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Molecular Identification and Antifungal Susceptibility of *Candida parapsilosis* sensu stricto, *Candida orthopsilosis*, and *Candida metapsilosis* in Sousse Region, Tunisia

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Abstract

Candida parapsilosis was recently reclassified into three distinct species, *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*. The aim of this study was to assess the prevalence and the antifungal susceptibility of these species among clinical isolates previously identified by phenotypical methods as *C. parapsilosis* in the laboratory of parasitology of Farhat Hached Hospital (Sousse, Tunisia). Ninety-six clinical isolates were identified by using a multiplex PCR targeting the gene encoding the ribosomal protein SO 40 (RPSO) and a PCR targeting the ITS region. Ninety one (94.8%) isolates were identified as *C. parapsilosis* sensu stricto, 3 (3.1%) as *C. metapsilosis*, and 2 (2.1%) as *C. orthopsilosis* based on PCR-ITS. These results were concordant with those achieved by Multiplex PCR-RPSO but the prevalence of *C. orthopsilosis* and *C. metapsilosis* seems to be very low in the region. Seventeen of the Candida isolates, *C. parapsilosis* sensu stricto (12), *C. metapsilosis* (3) and *C. orthopsilosis* (2) were assayed by ATB Fungus 3® to assess their susceptibility to Flucytosine, Amphotericin B, Fluconazole, Itraconazole and Voriconazole and by E-test® to assess their susceptibility to Caspofungin. All the isolates were susceptible to the antifungals tested.

Keywords: *Candida parapsilosis*; *Candida metapsilosis*; Molecular identification; Antifungal susceptibility; Tunisia

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Introduction

Candida parapsilosis is an emergent pathogen and has become the second or the third most common *Candida* species causing invasive and superficial candidiasis [1,2]. The molecular investigations of *C. parapsilosis* have led to the sequencing of its full genome, which showed high heterogeneity between strains otherwise phenotypically indistinguishable [3,4]. As a result, it was established that *C. parapsilosis* is a complex of three genetically distinct groups: group I, group II and group III [5,6]. Detailed molecular analyses have however, recently recognized *C. parapsilosis* groups as separate species named *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* respectively [7]. The differentiation of the three species of *C. parapsilosis* complex is clinically relevant because of their virulence and their susceptibility to antifungal agents. On the other hand, the prevalence of the species varies according to the regions [8-12]. A study conducted between 1995 and 2009 at the Farhat Hached teaching Hospital of Sousse, east-central Tunisia, revealed that *C. parapsilosis* sensu lato was the second most-frequent Candida species isolated from patients with candidemia, and that its incidence has dramatically increased over the study period [13]. The aim of the present study was to assess the frequency of each of the three species of *C. parapsilosis* complex in our region by using two PCR techniques: a PCR targeting the gene encoding the ribosomal protein SO 40 (RPSO) and a PCR targeting the ITS region, followed by the sequencing of the PCR product [14,15]. Additionally, the susceptibility of clinical isolates to antifungals by using ATB Fungus 3® test and E-test® was evaluated.

Materials and Methods

Strains: A total of 96 Candida strains were isolated from patients admitted to different care units of Farhat Hached Hospital of Sousse, Tunisia, or from outdoor patients referred to the laboratory

of parasitology-mycology of the same hospital between 2007 and 2013. Clinical sources of the 96 isolates are as listed in **Table 1**. All isolates were identified initially as *C. parapsilosis* sensu lato on the basis of morphological and biochemical criteria. Reference strains ATCC22019 and CECT13009 were used as controls for *C. parapsilosis*, while reference strains CECT13011 and CECT13010 were used as controls for *C. orthopsilosis* and *C. metapsilosis*, respectively. Isolates were kept at -80°C for long-term storage and prior to DNA extraction, isolates were grown in YPD (yeast peptone dextrose) medium at 35°C.

