Original Research Article

Molecular Characterisation of Isolates Belonging to *Candida Parapsilosis* Species Complex and Determination of Their Antifungal Resistance Profiles

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Abstract

Introduction: This study aimed to differentiate among the species belonging to the Candida parapsilosis complex, which is one of the leading causes of systemic mycoses through molecular characterisation and to determine their antifungal resistance pattern. ***Material and Methods:*** Ninety-five C. parapsilosis complex isolates identified using two yeast identification systems, BioMérieux ID 32C and Vitek 2™ with YST card, were included in this study. Molecular characterisation was achieved by polymerase chain reaction–restriction fragment length polymorphism analysis of the secondary alcohol dehydrogenase gene. Isolates that could not be identified using this technique were identified by internal transcribed spacer (ITS) sequencing. Antifungal susceptibility was evaluated using the Vitek 2™ AST-YS06 card. ***Results:*** Out of the 95 isolates, 94 were identified as C. parapsilosis sensu stricto, and only one isolate was identified as C. orthopsilosis; the latter result was achieved using ITS sequencing. C. orthopsilosis was susceptible against all tested antifungal agents. Among the C. parapsilosis sensu stricto isolates, three were found to be resistant: one to amphotericin B, two to fluconazole of which one showed intermediate resistance to voriconazole. ***Discussion:*** Isolation rates of C. orthopsilosis and C. metapsilosis in this study show that they are rare species. It was not possible to compare resistance among these three species due to very low isolation rates of C. orthopsilosis and C. metapsilosis. The use of molecular identification tests for these species in routine laboratory settings remains controversial. Nevertheless, their isolation rates and antifungal resistance patterns are important from the epidemiological viewpoint.
Keywords: *Candida parapsilosis*; *Candida orthopsilosis*; *Candida metapsilosis*; restriction fragment length polymorphism; antifungal susceptibility.

INTRODUCTION

The number of opportunistic fungal organisms has been increasing each year, but *Candida* still remains the most common disease-causing genus. Among the *Candida* species, non-albicans *Candida* species have increased in number. Worldwide, *Candida parapsilosis* complex has become the most commonly isolated non-albicans species from blood cultures [1-4]. It is not a coincidence that the isolation rate of *C. parapsilosis* complex has increased. This commensal species can easily spread in hospital environments when necessary infection control measures are not taken. It can multiply in total parenteral nutrition formulas as well as form biofilms on catheters and other invasive equipment; therefore, it can easily infect susceptible patients [1].

Even though the genetic diversity of *C. parapsilosis* had been known before, until 2005, it was characterised as a species complex which included three subspecies that could not be differentiated using phenotypic methods. In 2005, Tavanti et al. demonstrated that these three subspecies are sufficiently dissimilar to be named as three different species: *C. parapsilosis* sensu stricto, *C. metapsilosis* and *C. orthopsilosis* [5]. Since then, many studies have investigated if there are any differences in the virulence and antifungal susceptibility patterns among these new species [6, 7].

The aim of this study is to estimate the distribution rates of species belonging to the *C. parapsilosis* complex prevalent in Turkey and to determine the probable differences in their antifungal

susceptibility patterns, which can affect treatment. This study provides new epidemiological information which can be useful in a prophylactic and empirical antifungal treatment guide.

Experimental Section
Selection of Strains

This study includes 95 isolates of C. *parapsilosis* complex identified using the two yeast identification systems, BioMérieux ID 32C and Vitek 2™ with YST card. These isolates were found by screening various clinical samples obtained between the years 2009 and 2015 (Table-1). Strains were stored at −80°C in the brain heart infusion broth containing 3% glycerol. They were subcultured onto Sabouraud dextrose agar (SDA) and CHROMagar™ *Candida* agar plates and incubated at 37°C for 24 h.

Table-1: Distribution of sample types from which *C. parapsilosis* complex strains were isolated

Sample Type	*C. parapsilosis* complex (n)
Blood	33
Urine	19
Sputum	17
Oral swab	8
Abscess	4
Conjunctival swab	3
Tracheal Aspirate	2
Skin and Nail	2
Catheters	2
Rectal swab	2
Deep throat swab	1
Peritoneal fluid	1
Unknown	1

C. parapsilosis ATCC 22019, C. *metapsilosis* ATCC 96144, *C. orthopsilosis* ATCC 96139, *C. metapsilosis* CECT 13010 and *C. orthopsilosis* CECT 13011 were included in the study as reference strains.

Polymerase Chain Reaction (PCR)

The single-colony PCR method was chosen due to its ease of application and low cost [8]. In this method, a single yeast colony can be added directly into the amplification mix of PCR.

