Keywords: Candida parapsilosis complex; Candida parapsilosis sensu stricto; Oral dysbiosis; Immunological status

Introduction

Results on the distribution of the species in this complex are highly variable, although all the literature we reviewed reports Candida parapsilosis sensu stricto as having the highest prevalence worldwide, and Silva et al. [1] report it as the most frequent isolate in hematogenous infections. The distribution of Candida orthopsilosis and Candida metapsilosis varies widely according to geographic region, clinical service and anatomical site [2,3]. Indeed, Miranda et al. [4] claim that the exact importance of C. orthopsilosis and C. metapsilosis as human pathogens is as yet uncertain.

Little is known regarding the prevalence of species of the Candida parapsilosis complex in the oral cavity. The few papers published on the subject report variable results on its distribution in this specific ecological niche, and state that C. parapsilosis sensu stricto is the most commonly

outpatients and hospitalized immunocompromised patients in di erent clinical situations.

e sample consisted of 101 isolates which were successfully reconstituted, to be analyzed by end-point PCR with speci c primers. e following reference strains from the ATCC collection were used: C. parapsilosis (ATCC 22019), C. orthopsilosis (ATCC 96139) and C. metapsilosis (ATCC 96143), on which the same procedures were performed as on the clinical isolates.

For clinical correlation, patient·s clinical records and the dental data of the oral isolates were available.

For the in vitro susceptibility tests, we used Vitek2 automated susceptibility testing cards AST-YS07 to evaluate the response of 50 clinical isolates to the following antifungal agents: uconazole (FLC), voriconazole (VRC), caspofungin (CASPO), micafungin (MICA) and amphotericin B (AMB). To interpret the readings, we used the 2012 revision of species-speci c clinical breakpoints (CBPs) and epidemiological cuto value (ECV) (Pfaller and Diekema 2012) [9-12] (CLSI, M27-S4/2012). For quality controls for the study we used the following Candida strains: Candida kruse i ATCC 6258, C. parapsilosis ATCC 22019 and C. albicans ATCC 9002.

e following variables were analyzed: a) species of the parapsilosis complex; b) oral ecological niche; c) oral clinical status; d) intraoral appliances; e) immunological status; and f) response to antifungal agents.

Reconstitution of clinical isolates

Isolates were initially identi ed based on the color developed in the chromogenic medium, micro-morphology in 1%-Tween 80 milk agar and carbohydrate assimilation pro le using commercial systems API ID 32D and Vitek2 (BioMØrieux, France) [7,9].

To reconstitute the isolates, each strain was seeded in the following culture media: 1) BHI (brain-heart infusion) for metabolic activation of strains, incubated at 28℃-37℃ for 24-48 h [7]; 2) Di erential chromogenic solid medium for Candida (Chromagar), to ascertain the purity of the isolate and discard any contaminated strains, incubated at 28℃ for 24 hours [10]; 3) Sabouraud, to amplify colonies, incubated at 28℃-37℃ for 24 hours [10]; 4) YPD broth (yeast extract, peptone and glucose) to obtain a more robust culture, for 24 h with shaking at 37℃ [10].

Molecular characterization of clinical isolates by end-point PCR with speci c primers

Yeast DNA was obtained by breaking down the cell wall with zMd.6(do)16(.8 37℃)]TJ2Te(a)-5(k)j5(g)8(en)1(er)te aphorcup7.1(l)-3(a)1(s)5(t (CS-14(a)6(h)4(e;)13(e)ra)9(n)4(d dS14(b)6(e)-5(v)8(e))8
PS14(b)7(h)4(oe)13(o)11(tp7(l)-3(a)1(s)5(t)8(er)13(e)v8(er)16(i)e)-5(d))16(y)tp71(t)-5(i)al sicro-ctp71(y 73., f)9(n 6(er)w)-7(hic)6.1(h t
Pp12(r)13(o)11(t)-6ocol. e iA btained s reteted at

TØle -5(c)-7(tu-5(l)-2.9(a)8(9(r t)-6(y p12(in)1(g)a)3(s)d)12(n)4(e))16(y en)4(d-p)-9.9(o)12(nn)19(t PCR w)-3(1(in12.1(t)-6(h))5(p)-

For phylogenetic analysis we used the BIOEDIT so ware for editing sequence alignment and the MEGA 6 so ware for multiple alignment of sequences and phylogenetic analysis, for which the Neighbour joining algorithm was used. e tree was constructed with the reference ATCC sequences for *C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis* in addition to the sequences selected at random from the total which were positive to PCR with speci c primers.