DNA extraction: Yeast DNA was extracted as described by Tavanti et al. (2007) [16]. Briefly, yeasts grown in YPD medium at 35°C for 24 hours were harvested by centrifugation at 1500 rpm for 5 minutes, and lysed by vortexing for 5 minutes with 0.3 gr of glass beads (diameter 0.45 mm to 0.52 mm; Sigma-Aldrich, USA), 200 µl of lysis buffer (100 mmol/L Tris-Hcl, pH 8.0, 2% Triton X100 [vol/vol] and 1 mmol/L EDTA) and 200 µl of 1:1 (vol/vol) phenol-chloroform. After vortexing for 3 minutes, 200 µl of the extraction buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) were added to the lysate and the mixture centrifuged at 5000 rpm for 10 minutes. The aqueous phase was afterwards transferred to a new vial and the DNA precipitated by adding 1 ml of ethanol. After a 10 minutes centrifugation at 14000 rpm, the pellet was harvested and resuspended in 400 µl of extraction buffer with 5 µl of proteinase K (20 mg/ml) (Sigma-Aldirish, USA). The mixture was then incubated for 1 hour at 65°C and for 30 minutes at 72°C, and the DNA precipitated by the addition of 1 ml of isopropanol and 10 µl of 4 mol/L Ammonium acetate. After a 10 minutes centrifugation at 14000 rpm, the harvested DNA pellet was dried and redissolved in 50 µl of extraction buffer and cryopreserved at -20°C until use. Prior to the amplification procedure DNA concentrations and A 260 nm/A280 nm ratios were determined by means of the automated NanoDrop® ND.1000 spectrophotometer (BioRad, France). DNA was considered pure when the ratio A260nm/A280nm was between 1.8 and 2.0 one unit of optical density at 260 nm corresponding to 50 µg/ml.

Table 1 Distribution of tested clinical strains according to the site of isolation.

Site	Number (%)
Fingers tinea unguium	48 (50)
Toes tinea unguium	9 (9.37)
Blood	8 (8.33)
External otitis	7 (7.29)
Umbilical venous catheter	6 (6.25)
Inter toes intertrigo	5 (5.20)
Nose	2 (2.08)
Vagina	2 (2.08)
Central catheter	1 (1.04)
Hair	1 (1.04)
Sputum	1 (1.04)
Interdigital intertrigo	1 (1.04)
Mouth	1 (1.04)
Bronchial aspiration	1 (1.04)
Tinea cruris	1 (1.04)
Cerebrospinal fluid	1 (1.04)
Endotracheal tube	1 (1.04)
Total	**96 (100.00)**

Identification of isolates: Phenotypic identification: Initially, isolates were identified as *C. parapsilosis* on the basis of the following criteria: (i) characterization of colonies on chromogenic CAN2 medium (bioMérieux, France), (ii) microscopic features of colonies grown on PCB medium (BioRad, France) and (iii) sugar assimilation profiles with the Auxacolor 2® (BioRad, France) and ID32C (bioMérieux, France) kits.

Molecular identification: For this purpose we used two PCR methods.

PCR-RPSO: this method was carried out as described by Del Pilar Vercher et al. [14]. It is based on the amplification of the RPSO gene intron, by using 3 primers (presented in **Table 2**) that generate amplicons of 100 bp, 150 bp and 200 bp with *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* DNA extracts respectively. In the initial application reported by Del Pilar et al., the three primers were used separately in 3 PCR runs for each isolate [14]. In our own work, the technique was optimized in a way so that the primers were used simultaneously in a single PCR multiplex run. The PCR assay was performed in a total reaction volume of 25 µl containing 2.5 µl of 5X green Go Taq flexi buffer (Promega), 0.4 µmol/L of each primer, 400 µmol/L of 10 mmol/L dNTPs Mix (Promega), 2 mmol/L of 25 mmol/L $MgCl_2$ (Promega), 1.25 U of Go Taq Hot start polymerase (Promega), 11.75 µl of nuclease free water (Promega) and 100 ng of genomic DNA.

The amplification conditions were as follows: a 3 minutes initial denaturation step at 95°C, followed by 45 cycles at 94°C for 30 s (denaturation), 63°C for 30 s (annealing), and 72°C for 54 s (extension) and a final extension step of 10 minutes at 72°C. PCR was performed in a thermal cycler Perkin Elmer GeneAmp PCR System 2400.

