the other three primers. The PCR reaction carried out with a BioRad C1000$^{TM}$ Thermal Cycler (Bio-Rad, USA) consisted of a denaturation step at 95 °C for 5 min, followed by 34 cycles of 30 s at 94 °C, annealing for 40 s at 60 °C, elongation for 50 s at 72 °C, and a final 10 min

122 extension step at 72 °C. The DNA amplified products were separated by electrophoresis on
123 2% agarose gel stained with GelRed (Biotium, USA) for 180 min at 50 V.
124 *2.3.- Candida parapsilosis complex identification by secondary alcohol dehydrogenase*
125 *gene (SADH) analysis*
126 Clinical isolates of *C. parapsilosis* were analysed by polymerase chain reaction-restriction
127 fragment length polymorphism (PCR-RFLP) for the identification of *C. parapsilosis sensu*
128 *stricto*, *C. metapsilosis* and *C. orthopsilosis* species using specific primers for the region of
129 the *SADH* (Table 1) (Miranda-Zapico et al., 2011; Tavanti et al., 2005). Briefly, a mixture
130 containing BioMix™ Red (Bioline) and 0.4 μM of primers was subjected to PCR
131 amplification carried out with a BioRad C1000$^{TM}$ Thermal Cycler (Bio-Rad). The
132 amplification started with a denaturation step at 95 °C for 5 min, followed by 40 cycles of 1
133 min at 92 °C, 1 min at 45 °C and 1 min at 68 °C; and a final extension step of 7 min at 68
134 °C. The amplified fragments were digested with the restriction enzyme *Ban*I (New England
135 Biolabs, USA) for 2 h at 37 °C. The DNA fragments obtained were separated by
136 electrophoresis on GelRed stained agarose gel at 1.5 %, for 70 min at 90 V.
137 *2.4.-* In vitro *activity of fluconazole, itraconazole, miconazole and nystatin*
138 All oral isolates were evaluated by disk diffusion using tablets of 25 μg of fluconazole, 10
139 μg of itraconazole, 10 μg of miconazole and 50 μg of nystatin, (Rosco Diagnostica-
140 NeoSensitabs, Denmark) following a modification of the CLSI M44-A2 guidelines
141 (Clinical and Laboratory Standards Institute (CLSI), 2009) (Rementeria et al., 2007).
142 Mueller-Hinton agar medium (Difco) supplemented with 2% (w/v) glucose and 0.5 μg/l of
143 methylene blue was used for disk diffusion testing. Yeast cell suspensions of 0.5
144 McFarland (1-5 × $10^6$ CFU/ml, approximately) for each clinical isolate were prepared in
145 sterile saline water. Inocula were spread using sterile swabs onto Mueller-Hinton plates and

146 tablets were dispensed on the surface. In order to classify the clinical isolates in terms of
147 their susceptibilities to these antifungal agents, after 24 and 48 h incubation at 37 °C,
148 inhibition zone diameters endpoints were measured in millimetres using a calliper and
149 interpreted following the criteria published by the manufacturer. The susceptibility of
150 isolates was categorized according to inhibition zone diameter as follows: a) fluconazole ≥
151 19 mm, susceptible 15-18 mm, susceptible-dose dependent; and ≤ 14 mm, resistant; b)
152 itraconazole ≥ 23 mm, susceptible; zone diameter 14-22 mm, susceptible-dose dependent;
153 and ≤ 13 mm, resistant; c) miconazole ≥ 20 mm, susceptible; 12-19 mm, intermediate; and
154 ≤ 11 mm, resistant; d) nystatin ≥ 15 mm, susceptible; zone 10-14 mm, intermediate; and
155 <10 mm, resistant.

156 In addition, *in vitro* susceptibility to fluconazole was confirmed by microdilution antifungal
157 susceptibility testing as described in the document M27-A3 from the CLSI (Clinical and
158 Laboratory Standards Institute (CLSI), 2008). Stock solution of fluconazole (3200 µg/ml)
159 (Sigma-Aldrich, USA) was prepared in pure water and serial two-fold dilutions of the
160 antifungal were made on RPMI 1640 medium (Sigma-Aldrich) buffered to pH 7.0 with
161 0.165 M morpholinopropanesulfonic acid (MOPS, Sigma-Aldrich) and added into each
162 well of 96-well microplates. Antifungal concentrations ranged from 0.125 to 64 µg/ml and
163 inocula were adjusted to a final concentration of 1-5 × $10^3$ CFU/ml in RPMI medium.
164 Plates were then incubated at 37 °C for 24 and 48 h. Fluconazole MIC was considered as
165 the lowest concentration which caused ≥ 50% inhibition of growth ($MIC_2$) after 24 h of
166 growth compared to the growth without antifungal drug. Clinical breakpoints (CBP) are
167 often used to indicate those clinical isolates that are able to respond to treatment with a
168 given antimicrobial agent administered using the approved dosing regimen for that specific

