ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 11, pp. 14140-14148, November, 2016

International Journal of Recent Scientific Research

Research Article

MOLECULAR CHARACTERIZATION OF THE CANDIDA PARAPSILOSIS COMPLEX FROM SITES IN THE ORAL CAVITY AND OTHER HUMAN ECOLOGICAL NICHES

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ARTICLE INFO

Article History:

Received 15th August, 2016
Received in revised form 25th September, 2016
Accepted 28th October, 2016
Published online 28th November, 2016

Key Words:

Candida parapsilosis complex, *Candida parapsilosis* sensu stricto, Oral dysbiosis, Immunological status.

ABSTRACT

Candida parapsilosis is a complex made up of three species (*Candida parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*) which differ genetically. Of the three, sensu stricto has emerged over the past 10 years as an important hospital pathogen, and is currently the second most frequent yeast isolate in candidemias, after *C. albicans*, in addition to being considered the most pathogenic and prevalent in different human niches, both under immunocompetent and immunocompromised conditions. In Argentina and worldwide, no data is available on the distribution and behavior of the complex in oral cavity niches, and there is little information on its epidemiology. **Aim**: to characterize Argentine isolates of different species in the *C. parapsilosis* complex from different oral cavity sites and other ecological niches. **Methodology**: retrospective, cross-sectional, descriptive study on a collection of isolates which were previously identified by conventional methods as *parapsilosis* complex, in order to distinguish the species by using end-point PCR with specific primers derived from a single sequence in the ITS1-5.8SrRNA-ITS2 region. **Results**: 95% of the isolates were identified as *Cp*. sensu stricto, which was recovered with higher probability from oral mucosa sites, under pathological conditions, and in presence of intraoral appliances. Seventy-four percent of the strains were recovered under conditions of immunocompetence. Hundred percent had resistant phenotype to flucytosine. **Conclusions**: *Cp.* sensu stricto is a common species in different ecological niches in the Argentine population. It is more likely to be recovered under conditions of immunocompetence. Dysbiosis of the mouth favors the growth of *Cp*. sensu stricto, which under these conditions may become a source for cross-transmission of more or less virulent strains by direct person-to-person contact, and a potential source of candidemia or invasive infections through hematogenous dissemination of strains with increased pathogenicity.

INTRODUCTION

Candida parapsilosis is a complex made up of 3 species (*Candida parapsilosis* sensu stricto, *Candida orthopsilosis* and *Candida metapsilosis*) which are phenotypically indistinguishable although they differ genetically. They can only be distinguished by molecular techniques (Tavanti 2005)[1]. *Candida parapsilosis* has often been isolated from skin and fingernails of nurses and other healthcare professionals, where it forms part of the normal human commensal flora. It has also

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been isolated from medical devices such as intravascular catheters and parenteral nutrition lines, among others[2,3]. It has recently been considered as an important emerging pathogen, increasingly associated to a broad clinical spectrum of infections2, ranging from superficial infections such as vulvovaginitis, onychomycosis and oral candidiasis, to invasive forms such as candidemia, endocarditis, peritonitis, endophthalmitis, joint disorders, and meningitis, among others (Treviño Rangel, 2012)[4]. Of these, fungemia is the most frequent invasive disease caused by *C. parapsilosis*. The incidence of candidosis disseminated by this yeast has risen over the past 8 years in tertiary hospitals, especially in Latin America, increasing from 19% in 1991 - 2008, to 24 - 27% in 2009 - 20162.

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*.[5] 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 site6,[7]. Indeed, Miranda *et al*.[8] 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 recovered species in immunocompetent individuals both in Europe and in North America[5,9,10], while *C. metapsilosis* seems to prevail over *C. parapsilosis* sensu stricto in immunocompromised patients (Moris, *et al*. 2012)[11]

Candida parapsilosis is usually susceptible to antifungal agents, but there are reports of isolates with reduced susceptibility to azoles and echinocandins8. This has serious clinical implications, since these are the drugs used as first-line agents for the treatment of invasive micosis[5,12].