PCR-ITS: the PCR-ITS was performed by using the primers ITS1 (5'-CCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described by White et al. [15]. These primers amplify a 500 bp product that encompasses the entire ITS1, the 5.8s and the ITS2 gene regions. The PCR assay was carried out according to the procedure described by Pryce et al. (2006), in a total reaction volume of 50µl [17]. The PCR mixture contained 5 µl of 5x PCR buffer (5X green Go Taq flexi Buffer, Promega); 3 µl of 25 mmol/L $MgCl_2$; 3 µl of 10 µmol/L of each primer; 1 µl of 10 mmol/L of each dNTP (dNTP Mix, Promega); 2.5 U of Go Taq Hot start polymerase (Promega); and 29.5 µl of sterile distilled water (nuclease free water, Promega). The PCR was performed in a thermal cycler (Perkin Elmer GeneAmp PCR System 2400) as described by Turenne et al., with the following program: 94°C for 10 minutes (initial denaturation) followed by 94°C for 55 s (denaturation), 55°C for 60s (annealing), 72°C for 60s (extension) for 30 cycles, followed by 72°C for 10 minutes (final extension) [18].

Sequencing of the PCR-ITS products: All 96 PCR-ITS amplified products were sequenced by using the DNA Engine Tetrad 2 Peltier Thermal Cycler (BioRad, France) and the Cycle Sequencing ABI Dye Ter minutesator® kit (Applied Biosystems) according to the manufacturer's instructions. The amplified products were sequenced in a forward and reverse direction using ITS1 and ITS4 primer, respectively. The fluorescent fragments were

Table 2 Characteristics of primers used for PCR-RPSO.

Species	Primer name	Sequence	Amplicon size (bp)
C. parapsilosis	CP1	5'-AGGGATTGCCAATATGCCCA-3'	100
	CP2	5'-GTGACATTGTGTAGATCCTTG G-3'	
C. orthopsilosis	CO1	5'TTTCAATATGCCTAGAGCCACATTGTGAATC-3'	150
	CO2	5'-GCATTAGTTAGTATCGTCTTTTATTAAATA3'	
C. metapsilosis	CM1	5'-AATAGAGGAGATGTTTTATTTGAATTC-3'	200
	CM2	5'-GCAGAATCCGTAAGAACTGGGG-3'	

purified with the Big Dye X Terminator Purification Kit (Applied Biosystems), then sequenced by means of the ABI 3730xl DNA Analyzer sequencer (96 capillary type). The electrophoregrams were visualized by the Chromas Lite version 2.1.1 and the nucleotide sequences alignments performed with the (BLASTN 2.2.29+) BLAST for species identification.

Detection of PCR products: For both PCR products, the amplified products were detected by gel electrophoresis using a 2% agarose gel (UltraPure Agarose®, Invitrogen) in 0.5xTris-Acetate-EDTA buffer (UltraPure® 10X TAE buffer, Invitrogen), stained with 0.5 µg/ml ethidium bromide. The same buffer without ethidium bromide was used as electrophoresis buffer. A total volume of 5 µl of 100 bp ladder (Promega) was run in parallel with sample DNAs for PCR product band sizing. Electrophoresis conditions were 110v for 60 minutes in 0.5x TAE buffer. DNA bands were visualized by UV transillumination, photographed with the Molecular Imager® Gel Doc™ XR+ Imaging system and analyzed by using the Quantity One® software (BioRad, France).

Antifungal susceptibility testing: In order to evaluate the in vitro susceptibility profile of the clinical isolates *C. parapsilosis* complex strains to Flucytosine (Fc), Amphotericin B (Amb), Fluconazole (Flc), Itraconazole (Itc) and Voriconazole (VC) antifungals, 12 *C. parapsilosis* sensu stricto, 3 *C. metapsilosis* and 2 *C. orthopsilosis* were tested by using the ATB Fungus® kit (bioMérieux, France). The antifungals test was conducted according to the manufacturer's instructions, and reading taken after 24 hours. In addition, 17 Candida strains were tested against caspofungin (CS) by using the E-test® strips (BioMérieux, France). The test was performed by incubation of colonies of Candida isolates on agar plates containing 15 ml of casitone agar (BioRad, France). Plates were incubated at 35°C and read after 24 hours. For both ATB Fungus® and E-test®, the minimum inhibitory concentrations (CMIs) were determined for all antifungals (except FC) and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) recommendations.

Results

PCR: All tested isolates were successfully amplified using both PCR-RPSO and PCR-ITS.

Out of 96 *C. parapsilosis* clinical isolates tested with multiplex PCR-RPSO, 91 (94.8%) gave the single 100 bp band and were assigned to the most common *C. parapsilosis* species; 3 (3.1%) gave 2 bands of 100 bp and 150 bp respectively and were identified as *C. metapsilosis* and 2 (2.1%) gave 2 bands of 100 bp and 200 bp respectively, and were identified as *C. orthopsilosis*.