The secondary alcohol dehydrogenase (*SADH*) gene region, which is shared by the three subspecies of the *C. parapsilosis* complex, was amplified by PCR. The following forward and reverse primers, respectively, as defined by Tavanti *et al.*, were used: 5-GTTGATGCTGTTGGATTGT-3 and 5-CAATGCCAAATCTCCCAA-3 [5]. The amplification mix is represented in the Table-2. The amplification steps in the Biorad T100 thermal cycler were as follows: one denaturation cycle for 4 min at 95°C followed by 40 cycles of denaturation at 95°C for 0.5 min, annealing at 50°C for 1 min, elongation at 72°C for 1.5 min and a final extension step of 5 min at 72°C.

Table-2: Amplification mix

Component	Amount
Taq DNA Polymerase (5 U/μL)	0.125 μL
10X buffer solution $(NH_4)_2SO_4$ (within the polymerase kit)	2.5 μL
25 mM $MgCl_2$	1.5 μL
10 mM dNTP	0.5 μL
25 μM forward primer	0.5 μL
25 μM reverse primer	0.5 μL
Template DNA (yeast suspension)	2 μL
Water	17.375 μL
Total	25 μL

Restriction and Electrophoresis

A PCR product measuring 716 base pairs (bp) in length was identified electrophoretically using an agarose gel concentration of 1.7%. For restriction enzyme digestion, a mixture consisting of 5 μL PCR product, 8 μL water, 0.5 μL BanI restriction enzyme (New England Biolabs) and 1.5 μL buffer solution (provided with the enzyme kit) was incubated at 37°C for 1 h. Restriction products were separated from each other electrophoretically in a 1.7% agarose gel. C. *parapsilosis* sensu stricto yielded two pieces measuring 521 bp and 196 bp, while C. *metapsilosis* yielded four pieces measuring 370 bp, 188 bp, 93 bp and 60 bp. C. *orthopsilosis* did not split at all.

Antifungal Susceptibility Testing

Antifungal susceptibility testing was performed using the Vitek 2™ AST-YS06

(BioMérieux) card according to the manufacturer recommendations. MIC ranges of the antifungal agents present in the Vitek 2™ AST-06 card are shown in Table-3. Results were evaluated after incubation for 24 h at 35°C. Antifungal susceptibility results were separately evaluated using EUCAST and CLSI criteria.

Table-3: Vitek 2™ AST-YS06 card content

Antifungal agent	MIC interval*	
	S	R
	≤	≥
Amphotericin B	0.25	16
Caspofungin	0.25	4
Fluconazole	1	64
Flucytosine	1	64
Voriconazole	0.12	8

* All values in µg/mL

Sequencing and Phylogenetic Tree

One strain could not be identified using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. Internal transcribed spacer (ITS) regions of these strains were amplified using PCR as specified in the literature [9]. PCR product was purified using the High Pure PCR Product Purification kit (Roche, Mannheim, Germany). After the purification step, bidirectional DNA sequence analysis was performed using the dye terminator method by employing the Applied Biosystems Big Dye v1.1 (Applied Biosystems, Foster City, USA) kit. Acquired data was evaluated using Applied Biosystems Sequencing Analysis v5.3.1 software.

RESULTS

Identification results of C. parapsilosis complex strains

Only one strain was identified as *C. orthopsilosis* by sequencing because PCR–RFLP experiment failed to identify it, while the rest of the 94 strains were identified as *C. parapsilosis* sensu stricto.

Antifungal susceptibility results of C. parapsilosis complex strains

All of the strains were susceptible to caspofungin according to CLSI M60 breakpoints. In EUCAST version 9.0, interpretation of caspofungin was combined with interpretations of micafungin and anidulafungin. Because Vitek 2™ AST-06 card lacks these antifungal agents, it was not possible to make an interpretation for caspofungin according to EUCAST criteria. Because there were no established breakpoints for flucytosine in CLSI M60 and EUCAST version 9.0 antifungal breakpoint tables, it was not possible to make an interpretation.

Two strains were resistant against fluconazole according to both EUCAST version 9.0 and CLSI M60 criteria. One of them showed intermediate resistance against voriconazole. One strain was found to be resistant against amphotericin B according to EUCAST version 9.0 criteria. CLSI antifungal susceptibility criteria did not include any breakpoints for amphotericin B; therefore, it was not possible to make an interpretation. MIC values and sample types of resistant strains can be seen in Table-4.