Results

Of the total isolates upon which molecular analysis was performed, 96 (95%) were positive for the species *C. parapsilosis sensu stricto* (Table 2) according to the end-point PCR method with the pair of speci c primers CPAR-CPAF, providing 379 bp amplicons (Figures 2-5). is band pattern is compatible to the one published by Asadzadeh et al. in the Journal of Medical Microbiology in 2009 [10]. e 5 remaining strains were negative for all three species of the parapsilosis complex, so they could only be

ere is almost 4 times more probability of recovering C. parapsilosis of buccal cavity in pathological conditions than in health condition. e di erence was statistically signi cant and clinically relevant (Table 4).

e probability of recovering C. parapsilosis from the oral cavity with

each drug tested. e highest frequency of resistance and/or reduced susceptibility to FLC was obtained. For AMB and FLUCY, resistance frequency in the 50 strains is undetermined (ND) because there is no validated species-speci c cuto point. However, according to the epidemiological cuto point (Pfaller and Diekema 2012) [12], all 50 strains were wild type to AMB and no wild type to FLUCY (Figure 6).

Analysis of the distribution of strains with resistant phenotype, as determined by ECV, according to the niche from which they were isolated, showed that the largest percentage came from skin and blood (Figure 7).

Discussion

Many studies around the world report that within the complex, C. parapsilosis sensu stricto is the species most frequently recovered from clinical isolates in both pathological and commensal conditions [1-6,13,14]. However, there are few studies reporting prevalence and distribution of the species in this complex under conditions of oral health and disease. So far, C. parapsilosis sensu stricto is known to be the most prevalent species in oral cavity niches in conditions of immunocompetence, regardless of geographical region, as reported by studies from USA [5], Portugal [1], Turkey [6] and China [2]. One study from Brazil investigated the distribution of the species in this complex within the oral cavity of patients with chronic immunode ciency due to HIV, in whom C. metapsilosis was the most frequently isolated species, followed by C. parapsilosis sensu stricto, although the di erence was not statistically signi cant and the sample was very small [15].

Figure 7: The distribution suggests that the probability of retrieving C. parapsilosis strains with resistant phenotype is greater from the skin and blood niches.

DRUG	MIC (ug/ml)	CBPs			ECV	
		S	DDS/IS	R	WT	NoWT

In our study, only C. parapsilosis sensu stricto was isolated, with 100% prevalence in all samples recruited from oral cavity and other human ecological niches, predominating under conditions of immunocompetence. is result is similar to those reported by other authors such as Lotfali et al. [16] and Odds et al. [17-25], but in contrast to the results of most papers on the subject, which report recovery of C. orthopsilosis and C. metapsilosis in both hemocultures and various clinical samples (Tables 9 and 10).

Our study found that the probability of recovering C. parapsilosis sensu stricto from oral cavity is higher under pathological conditions, in agreement with a Chilean study published in 2008 [26], which reports a lower prevalence of yeasts, as re ected by a lower count of colony-forming units (CFU/ml) of Candida species, among periodontally healthy subjects than among periodontally a ected subjects, with statistically signi cant di erence. We also found that C. parapsilosis is more o en recovered in presence of prosthetic or orthodontic devices. is is consistent with Jewtuchowicz et al. [27] who found higher prevalence of Candida genus yeasts from subgingival niches in immunocompetent non-smokers with periodontal condition who wore prosthetic devices than in non-users, identifying both albicans and non-albicans species, and recovering C. parapsilosis in the latter group. Our study also found that colonization by C. parapsilosis sensu stricto is more common in oral mucosa than in gingival sulcus. is is in agreement with Urzoea et al. [26], who showed that it is more common to recover Candida parapsilosis from oral mucosa sites than from subgingival niches, in conditions of both health and disease; with C. albicans y C. dubliniensis being more frequently isolated from the subgingival niche [26]. It should be highlighted that there is no published paper speci cally studying the parapsilosis complex in relation to the three variables analyzed. However, a review of PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) did show that to date, recovery of C. parapsilosis and other non-Candida albicans species such

Author	C. parapsilosis	C. orthopsilosis	C. metapsilosis	Study country	Reference
	N (%)	N (%)	N(%)		
Moris et al.	7 (46.7)	0	8 (53.3)	Brazil (2014)	15
Enger et al.	9 (64.3)	5 (35.7)	0	Global (2001)	32
Silva et al.	65 (94.2)	-	4 (5.8)	Portugal (2009)	1
This study	34 (100)	0	0	Argentina	33

Table 10: Distribution of C. parapsilosis complex species in oral cavity studies [1,4,15,32,33].

as C. tropicalis, C. dubliniensis and C. glabrata is greater in subjects with periodontal disease [26,28], poorly controlled diabetics [29]; female subjects who use oral contraceptives [30]; and male subjects who use androgenic steroids [31,32]. Based on our review of this database, we can say that ours is the rst study in Argentina and the American continent to study the distribution and behavior of this complex in the oral cavity based on a collection of more than 20 isolates (Table 10).

Seventy-four percent of the strains came from immunocompetent subjects. is result is consistent with Constante et al. [33], who report that C. orthopsilosis is able to produce disease mainly in immunodepressed patients. C. parapsilosis may thus be the most pathogenic species in the complex, since it has shown that it is able to produce disease especially in conditions of immunocompetence.