An example of the electrophoregram given by some clinical strains and by the reference strains is shown in **Figure 1**.

Out of the 3 *C. metapsilosis* isolates, two were collected from toes' onychomycosis and one from a venous umbilical catheter.

Both *C. orthopsilosis* isolates were collected from fingers' onychomycosis. *C. parapsilosis* was isolated from all types of sites and/or lesions, namely: fingers tinea unguium (46), blood (8), toes tinea unguium (7), external otitis (7), umbilical venous catheter (5), inter toes intertrigo (5), nose (2), vagina (2), central catheter (1), hair (1), sputum (1), intertrigo (1), mouth (1), bronchial aspiration (1), tinea cruris (1), cerebrospinal fluid (1), endotracheal tube (1).

All of the 96 *C. parapsilosis* sensu lato clinical strains gave the expected 500 bp band in PCR-ITS. In order to confirm their characterization at the species level, the ITS1/ITS2 region and the 5.8 rRNA gene previously amplified by PCR-ITS, were sequenced

After analysis of forward and reverse sequences by nucleotide BLAST search, 91 isolates showed 96-99% similarity with the *C. parapsilosis* sequence, 3 isolates showed 99% similarity with the *C. metapsilosis* sequence, and 2 isolates showed 99% similarity with the *C. orthopsilosis* sequence.

Thus, according to PCR-RPSO and DNA sequencing of ITS region, 91 isolates were identified as *C. parapsilosis* sensu stricto; three isolates were identified as *C. metapsilosis* and two isolates identified as *C. orthopsilosis*. No discordant results were observed between the two techniques.

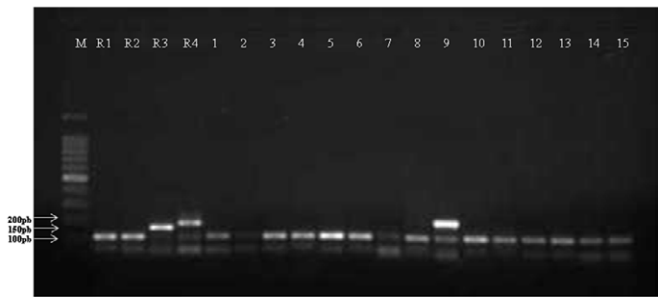

Figure 1 Results of the multiplex PCR-RPSO with 15 clinical strains and the 4 reference strains. LANE M: MOLECULAR SIZE MARKER (100 PB); Lane M: Molecular size marker (100 pb); Lane R1: *C. parapsilosis* CECT13009, Lane R2: *C. parapsilosis* ATCC22019, Lane R3: *C. orthopsilosis* CECT13011, Lane R4: *C. metapsilosis* CECT13010; Lanes 1 to 15: Clinical strains. Lanes 1-8 and 10-15: Amplification of *C. parapsilosis* sensu stricto strains showing the unique 100 pb band. Lane R3: Amplification of *C. orthopsilosis* strain showing bove 100 pb band and 150 pb band. Lanes R4-R9: amplification of *C. metapsilosis* strains showing above 100 pb band and 200 pb band.

Antifungal susceptibility: The 17 tested strains were susceptible to all antifungal agents. MICs of AMB, FLC, ITC and VC as determined by ATB Fungus® were <0.5 µg/ml, <1 µg/ml, <0.125 µg/ml and <0.125 µg/ml, respectively for all 3 species and all isolates.

MICs of CS as determined by E-test® were 0.047 µg/ml and 0.25 µg/ml for the first and second isolates of *C. orthopsilosis*, respectively; and 0.064 µg/ml for all 3 isolates of *C. metapsilosis*. MICs ranged between 0.016 µg/ml and 0.19 µg/ml for the 91 *C. parapsilosis* sensu stricto isolates.

Discussion

Many molecular methods have been adapted to the identification at the species level of *C. parapsilosis* clinical isolates, and among them are PCR-RPSO and PCR-ITS, which are the most widely used [14,15]. Multiplex PCR-RPSO is highly recommended for the molecular identification of the three cryptic species owing to its simplicity, rapidity and cost, especially in laboratories with low income. Thus, in the present study, molecular analysis of *C. parapsilosis* isolates led to the separation of the species into three groups further reclassified as separate species, *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*, respectively [7]. But the three species were phenotypically indistinguishable, in spite of the fact that recent studies implicated the three recently described species in human diseases, including bloodstream infection [11,12,19-22].