drug (Pfaller & Diekema, 2012a; Turnidge & Paterson, 2007). In this study, the CBP used were the recommended in the M27-S4 supplement of CLSI (Clinical and Laboratory Standards Institute (CLSI), 2012) and are as follow: for fluconazole against *C. glabrata*, susceptible-dose dependent ≤ 32 µg/ml and resistant ≥ 64 µg/ml; and against *C. parapsilosis*, susceptible ≤ 2 µg/ml, susceptible-dose dependent 4 µg/ml, and resistant ≥ 8 µg/ml. Moreover, epidemiological cut-off values (ECV) which can be the most sensitive measure of the emergence of strains with decreased susceptibility to a given agent, were also used to categorize wild-type (WT- those without mutational or acquired resistance mechanisms) and non-wild-type isolates (NWT- those having mutational or acquired resistance mechanisms) since resistance of oral *Candida* isolates to fluconazole has not been defined. The MIC for fluconazole to separate NWT isolates of *C. parapsilosis* was 2 µg/ml, and 32 µg/ml for *C. glabrata* (Pfaller & Diekema, 2012a).

*2.6.- Quality control*

Type strains obtained from the American Type Culture Collection (ATCC), the National Collection of Yeast Cultures (NCYC) and the Central Bureau voor Schimmel cultures (CBS) were used as quality control for the molecular identification and *in vitro* antifungal susceptibility testing: *C. albicans* ATCC 64548, *C. albicans* ATCC 64550, *C. bracarensis* NCYC 3133, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *C. metapsilosis* ATCC 96144, *C. nivariensis* CBS 9984, *C. orthopsilosis* ATCC 96141, *C. parapsilosis sensu stricto* ATCC 22019.

## 3.- Results

*3.1.- Species identification*

192 During 2003 to 2013, a total of 1328 clinical isolates were recovered from 1126 episodes of
193 clinical oral candidiasis in patients attending at the Dental Clinic Service at UPV/EHU.
194 *Candida albicans* was the most prevalent species (928 out of 1328 isolates, 70.4%)
195 followed by *C. glabrata* (114 out of 1328 isolates, 8.6%), *C. parapsilosis* (97 out of 1328
196 isolates, 7.4%) and *C. tropicalis* (43 out of 1328 isolates, 3.3%). Mixed cultures of more
197 than one isolate, were obtained from a total of 173 out of 1126 episodes (15.3%) and *C.*
198 *glabrata* or *C. parapsilosis* complexes were present in 126 of these episodes (72.8%).

199 All isolates previously identified as *C. glabrata* by conventional mycological methods were
200 identified as *C. glabrata sensu stricto* by multiplex-PCR (Figure 1). Of interest was than *C.*
201 *bracarensis* and *C. nivariensis* were not detected in the oral specimens from these patients.
202 Regarding the 97 *C. parapsilosis* isolates, 93 out of 97 isolates were identified as *C.*
203 *parapsilosis sensu stricto* (95.9%), three as *C. orthopsilosis* (3.1%) and one as *C.*
204 *metapsilosis* (1%) by PCR-RFLP (Figure 2).

205 Most *C. glabrata* isolates were yielded as mixed cultures (83 out of 114 isolates, 72.8%)
206 (Table 2). There were associations of up to four species of *Candida* and the most frequent
207 was *C. albicans* (74 out of 114 total isolates, 64.9%) plus *C. glabrata* and *C. tropicalis* (11
208 out of 114 isolates, 9.6%). Regarding to the isolates of *C. parapsilosis* complex, the
209 presence as pure culture (51 out of 97 isolates, 52.6%) was slightly higher than with other
210 yeast species (46 out of 97 isolates, 47.4 %). *Candida parapsilosis* was found together with
211 *C. albicans* in most cases (36 out of 97 total isolates, 37.1%). In eight of these 36 isolates
212 (22.2%) both species were yielded together with other *Candida* species. Conversely, *C.*
213 *metapsilosis* and *C. orthopsilosis* were always isolated as pure cultures.