Despite the importance of *C. parapsilosis* as a hospital pathogenic fungus, there are few studies, particularly in Latin America, on global epidemiology or on response to antifungal agents of species in this complex, and still less is known regarding its distribution in oral cavity niches. The oral cavity may be a source of candidemias caused by this fungus in patients with risk factors. In Argentina, there is very little information on the distribution and response to antifungal agents of the species in the complex, hence, our aim is to study the prevalence, distribution in the mouth and other ecological niches, and response to antifungal agents of the 3 species in the *parapsilosis* complex, from a collection of clinical isolates obtained in previous research studies and stored at the Mycological Center at the Buenos Aires University (UBA) School of Medicine.

MATERIALS AND METHODS

This was a retrospective, cross-sectional, descriptive study using 150 clinical isolates of the *parapsilosis* complex from oral cavity and other ecological niches, stored in the collection at the IMPAM Mycology Center, School of Medicine, Buenos Aires University (UBA), which were collected during previous research projects from immunocompetent outpatients and hospitalized immunocompromised patients in different clinical situations. The sample consisted of 101 isolates which were successfully reconstituted, to be analyzed by end-point PCR with specific primers. The 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, patients' 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: fluconazole (FLC), voriconazole (VRC), caspofungin (CASPO), micafungin (MICA) and amphotericin B (AMB). To interpret the readings, we used the 2012 revision of species-specific clinical breakpoints (CBPs) and epidemiological cutoff value (ECV) (Pfaller and Diekema 2012)[15]. 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.**

The 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 identified based on the color developed in the chromogenic medium, micro-morphology in 1%-Tween 80 milk agar and carbohydrate assimilation profile using commercial systems API ID 32D and Vitek2 (® BioMérieux, France)[4,11].

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°C - 37ºC for 24 - 48 hours; 2) Differential chromogenic solid medium for Candida (Chromagar), to ascertain the purity of the isolate and discard any contaminated strains, incubated at 28ºC for 24 hours; 3) Sabouraud, to amplify colonies, incubated at 28°C - 37ºC for 24 hours; 4) YPD broth (yeast extract, peptone and glucose) to obtain a more robust culture, for 24 hours with shaking at 37ºC.

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

Yeast DNA was obtained by breaking down the cell wall with zymolyase to generate spheroplasts (Scherer and Stevens 1987)[13]. Spheroplasts were verified by optical microscopy, after which a column (QUIAM Blood DNA Kit) was used for purifying, following the Qiagen protocol. The DNA obtained was preserved at -20ºC. Molecular typing was done by end-point PCR with specific primers derived from unique sequences contained in the internal transcriptional spacer 1 **(ITS 1)-5.8 rRNA-(ITS2)** of the fungal ribosomal DNA, which enable species-specific sequences for *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis* to be retrieved separately (Table 1).

Table 1 Primers used for rapid identification at species level of the *C. parapsilosis* complex. Source: Asadzadeh M, *et al*. (2009)14.

Primer	Target gene	Direction	Species specificity	Sequence	Amplification size
CPAF	ITS 1	Forward	*C. parapsilosis*	TTTGCTTTGGTAGGCCTTCTA	379pb
CPAR	ITS 2	Reverse		GAGGTCGAATTTGGAAGAAGT	
CORF	ITS 1	Forward	*C. orthopsilosis*	TTTGGTGGCCCACGGCCT	367pb
CORR	ITS 2	Reverse		TGAGGTCGAATTTGGAAGAATT	
CMEF	ITS 1	Forward	*C. methapsilosis*	TTTGGTGGGCCCACGGCT	374pb
CMER	ITS 2	Reverse		GAGGTCGAATTTGGAAGAATGT	