In PCR-RPSO, which targets the gene encoding the SO40 S ribosomal protein, the size of the amplified fragment is different according to the species, and this allows a precise identification of each of the three species of the complex. However, isolates were tested in three separate runs, each using one different primers couple [14]. In contrast, in the present study, a PCR-RPSO method was developed where the three primers were used simultaneously in the same run, allowing a much more rapid and less costly identification procedure. The RPSO gene was successfully amplified in all 96 clinical isolates, as well as in the four reference strains tested. According to the patterns obtained, 91 (94.8%) isolates were identified as *C. parapsilosis* sensu stricto, three (3.1%) as *C. metapsilosis* and two (2.1%) as *C. orthopsilosis*. For a further confirmation of these findings, all the clinical isolates were subjected to PCR-ITS and the amplification products submitted for sequencing. It is worth noting that the PCR-ITS which targets the 5.8s ribosomal gene together with the ITS1 and ITS2 flanking regions and the sequencing of the amplified products are considered as the gold standard in the distinction between the three species of *C. parapsilosis* complex [15,18,23]. Results of this study showed a very high concordance between PCR-RPSO and sequences of PCR-ITS amplified products. Nevertheless, PCR-RPSO was much more adapted to the hospital setting owing to its advantages in terms of simplicity, rapidity and cost especially when used in the multiplex variant we developed in the present work. Regarding the frequency of each of the three clinical species among the isolates investigated in our study, present findings are in accordance with nearly all previous reports where the prevalence of *C. orthopsilosis* and *C. metapsilosis* is less than 10% even though differences between series and regions have been described [7,8,11,12,16,19-21]. The much higher frequency of the isolation from clinical specimens of *C. parapsilosis* sensu stricto than *C. orthopsilosis* and *C. metapsilosis* has been attributed, among other factors, to its easier transmission from person to person and to its better adaptation to human commensal environment than its two relatives [5,6]. We didn't observe any resistance to all antifungals tested in our 17 isolates. MICs of AMB, FLC, ITC and VC were low for all isolates of the three species. This finding is in accordance with most of previous reports [24,25]. However, the low number of isolates tested in our study makes it difficult to determine any significant conclusion especially concerning caspofungin. Indeed, *C. parapsilosis* sensu stricto and *C. orthopsilosis* isolates have been associated with lower susceptibility to and higher MICs of CS as compared to *C. metapsilosis* and other Candida species and MICs of micafungin have been reported to be higher towards *C. parapsilosis* sensu stricto when compared to both *C. orthopsilosis* and *C. metapsilosis* isolates [23]. Several recent studies highlighted the clinical relevance of both *C. orthopsilosis* and *C. metapsilosis*. *C. orthopsilosis* has been recovered from blood, indwelling catheters, urine, lungs, skin and genital apparatus [8,11,12,19,22]; and *C. metapsilosis* was reported to have caused up to 6.9% of all *C. parapsilosis* sensu lato fungemia cases in Spain [20]. Out of the five non *C. parapsilosis* sensu stricto isolates identified in this study, four were collected from superficial sites (nails). The strain identified as *C. metapsilosis* from an umbilical catheter may indicate the invasiveness of the strain, with a further diffusion through blood. Even though none can be considered to be resistant to CS, the relatively high MICs shown by 17 of the isolates is somewhat in agreement with the findings mentioned earlier. Concerning the commonly used azoles, *C. parapsilosis* sensu stricto and to a lesser extent *C. metapsilosis* isolates have been shown to be less sensitive to FLC. Resistance to FC which has been described among *C. parapsilosis* isolates is unlikely to be a major concern when considering therapeutic use of this drug in candidiasis [23]. These differences in the activity of antifungals could have clinical relevance and affect the therapeutic choices. They emphasize the need for accurate species identification and antifungal susceptibility testing of *C. parapsilosis* strains isolated from patients with invasive candidiasis. However, molecular methods may not be adapted to the identification of isolates in routine; so the development of new tests for phenotypical identification is highly needed. Recently, several studies have reported the efficiency of MALDI-TOF MS for fast and accurate identification of *C. parapsilosis* sensu stricto, *C. metapsilosis* and *C. orthopsilosis* [26-28].

Conflict of Interest

The author has no conflict of interest.

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