214 *3.3.- Antifungal susceptibility testing*

Table 3 shows the *in vitro* antifungal susceptibility of the 114 isolates of *C. glabrata*, 93 isolates of *C. parapsilosis*, three isolates of *C. orthopsilosis* and one of *C. metapsilosis*. Figure 3 shows the isolates distribution regarding to the zone diameters obtained by disk diffusion. The reference strains used as quality controls presented the expected values (data not shown).

Nystatin showed an excellent activity against all isolates. Most isolates of *C. glabrata* were susceptible to miconazole (113 out of 114 isolates, 99.2%), and only one was intermediate (0.8%). Eight (7%) and seven (6.1%) out of 114 *C. glabrata* isolates were susceptible-dose dependent to itraconazole and fluconazole, respectively. Moreover, 14 (12.3%) and three (2.6%) *C. glabrata* isolates were resistant to fluconazole and itraconazole, respectively. On the other hand, all *C. parapsilosis* isolates were susceptible to fluconazole. However, half of *C. parapsilosis* isolates were intermediate to miconazole (46 out of 93 isolates, 49.5%) and one was resistant to this drug (1.1%). Susceptibility-dose dependent to itraconazole was detected in four *C. parapsilosis* isolates (4.3%). *Candida metapsilosis* and *C. orthopsilosis* isolates were susceptible to all antifungal agents tested.

Fluconazole activity was also tested by microdilution method for 35 *C. glabrata* with different *in vitro* susceptibilities to this antifungal agent by disk diffusion method. These isolates were classified by disk diffusion method as 13 resistant, seven susceptible-dose dependent and 15 susceptible isolates. All susceptible and susceptible-dose dependent isolates by disk diffusion method were susceptible-dose dependent by microdilution method. Conversely, only one out of 13 resistant isolates by disk diffusion method was found to be fluconazole resistant by microdilution assay (7.7%). This resistant isolate was found as pure culture, and fluconazole MIC for this isolate was 64 μg/ml. The remaining 12

resistant isolates by disk diffusion method were found to be susceptible-dose dependent by microdilution assay. According to the CLSI interpretation criteria for microdilution assay and fluconazole, *C. glabrata* cannot be classified as susceptible, only resistant or susceptible-dose dependent. Therefore, in an attempt to analyze these 12 resistant isolates only by disk diffusion, ECVs were considered and, it was found that two isolates, classified as resistant by disk diffusion method, were categorized as NWT with MICs of 32 and 64 µg/ml and the remaining isolates were categorized as WT.

Azole cross-resistance was observed in isolates of *C. glabrata* and *C. parapsilosis*. Three out of 14 *C. glabrata* isolates resistant to fluconazole by diffusion method were also resistant to itraconazole. One of these three isolates were intermediate and the other two were susceptible to miconazole. Three out of 14 fluconazole resistant isolates were also susceptible-dose dependent to itraconazole. On the other hand, one miconazole resistant isolate of *C. parapsilosis* was susceptible-dose dependent to itraconazole and was separated from a mixed culture along with *C. albicans*. Moreover, two itraconazole susceptible-dose dependent *C. parapsilosis* isolates also were intermediate to miconazole.

## 4.- Discussion

*Candida albicans* is the major aetiological agent of oral candidiasis but *C. glabrata* and *C. parapsilosis* are considered emerging causes of this disease presenting decreased susceptibilities to current antifungal drugs (Pfaller et al., 2012b; Sadeghi et al., 2018; Samaranayake et al., 2009). There are limited studies on the presence of species from *C. glabrata* and *C. parapsilosis* complexes in oral cavity (Borman et al., 2008; Jahanshiri et al., 2018; Wahyuningsih et al., 2008). In the present study, more than 15% of oral isolates belonged to *C. glabrata* (8.6%) and *C. parapsilosis* (7.4%) species complexes and were