Validation of PCR results with Sanger sequencing

The results of amplification with specific primers were validated with Sanger sequencing, for which an end-point PCR was performed using the pan-fungal primers ITS 1**(forward: TCCGTAGGTGAACCTGCGG)** and ITS 4**(reverse: TCTTTTCCTCCGCTTATTGATATG)** to amplify and then sequence the ITS1-ITS4 region of ribosomal RNA gene 28S, as described by White *et al*.6

These primers amplified a 536 bp fragment from a fungal ribosomal DNA region (Fig. 1). The PCR cycles were performed in a MiniCyclerTM, MJ Research INC thermal cycler, under the following protocol: one 5-minute cycle at 95º, followed by 30 3-stage cycles (20 seconds at 95º C//15 seconds at 55º C// 65 seconds at 72º C), and finally, one 5-minute cycle at 72º C.

The amplified fragments were purified using a commercial QIAquick PCR purification Kit (Qiagen), and sequenced using an ABI Prism 3730xl DNA analyzer (AppliedBiosystems, BsAs-Argentina) with the primers ITS1 and ITS4.

Sequence identification and phylogenetic analysis

The sequences obtained were analyzed with the algorithm for sequence comparison BLAST (Basic Local AlignmentSearchTool)/(http://www.ncbi.nlm.nih.gov/BLAST). To choose the best alignment between query and target sequence, we considered: a) % of identity; b) positive %; c) query coverage; d) and e-value.

For phylogenetic analysis we used the **BIOEDIT** software for editing sequence alignment and the **MEGA 6** software for multiple alignment of sequences and phylogenetic analysis, for which the Neighborjoining algorithm was used. The 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 specific 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 specific primers CPAR-CPAF, providing 379 bp amplicons (Figs. 2, 3, 5). This band pattern is compatible to the one published by Asadzadeh *et al*. in the Journal of Medical of Microbiology in 200914. The 5 remaining strains were negative for all three species of the *parapsilosis* complex, so they could only be identified by sequencing followed by bioinformatic analysis.

Table 2 Distribution of frequencies for the species in the *parapsilosis* complex in the collection of clinical isolates.

Species	AF	RF	PF	CI95%
C. parapsilosis	96	0.95	95%	94.9-95%
C. orthopsilosis	0	0	0	0
C. metapsilosis	0	0	0	0
Others	5	0.05	5%	4.6-5.4%
Total	**101**	**1**	**100%**	

Note: **AF** (absolute frequency); **RF** (relative frequency); **PF** (percentage frequency); **CI95** (95% confidence interval). **Others:** *Candida* species different from the *parapsilosis* complex.

DNA sequencing study in the ITS region

Of the 96 strains identified by PCR as *C. parapsilosis*, 3 strains were selected at random to validate by sequencing. The 5 strains that were negative for the 3 species of the *C. parapsilosis* complex were also sequenced to enable their identification. The alignment determined 100% identical sequence for *C. parapsilosis* sensu stricto for all 3 strains (Cp1; Cp17; Cp10.2) selected at random, and which were positive for PCR for said species. Phylogenetic analysis using MEGA 6 software confirmed the result obtained in BLAST, showing that the unknown strains and reference strain ATCC 22019 were 100% identical, with *C. orthopsilosis* and *C. metapsilosis* being more closely related to each other than they were to *C. parapsilosis* (Fig.1).

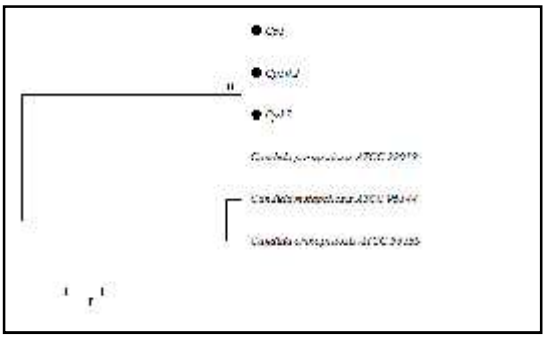

Fig. 1 Phylogram constructed with reference strains and 3 unknown strains.