Resistance or tolerance to antifungal agents in species in the parapsilosis complex is a growing problem in the context of usual and new antifungal agents. According to some studies, C. parapsilosis sensu stricto isolates are less susceptible than C. orthopsilosis and C. methapsilosis isolates to some antifungal agents used in the treatment of candidiasis, such as amphotericin (AMB), uconazole (FLC), itraconazole (ITC) and caspofungin (CASPO). Among the azole-derived agents analyzed in our study, FLC was the least active, with C. parapsilosis sensu stricto strains showing resistance and/or reduced susceptibility to it. In agreement with this, Silva et al. [9] found an MIC for FLC equal to 16ug/mL for a C. parapsilosis sensu stricto isolate, and similar results have been reported by Gomez-Lopez et al. [34]. In contrast, Ataides et al. [13] and Ruiz et al. [35] report resistance in a C. parapsilosis sensu stricto isolate to ITC but not to FLC. In this regard, Van Asbeck et al. [14] suggest that the di erences in susceptibility to FLC may also re ect di erent a nities for azoles in the key enzyme that synthesizes ergosterol, 14- -demethylase or for other enzymes in this pathway. Interestingly, MIC50 and MIC90 for both azoles (Table 11) found for the 50 study strains are comparatively higher than values reported for other regions such as Turkey [6], Spain [4], and Argentina [36]. is demonstrates geographic variability regarding the way in which species in this complex respond to antifungal drugs.

Two isolates were resistant to CASPO in our study. is is consistent with the worldwide trend, since many papers have reported that the MIC for CASPO in C. parapsilosis sensu stricto is higher than that for the other two species in the complex. Mutations in the FKS gene have been found to be associated with resistance to CASPO, as shown by the increase in MIC in mutant isolates compared to non-mutant or wild-types [37,38]. In contrast to the results with CASPO; all 50 strains were sensitive to MICA, and the MIC50 and MIC90 for both echinocandins was comparatively lower than reported in other parts of the world such as Turkey [39,10], Spain [8]. But this response was similar to that reported by Lockhart [3] and Canton [40].

Based on ECV, all strains were susceptible to AMB, in agreement with other studies [16,18]. However, Ataides et al. [13] and Lockhart et al. [3], reported resistance of the species C. parapsilosis sensu stricto to AMB. e response of the parapsilosis complex species to AMB varies considerably among regions, which may be determined by intra-species genotype variability.

In our study, 100% of the isolates presented MIC values lower than or equal to 1ug/mL FLUCY. As with AMB, there is no consensus on the cuto point for FLUCY for the species Candida parapsilosis Nevertheless, considering the ECV classi cation proposed by Pfaller and Diekema in 2012 [12], we found that 100% of the strains were resistant to FLUCY, since according to ECV, an MIC value for FLUCY equal to or less than 0.5 µg/ml corresponds to a wild-type (WT) strain, while an MIC value greater than 0.5 µg/ml indicates a Non-WT strain which has developed resistance mechanisms. However, the British Society for Mycopathology [39] establishes as ucytosine- sensitive isolates those which have an MIC to ucytosine equal to or less than 1 µg/ml. Our study found an MIC value for ucytosine for the 50 strains which is higher than those reported by Silva et al. [1], Miranda et al. [4] and Cantón et al. [40] in Europe. But in India, Bhatt et al. [41] found a high resistance rate to ucytosine in C. parapsilosis isolates.

Due to the fact that we did not recover C. orthopsilosis or C. metapsilosis, we were unable to establish di erences in the response pro les among the 3 species in this complex.

Conclusions

- In Argentina, C. parapsilosis sensu stricto may be the most prevalent species in the complex in di erent human ecological niches, under conditions of both health and disease, being more likely to be retrieved in situations of immunocompetence.
- C. parapsilosis sensu stricto is a common species in oral mucosa, predominating in pathological conditions and in presence of prosthetic devices.
- In the Argentine population, C. orthopsilosis and C. metapsilosis are probably two uncommon species in di erent human ecological niches, under both pathological and commensal conditions.
- Subgingival sites in the oral cavity may constitute an unfavorable microenvironment for colonization and development of C. parapsilosis
- e response of C. parapsilosis sensu stricto to antifungal agents seems to depend on the strain and the geographic region.

- Mouth and skin may be reservoirs for parapsilosis strains with resistant phenotype and/or reduced susceptibility to the group of azoles, echinocandins and ucytosine.

Recommendations

Depending on the limitations of this study, we suggest:

- Repeating this study design but in a prospective model and with a larger sample size to minimize the random error inherent to the process.
- Validate the results of in vitro antifungal sensitivity with the reference method (CLSI M27-S4 / 2008)
- Evaluate sensitivity and speci city of the molecular technique employed in this study to discriminate at species level, by comparing it to the Gold standard in a large number of samples.
- Study the impact of the oral microenvironment in dysbiosis on the virulence of Candida parapsilosis sensu stricto.

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