present in 72.8% of mixed cultures. This fact remarks the importance of these species in oral pathology and should be considered for therapeutical approach. Moreover, in the current study, inside the *C. parapsilosis* complex, *C. orthopsilosis* and *C. metapsilosis* were yielded as pure cultures from a low number of oral cavities of patients as it has been described by other authors (Ge et al., 2012; Moris et al., 2012). This event highlights the necessity of achieving a correct identification of the isolates involved in oral candidiasis because of the differences in virulence and susceptibility patterns of these species in comparison to *C. parapsilosis*. However, *C. nivariensis* and *C. bracarensis* were not present in oral specimens of patients suffering from clinical oral candidiasis in the current study. Previous studies have reported a low prevalence of *C. nivariensis* and *C. bracarensis* in the oral cavity (Borman et al., 2008; Lockhart et al., 2009; Wahyuningsih et al., 2008), the female genitourinary system (Li, Shan, Fan, & Liu, 2014; Sharma et al., 2013), or blood cultures (Li et al., 2014; López-Soria et al., 2013; Miranda-Zapico et al., 2011). The species variability found in oral cavity can be wide due to population, dietary or geographical reasons (Lockhart et al., 1999; Sharifzadeh et al., 2013).

*Candida albicans* can be co-isolated with other *Candida* species as it has previously been reported in other studies (Kleinegger, Lockhart, Vargas, & Soll, 1996; Qi, Hu, & Zhou, 2005; Zaremba et al., 2006). In the current study, mixed cultures were present in 173 out of 1126 episodes (15.3%). The most common association found was *C. albicans* and *C. glabrata* (64.9%), as it has been reported previously in other studies (Coco et al., 2008; Martins et al., 2010; Muadcheingka & Tantivitayakul, 2015; Zomorodian et al., 2011), followed by the association between *C. albicans* and *C. parapsilosis* with a frequency of 37.1%. Other authors reported a lower mixed colonization with other species different of *C. albicans*; however, the presence of multiple *Candida* species may contribute to their

285 permanence in oral cavity and in case of causing oral candidiasis, a more complicate or
286 recalcitrant episode (Lockhart et al., 1999; Martins et al., 2010).
287 In this study, the most frequent association with more than two species was composed by
288 *C. albicans*, *C. glabrata* and *C. tropicalis*, as it is also reported by other authors
289 (Muadcheingka & Tantivitayakul, 2015; Pereira et al., 2013; Sanita et al., 2011; Rabelo,
290 Noborikawa, Silva-Siqueira, Silveira, & Lotufo, 2011). The presence of two or more
291 species of *Candida* in oral specimens from a patient suffering from candidiasis is difficult
292 to interpret. Probably, the apparently less pathogenic species could be an adjuvant pathogen
293 or merely a colonizer.
294 Nystatin was the most active antifungal agent *in vitro* against *Candida*. This polyene is one
295 of the first choices of treatment for mucosal and superficial candidiasis (Carrillo-Muñoz et
296 al., 2010; das Neves et al., 2008; García-Cuesta et al., 2014; Niimi, Firth, & Cannon, 2010).
297 Resistance to nystatin is infrequent and it has been attributed to alterations in cell
298 membrane (Kathiravan et al., 2012; Marcos-Arias et al., 2012; Mohamadi et al., 2014).
299 Miconazole showed good activity against *C. glabrata* and *C. parapsilosis*. Different
300 formulations of miconazole have been used such as gel or mucoadhesive buccal tablets
301 (Bensadoun et al., 2008; Khozeimeh, Shahtalebi, Noori, & Savabi, 2010; Miki, Ohtani, &
302 Sawada, 2011; Vázquez & Sobel, 2012) and, although some resistant isolates of *Candida*
303 have been reported for miconazole (Kuriyama et al., 2005; Manfredi et al., 2006; Marcos-
304 Arias et al., 2012), this antifungal agent exerts great inhibitory activity against most
305 *Candida* species (Bensadoun et al., 2008; Isham & Ghannoum, 2010; Khozeimeh et al.,
306 2010; Niimi et al., 2010; Thevissen et al., 2007; Van Roey, Haxaire, Kamya, Lwanga, &
307 Katabira, 2004).

Fluconazole is a common antifungal agent used for most oral candidiasis and has been also used for systemic *Candida* infections due to its reduced toxicity, efficacy and good tolerance (Maertens & Boogaerts, 2005). However, the widespread use of this antifungal agent has likely promoted the higher resistance rates observed (Fakhim et al., 2017; Jahanshiri et al., 2018; Silva et al., 2012). In the present study, fluconazole was very effective against *C. parapsilosis* but its activity was not as good against *C. glabrata*. Some authors have reported that the latter species develops resistance to fluconazole during therapy and, in general, presents intrinsically low susceptibility to triazoles (Arendrup et al., 2013; Pemán et al., 2012; M. A. Pfaller & Diekema, 2007; Quindós, 2014; Tscherner et al., 2011).