The BLAST analysis of the 5 strains that were negative according to PCR for the three strains in the complex determined the following identification:
Cp36A: 100% identical to *C. albicans*
Cp53.1: 100% identical to *C. albicans*
Cp78/2.2: 100% identical to *C. pararugosa*
Cp78PA: 100% identical to *Kluyveromyces marxianus*
Cp381: 100% identical to *C. albicans*

Prevalence and distribution of species in the parapsilosis complex according to ecological niche

Oral cavity

Thirty-eight isolates were successfully recovered from oral cavity at the following sites: tongue, cheek, palate, subgingival

mucosa and peri-implant mucosa. Of the total *C. parapsilosis* strains isolated from mouth, 34 (89.5%) were positive for the species *C. parapsilosis* sensu stricto (Fig.9), while 4 were negative for the three species in the complex.

Fig 2 Electrophoretic run for PCR products from 17 samples from different sites in the oral cavity, showing a 379 bp band pattern.

Note: Mp: weight marker; C+: positive control; C-: negative control.

Oral niche: out of the 34 positive strains for the sensu stricto species, 24 were from different sites in the oral mucosa (tongue, cheek mucosa, palate, sulcus bottom) and 10 were from the gingival sulcus, with statistically significant difference (Table 3).

Table 3 Probability per ecological niche

Type of oral niche	*C. parapsilosis* sensu strictoN (%)
Oral mucosa	24 (70.6)
Gingival sulcus	10 (29.4)
Total	34 (100)

Fisher's test: 0.0007136 (p: <0.05)
PR=2.4 (CI95%: 1.7-3.1)

Patient's oral status at the time the sample was taken (based on data provided by the clinical record): of the 34 strains which were positive for *C. parapsilosis* sensu stricto, 27 were isolated under pathological conditions corresponding to clinical forms of gingivitis and periodontitis, and 7 (7/34) under conditions of oral health, with statistically significant difference (Table 4).

Table 4 Probability per oral clinical condition

Oral condition	C. *parapsilosis* sensu strictoN(%)
With SF	27 (79.4)
Without SF	7 (20.6)
Total	34(100)

Note: SF: septic foci.
Fisher's test: 0.000001083 (p: <0.05)
PR: 3.9 (IC95%: 3.1 – 4.7)

Use of intraoral appliances: out of the 34 strains characterized as *C. parapsilosis* sensu stricto, 25 were from patients with some kind of intraoral device (orthodontics, removable/fixed partial/full prosthesis) and 9 were isolated from patients without intraoral appliances. The difference was statistically significant (Table 5).

Table 5 Probability according to presence/absence of intraoral appliances

Intraoral appliances	C. *parapsilosis* sensu strictoN(%)
With	25 (73.5)
Without	9 (26.5)
Total	34(100)

Note: AI: intraoral appliance
Fisher's test: 0.000109359 (p: <0.05)
PR: 2.8 (IC95%: 2 – 3.6)

All (100%) isolates from oral cavity were obtained from immunocompetent patients.

Other niches

Blood: 25 (25/101) isolates from patients with candidemias were analyzed, of which 24 (96%) were positive for the species *C. parapsilosis* sensu stricto. Only one isolate was negative for the three species in the complex, which was identified by sequencing as *C. albicans* (Fig. 10).

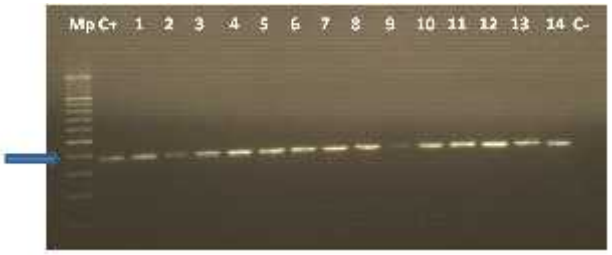

Fig. 3 Electrophoretic run of the PCR products from blood samples, showings a 379 bp band pattern.