Table-4: MIC values in µg/mL and sample types of resistant strains

Strain number	Sample Type	Amphotericin B (µg/mL)	Caspofungin (µg/mL)	Fluconazole (µg/mL)	Flucytosine (µg/mL)	Voriconazole (µg/mL)
8	Blood	**4**	0.5	2	≤1	≤0.12
69	Blood	≤0.25	0.5	**32**	≤1	**0.25**
70	Urine	≤0.25	0.5	**8**	≤1	≤0.12

The *C. orthopsilosis* strain was found to be susceptible to all the antifungal agents tested. The MIC values of amphotericin B, caspofungin, fluconazole, flucytosine and voriconazole were measured as ≤0.25 µg/mL, ≤0.25 µg/mL, ≤1 µg/mL, ≤1 µg/mL and ≤0.12 µg/mL, respectively.

Sequencing Results

The strain number 2, whose PCR result was unclear, was identified as C. orthopsilosis. Using the basic local alignment search tool (BLAST), this strain number 2 was recorded to have a 100% match with one strain and 99% match with many strains.

DISCUSSION

Importance of the correct identification at species level has increased as the rate of infections caused by yeasts other than *C. albicans* increased. Species-level identification gives preliminary information about antifungal susceptibility which makes the need for accurate identification crucial. Identification and distribution of *C. parapsilosis*

complex species were performed using different methods. Isolation rates of *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis* range between 71%–100%, 0%–26% and 0%–28%, respectively, in different studies [5, 10, 11]. Lockhart *et al*., determined the species distribution of 1929 clinical *C. parapsilosis* complex isolates collected worldwide and found that 92% of them were *C. parapsilosis* sensu stricto, 6% were *C. orthopsilosis* and 2% were *C. metapsilosis* [7]. Two studies from Turkey found similar rates with *C. parapsilosis* sensu stricto being the most common species having an isolation rate of >90% [12, 13].

PCR–RFLP was the chosen method for the present study because it is a simple, inexpensive and relatively rapid method. This method like all other similar methods has certain limitations. Optimisation of conventional PCR requires considerable time and manual work during the experiment, which increases the possibility of errors. This method has lower sensitivity than the sequencing technique. In the present study, it was not possible to obtain the PCR product of

a strain which was identified as *C. orthopsilosis* using ITS sequencing. *C. orthopsilosis* is known to be genetically diverse, thereby having polymorphisms at target locations [14, 15]. It is possible that primers were not able to bind to their target and thus generate a product.

Antifungal susceptibility experiments on *C. parapsilosis* complex were focused more toward echinocandin-group antifungal agents. The reason for this is that wild-type *C. parapsilosis* complex species have reduced susceptibility to echinocandins [16, 17]. In our study, all of the strains were susceptible to caspofungin.

Another important class of antifungal agents used in the treatment of *C. parapsilosis* infections is triazoles. Voriconazole and posaconazole are effective against all the three subspecies of the complex [18]. Results of fluconazole are more equivocal. Several studies have reported that *C. orthopsilosis* and *C. metapsilosis* have higher fluconazole MIC values than *C. parapsilosis* sensu stricto and may display resistance against this antifungal [2, 8, 19, 20]. In our study, two isolates were resistant against fluconazole and one of them also showed resistance against voriconazole.

In studies which investigated the susceptibility of *C. parapsilosis* complex to amphotericin B, MIC values of *C. parapsilosis* sensu stricto strains were found to be higher than those of *C. orthopsilosis* and *C. metapsilosis* strains, and some studies have reported the resistance among *C. parapsilosis* sensu stricto strains to this drug [7, 18-21]. In our study, only one isolate showed resistance against amphotericin B.

The chosen antifungal susceptibility method for this study was the use of Vitek 2™ AST-YS06 (BioMérieux) cards. Vitek 2™ system was preferred because it is both rapid and accurate with high reproducibility. MIC is determined objectively in this method which is another advantage. Studies comparing this method with broth microdilution reference method for *C. parapsilosis* complex isolates showed very high rates of essential and categorical agreement and concluded that this method can be used as an alternative to the broth microdilution method [22-26].

CONCLUSION

Separate identification of C. parapsilosis complex species using molecular methods is not suitable in routine laboratory settings. One contributing reason is that the combined isolation rates of C. orthopsilosis and C. metapsilosis are <10% on an average. Another more important reason is that the difference in antifungal susceptibility of C. orthopsilosis and C. metapsilosis strains compared with C. parapsilosis sensu stricto strain remains controversial. Nonetheless, isolation rates and antifungal susceptibility results of these three species should be closed monitored for determining changes in epidemiological patterns.

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