Regarding to itraconazole activity, resistance and dose dependent susceptibility was observed in less than the 10% of the isolates of *C. glabrata* and *C. parapsilosis sensu stricto*. This azole has been indicated as good alternative for fluconazole resistant *Candida* isolates (Oude Lashof et al., 2004) and it has also been successfully used to treat patients with oropharyngeal candidiasis (Koks, Meenhorst, Bult, & Beijnen, 2002) and denture stomatitis (Maertens & Boogaerts, 2005).

Despite the high effectiveness of the antifungal agents tested, the azole cross-resistance observed by disk diffusion requires consideration. Six of 14 isolates of the fluconazole resistant *C. glabrata* isolates by disk diffusion method presented azole cross-resistance. Cross-resistance was mainly observed against fluconazole and itraconazole but in one isolate was extended to miconazole. Interestingly, three azole cross-resistant *C. glabrata* were isolated in association with *C. albicans* suggesting that the treatment may be effective against *C. albicans* but not against *C. glabrata*, and could result in an increase of the oral burden with this resistant species, maintaining an oral candidiasis recalcitrant to the

antifungal therapy. It has been reported that the co-infection or prior infection with *C. albicans* might advantage *C. glabrata* infection (Tati et al 2016). Moreover, two *C. glabrata* isolates were cross-resistant to fluconazole and itraconazole, one of them in association with *C. krusei*; which has known intrinsic resistance to azoles (Arendrup et al., 2013; Pemán et al., 2012). Azole cross-resistance was also observed to itraconazole and miconazole in three *C. parapsilosis sensu stricto* isolates from which one was in association with *C. albicans*. Multi-species colonization can contribute to increase both the interaction with surfaces in oral cavity and the risk of being resistant to the treatment (Martins et al., 2010).

Increased isolation of non-*C. albicans* species could be related to the use of more sensitive techniques that has allowed an accurate identification of species that always have been present in oral cavity (Dahiya et al., 2003; Fakhim et al., 2018; Muadcheingka & Tantivitayakul, 2015). Alternatively, a real increase of these species can be associated to changes in the oral environment by the use of antifungal drugs or other antimicrobial compounds, such as chlorhexidine or triclosan. In this regard, this study highlights that the development and implementation of accurate identification techniques would contribute to enhancing the knowledge of the oral candidiasis aetiology and, therefore, to the best choice for the most appropriate treatment. The present study reports the increase in the frequency of *C. glabrata*, *C. parapsilosis* and their close-related species in oral candidiasis. Furthermore, the important rate of antifungal resistance observed is a clinical challenge that makes it necessary to study the in vitro susceptibility of oral *Candida* isolates to guide the selection of the most appropriate treatment.

**Disclosure**

All authors have read and approved the final article. Also, authors declare that they have no conflict of interests related to the present study.

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609

610 **LEGENDS FOR THE FIGURES**

611 **Figure 1.** 5.8S rRNA gene and the internal transcribed spacer (ITS1) amplification by
612 multiplex-PCR of oral isolates of *C. glabrata*. Lanes: *M* 100-bp DNA ladder, *1 to 15* oral
613 isolates, *Cg C. glabrata* ATCC 90030, *Cb C. bracarensis* NCYC 3133, *Cn C. nivariensis*
614 CBS 9984, -*C* negative control.

615 **Figure 2.** Secondary alcohol dehydrogenase gene (SADH) restriction profiles by PCR-
616 RFLP of oral isolates of *C. parapsilosis* complex. Lanes: *M* 100-bp DNA ladder, *1 to 15*
617 oral isolates, *Cp C. parapsilosis sensu stricto* ATCC 22019*, Cm C. metapsilosis* ATCC
618 96144, *Co C. orthopsilosis* ATCC 96141, -*C* negative control.

619 **Figure 3.** Isolates distribution according to zone inhibition diameter (millimetres) for
620 fluconazole, itraconazole, miconazole, and nystatin by disk diffusion test: (a) isolates of

621 *Candida glabrata*; (b) isolates of *Candida parapsilosis* complex. Zone diameters endpoints
622 are indicated as susceptible (S), susceptible-dose dependent (S-DD), intermediate (I) and
623 resistant (R) for each antifungal drug. The distribution of the two non-wild-type isolates
624 (NWT) for fluconazole is represented by asterisk.