Note: Mp: weight marker; C+: positive control; C-: negative control.

Out of the total isolates from blood characterized as *C. parapsilosis* sensu stricto, 5 (20.8%) were from immunocompetent subjects and 19 (79.2%) from immunocompromised patients (Fig. 4).

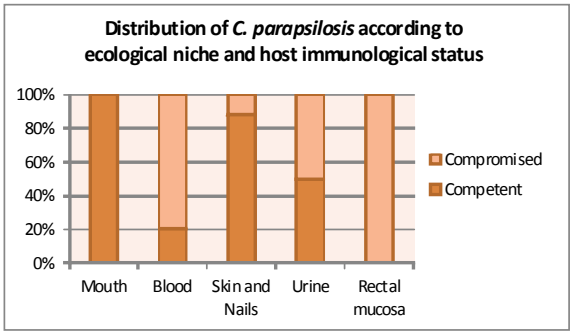

Fig. 4 Proportion of *C. parapsilosis* isolates according to host immunological status per ecological niche.

Skin and nails: 35 isolates were analyzed, of which 100% were identified as *C. parapsilosis* sensu stricto, with 31 (88.6%) coming from immunocompetent patients (Figs.4, 5).
Urine: two isolates were retrieved from urine, and confirmed as *C. parapsilosis* sensu stricto, one of which was from an immunocompetent and the other from an inmmunocompromised subject (Fig. 4).
Rectal mucosa: only one isolate was retrieved from rectal mucosa. It was identified as C. *parapsilosis* sensu stricto. It came from an immunocompromised patient (Fig. 4).

Fig. 5 Electrophoretic run of PCR product from skin/nail samples

Note: Mp: weight marker; C+: positive control; C-: negative control.

Host immunological status at the time when the isolate was obtained: *C. parapsilosis* was found to have a higher probability of being retrieved from immunocompetent than immunocompromised patients, with statistically significant difference (Table 6).

Table 6 Bivariate distribution of the *parapsilosis* complex according to host immunological status

Immunological status	*C. parapsilosis* sensu stricto N(%)
Competent	71(74)
Compromised	25(26)
Total	96(100)

p=0.000
PR= 2.84 (CI95%=2.34-3.34)

Antifungal susceptibility profile

Fifty of the 101 strains characterized as *C. parapsilosis* sensu stricto were selected to test for susceptibility to six antifungal agents which are regularly used in clinical practice: FLC, VRC, CASPO, MICA, AMB and FLUCY (Table 7).

According to the species-specific cutoff point proposed by the CLSI Subcommittee in 2012, only 3 (6%) of the 50 strains showed a phenotype resistant to fluconazole, two of which were resistant to both FLC and CASPO. Five strains showed dose-dependent susceptibility to FLC (Table 7). It was not possible to classify the strains regarding amphotericin B because there is no validated clinical cutoff point. However, considering the epidemiological cutoff value (ECV) proposed for AMB and the species *parapsilosis*, 100% of the strains were wild-type phenotype (Fig. 6). Since there is no validated CBP for flucytosine in this *Candida* species, the response of the 50 strains to it would be considered as undetermined (ND) (Table 7). However, considering the ECV, 100% of the strains were non wild-type to flucytosine, i.e. strains which have developed resistance mechanism to flucytosine (Fig. 6).

Table 7 Antifungal susceptibility profile of 50 strains of *C. parapsilosis* sensu stricto

Drug	S N(%)	IS N(%)	DDS N(%)	R N(%)	WT N(%)	noWT N(%)
FLC	42(84)	-	5(10)	3(6)	42(84)	8(16)
VRC	49(98)	1(2)	-	-	49(98)	1(2)
CASPO	48(96)	-	-	2(4)	48(96)	2(4)
MICA	50(100)	-	-	-	50(100)	0
AMB	ND	ND	ND	ND	50(100)	0
FLUCY	ND	ND	ND	ND	0	50(100)

Note: S: sensitive; IS: intermediate sensitivity; DDS: dose-dependent sensitivity; R: resistant; WT: wild-type strain; noWT: non wild-type strain.

Table 11 shows the minimum inhibitory concentration (MIC) needed to inhibit 50 and 90% of the strains, and the MIC range for each drug tested. The 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-specific cutoff point. However, according to the epidemiological cutoff point (Pfaller and Diekema 2012)[15], all 50 strains were sensitive to both AMB and FLUCY.

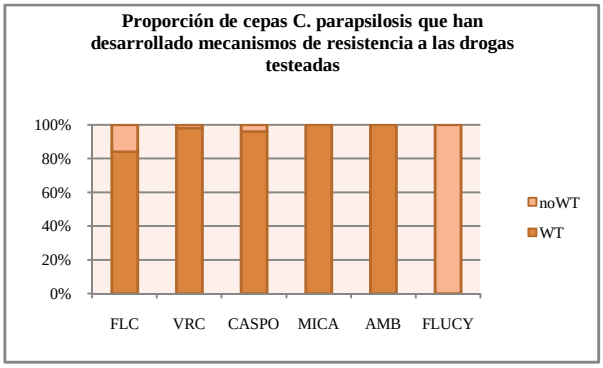

Fig.6 The antifungal agents showing least activity against the strains assessed were FLUCY and FLC.
Proportion of *C. parapsilosis* strains that have developed resistance mechanisms against the drugs tested

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 (Fig. 7).

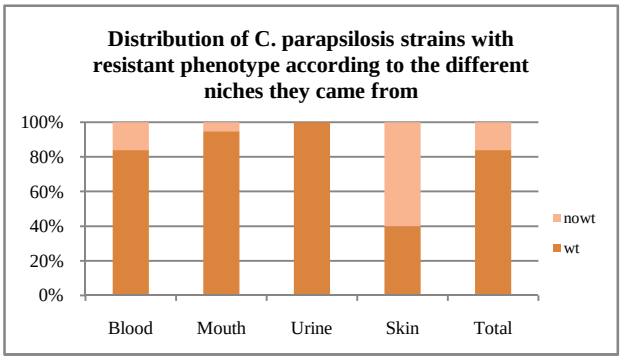

Fig. 7 The distribution suggests that the probability of retrieving *C. parapsilosis* strains with resistant phenotype is greater from the skin and blood niches.

For oral cavity, the response to antifungal agents of 19 isolates was assessed, of which only one strain from tongue had high MIC values for FLC, VRC and CASPO (Table 8). In this particular case, the MIC for FLC corresponded to dose-dependent sensitivity, while the MIC for VRC and CASPO corresponded to intermediate sensitivity to the drug. According to the epidemiological cutoff value (ECV) proposed in 2012 by the CLSI Subcommittee[15], this tongue-derived strain corresponds to a non wild-type strain (No-WT) which has developed resistance mechanisms (Table 8). The frequency of resistance and/or reduced susceptibility to the antifungal agents tested in this specific ecological niche was 5.3% (Fig. 7)

Table 8 Response profile to FLC, VRC and CASPO for a strain from tongue which showed reduced susceptibility.

DRUG	MIC (ug/ml)	CBPs			ECV	
		S	DDS/IS	R	WT	NoWT
FLC	4		x			X
VRC	0.25		x			X
CASPO	4		x			X

DISCUSSION

Many studies around the world report that within the complex, *C. parapsilosis* sensu stricto is the species most frequently recovered from clinical isolates from immunocompetent and immunocompromised patients, in both pathological and

commensal conditions[6,8,5,7,10,16,17]. 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[9], Portugal[5], Turkey[10] and China[6]. One study from Brazil investigated the distribution of the species in this complex within the oral cavity of patients with chronic immunodeficiency due to HIV, in whom *C. metapsilosis* was the most frequently isolated species, followed by *C. parapsilosis* sensu stricto, although the difference was not statistically significant and the sample was very small[18].

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, in both immunocompetent and immunocompromised subjects. This result is similar to those reported by other authors such as Lotfali E, *et al*. (2016)[19] and Tosun *et al*. (2012)[10], 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).

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 as *C. tropicalis*, *C. dubliniensis* and *C. glabrata* is greater in subjects with periodontal disease[28,30], poorly controlled diabetics[31]; female subjects who use oral contraceptives[32]; and male subjects who use androgenic steroids[33]. Based on our review of this database, we can say that ours is the first 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. This result is consistent with Constante *et al*.[36], 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.

Table 9 Distribution of species from the *Candida parapsilosis* complex, summarized from published papers

Ecological niche	*C. parapsilosis*	*C. orthopsilosis*	*C. metapsilosis*	Study country	Reference
Various clinical samples	18 (90.0%)	0	2 (10%)	Hungary	20
Hemocultures	60 (95%)	1(2%)	2(3%)	Italy	21
Hemocultures	67 (85.9%)	5 (6.4%)	6 (7.7%)	Spain	22
Various clinical samples	111 (91.0%)	10 (8.2%)	1 (0.8%)	Spain	23
Invasive clinical samples	1762 (92.1%)	117 (6.1)	34 (1.8%)	Global	7
Hemocultures	29 (70.7%)	10 (24.4%)	2 (4.9%)	Malaysia	24
Various clinical samples	109 (95.6%)	5 (4.4%)	0	Kuwait	14
Varias muestras clínicas	160 (94.6%)	4 (2.4%)	5 (2.9%)	Portugal	5
Hemocultures	126 (88.1%)	13 (9%)	4 (2.8%)	Brazil	16
Hemocultures	75 (95.0%)	2 (2.5%)	2 (2.5%)	Denmark	25
Invasive clinical samples	112 (53.3%)	30 (14.3%)	56 (26.7%)	China	26
Various clinical samples	81 (83.5%)	7 (7.2%)	9 (9.3%)	Taiwan	27
Various clinical samples	41 (71.9%)	0	16 (28.1%)	Eastern China	6
Various clinical samples	96 (100%)	-	-	Argentina	This study

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[28], which reports a lower prevalence of yeasts, as reflected by a lower count of colony-forming units (CFU/ml) of *Candida* species, among periodontally healthy subjects than among periodontally affected subjects, with statistically significant difference. We also found that *C. parapsilosis* is more often recovered in presence of prosthetic or orthodontic devices. This is consistent with Jewtuchowicz V, *et al*. (2007),[29] 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. This is in agreement with Urzúa B *et al*.[28], 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[28]. It should be highlighted that there is no published paper specifically studying the *parapsilosis* complex

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.

Table 10 Distribution of *C. parapsilosis* complex species in oral cavity niches, summarized from published studies

Author	*C. parapsilosis* N (%)	*C. orthopsilosis* N (%)	*C. metapsilosis* N(%)	Study country	Reference
Ghannoum *et al.*	3(75)	0	1(25)	USA (2010)	9
Ge *et al.*	2(66.7)	0	1(33.3)	China (2012)	6
Moris *et al.*	7(46.7)	0	8(53.3)	Brazil (2014)	18
Enger *et al.*	9(64.3)	5(35.7)	0	Global (2001)	34
Silva *et al.*	65(94.2)	-	4(5.8)	Portugal (2009)	5
Tosun *et al.*	2(100)	0	0	Turkey (2012)	10
This study	34(100)	0	0	Argentina	35

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), fluconazole (FLC), itraconazole (ITC) and caspofungin (CASPO). Among the azole-derived agents analyzed in our study, FLC was the least active, with 8 *C. parapsilosis* sensu stricto strains showing resistance and/or reduced susceptibility

to it. In agreement with this, Silva *et al.*[4] 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.*[38] In contrast, Ataides *et al.*[16] and Ruiz *et al.*[38] report resistance in a *C. parapsilosis* sensu stricto isolate to ITC but not to FLC. In this regard, Van Asbeck *et al.*[17] suggest that the differences in susceptibility to FLC may also reflect different affinities for azoles in the key enzyme that synthesizes ergosterol, 14-α-demethylase or for other enzymes in this pathway. Interestingly, MIC_{50} and MIC_{90} for both azoles (Table 11) found for the 50 study strains are comparatively higher than values reported for other regions such as Turkey (Tosun, *et al.*)[10] and Spain (Miranda, *et al.*)[8]. This demonstrates geographic variability regarding the way in which species in this complex respond to antifungal drugs.

Echinocandins: two isolates were resistant to CASPO in our study. This 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[39,40]. In contrast to the results with CASPO; all 50 strains were sensitive to MICA, and the MIC_{50} and MIC_{90} for both echinocandins (Table 16) was comparatively lower than reported in other parts of the world such as Turkey (Tosun, *et al.*)[10] and Spain (Miranda, *et al.*)[8].

Table 11 MIC_{50} and MIC_{90} values for each drug tested for the 50 study strains.

DRUG	MIC_{50} (ug/ml)	MIC_{90} (ug/ml)	MIC RANGE (ug/ml)	Resistance Frequency
FLC	<=1	4	<=1-64	16%
VRC	<=0.12	<=0.12	<=0.12-<=0.25	2%
CASPO	0.5	1	0.5->=4	4%
MICA	0.5	1	0.5-1	0%
AMB	<=0.25	0.5	0.25-0.5	ND
FLUCY	<=1	<=1	<=1	ND

Note: ND=undetermined.

Amphotericin B: Based on ECV, all strains were susceptible to AMB, in agreement with other studies[19,20]. However, Ataides *et al.*[16] and Lockhart *et al.*[7], reported resistance of the species *C. parapsilosis* sensu stricto to AMB. The response of the *parapsilosis* complex species to AMB varies considerably among regions, which may be determined by intra-species genotype variability.

Flucytosine: 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 cutoff point for FLUCY for the species *Candida parapsilosis*. Nevertheless, considering the ECV classification proposed by Pfaller and Diekema in 2012[15], 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 0.5ug/ml corresponds to a wild-type (WT) strain, while an MIC value greater than 0.5ug/ml indicates a Non-WT strain which has developed resistance mechanisms. However, the British Society for Mycopathology[21] establishes as flucytosine-"sensitive" isolates those which have an MIC to flucytosine equal to or less than 1ug/ml. Our study found an MIC value for flucytosine for the 50 strains which is higher than those reported by Silva *et al.*[5], Miranda *et al.*[8] and Cantón *et al.*[42] in Europe, while in India, Bhatt M *et al.*[43] found a high resistance rate to flucytosine in *C. parapsilosis* isolates.

Due to the fact that we did not recover *C. orthopsilosis* or *C. metapsilosis*, we were unable to establish differences in the response profiles among the 3 species in this complex.

CONCLUSIONS

- In Argentina, *C. parapsilosis* sensu stricto may be the most prevalent species in the complex in different 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 different human ecological niches, under both pathological and commensal conditions.
- Subgingival sites in the oral cavity may constitute an unfavorable microenvironment for colonization and development by *C. parapsilosis*.
- The 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 *C. parapsilsois* strains with resistant phenotype and/or reduced susceptibility to the group of azoles, echinocandins and flucytosine.

Recommendations

- We recommend repeating this study design but in a prospective model and with a larger sample size to minimize the random error inherent to the process.
- Evaluate sensitivity and specificity 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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