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ORIGINAL PAPER

Genetic Diversity and Antifungal Susceptibility of *Candida parapsilosis* Sensu Stricto Isolated from Bloodstream Infections in Turkish Patients

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Abstract *Candida parapsilosis* sensu stricto is an emerging cause of hospital-acquired *Candida* infections, predominantly in southern Europe, South America, and Asia. We investigated the genetic diversity and antifungal susceptibility profile of 170 independent *C. parapsilosis* sensu stricto strains obtained from patients with candidemia who were treated at the Ege University Hospital in Izmir, Turkey, between 2006 and 2014. The identity of each strain was confirmed via PCR amplification and digestion of the secondary alcohol dehydrogenase-encoding gene. The 24-h geometric mean minimum inhibitory concentrations of the antifungal agents, in increasing order, were as follows: posaconazole, 0.10 µg/mL; voriconazole, 0.21 µg/mL; caspofungin, 0.38 µg/mL; amphotericin B, 0.61 µg/mL; anidulafungin, 0.68 µg/mL; and fluconazole, 2.95 µg/mL. Microsatellite genotyping of the isolates (using fluorescently labeled primers and a panel of four different short-nucleotide repeat fragments) identified 25, 17, 17, and 8 different allelic genotypes at the *CP*6, *B*5, *CP*4, and *CP*1 locus, respectively. Posaconazole, caspofungin, and amphotericin B showed the greatest in vitro activity of the tested systemic azole, echinocandin, and polyene agents, respectively, and the observed antifungal susceptibility of the isolates was shown to be independent of their isolation source. We obtained a combined discriminatory power of 0.99 with a total of 130 genotypes for 170 isolates tested. Finally, microsatellite profiling analysis confirmed

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the presence of identical genotype between separate isolates, supporting that effective surveillance and infection-prevention programs are essential to limit the impact of *C. parapsilosis* sensu stricto on hospitalized patients' health.

Keywords *Candida parapsilosis* · Microsatellite genotyping · Antifungal susceptibility · Posaconazole · Caspofungin · Amphotericin B

Introduction

Candida yeast species are a major cause of hospital-acquired infections that are associated with high morbidity and mortality rates, prolonged hospitalization periods, and increased healthcare costs [1, 2]. While *C. albicans* was previously thought to be the pathogen most often responsible for nosocomial bloodstream infections in both children and adults, other pathogenic *Candida* species have been identified in this context during the past two decades. For example, *C. glabrata* complex, *C. parapsilosis* complex, and *C. tropicalis* are often identified in affected patients, while *C. dubliniensis*, *C. lusitaniae*, *C. kefyr*, and *C. guilliermondii* are less frequently observed [3–5]. In addition, *Candida auris* was recently reported as a rapidly emerging cause of hospital-acquired multidrug-resistant infections worldwide [6].

Given that the various *Candida* species exhibit different rates of susceptibility to antifungal agents, their distribution in each context significantly affects the treatment modalities [7–10]. Notably, the epidemiology of *C. parapsilosis* strains in the hospital environment is unique. Compared with other *Candida* species, *C. parapsilosis* strains exhibit an increased propensity to both adhere to physical surfaces and colonize human skin; thus, they readily cause nosocomial and opportunistic infections in southern Europe, South America, and Asia [1, 2, 11–13]. *C. parapsilosis* complex (sensu lato) comprises three closely related species: *C. parapsilosis* sensu stricto, *Candida orthopsilosis*, and *Candida metapsilosis* [14, 15]. Of these, *C. parapsilosis* sensu stricto exhibits the highest virulence potential and is most frequently identified in infected patients [14, 15]. In fact, infections caused by *C. parapsilosis* sensu stricto predominantly affect severely ill patients in intensive care units, low-birth-weight neonates, and/or patients receiving parenteral nutrition [14, 16]. Furthermore, there are rising concerns regarding optimal antifungal regimens for *C. parapsilosis* bloodstream infection, since it is resistant to both azole and echinocandin antifungal agents as a result of its ability to form biofilms [1, 2, 17–19].

In a previous study in our university hospital, we found that *C. parapsilosis* was the second most common (28.3%) *Candida* species isolated from blood cultures [20]. Thus, the present study investigated the genetic diversity, and antifungal susceptibility profile of *C. parapsilosis* sensu stricto bloodstream isolates obtained at the Ege University Hospital, Izmir, Turkey.

Materials and Methods

Fungal Isolates

A collection of 170 independent *C. parapsilosis* strains was isolated from patient blood samples collected at the Ege University Hospital, Izmir,

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Turkey, between 2006 and 2014. Only one isolate per patient was included. All isolates were sub-cultured (37 °C, 48 h) onto Sabouraud glucose agar (Merck, Darmstadt, Germany) before undergoing species identification, genotyping, and antifungal susceptibility analysis.

Species Identification

The identity of each strain was then confirmed at the species level via PCR amplification and digestion of the secondary alcohol dehydrogenase-encoding (*SADH*) gene, as previously described [21].

Genomic DNA was extracted from all isolates, as previously described [22], and PCR-amplified in 25-µL reaction volumes containing 1 × PCR buffer (20 mM of Tris HCl, pH 8.4, and 50 mM of KCl), 0.5 mM of each dNTP (Fermentas, Massachusetts, MA, USA), 0.5 mM of forward (5′-GTT GAT GCT GTT GGA TTG T-3′) and reverse (5′-CAA TGC CAA ATC TCC CAA-3′) primersprimers, 1.5 mM of $MgCl_2$ (Fermentas), 3 µL of genomic DNA, and 1.25 U of Taq DNA polymerase (New England BioLabs, Herts, UK). The identification of all study isolates as *C. parapsilosis* sensu stricto (rather than *C. metapsilosis* and/or *C. orthopsilosis*) was then confirmed via PCR-restriction fragment length polymorphism (RFLP) analysis. The *SADH* PCR products were digested using the fast digest restriction enzyme *NlaIII* (Thermo Scientific, Waltham, MA, USA), for 5–15 min at 37 °C. The reaction was performed in a 30-µL reaction volume containing 1 µL (10 U) of restriction enzyme, 3 µL of 10 × FastDigest® Green buffer (supplied), 10 µL (∼ 0.2 µg) of PCR product, and 16 µL of molecular-grade water. Finally, 5 µL of the digested product was separated via electrophoresis using a 2% agarose gel [21]. *Candida parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96139, and *C. metapsilosis* ATCC 96144 reference strains were used as controls. With the *Nla*III enzyme digestion, the following fragment sizes were expected: *C. parapsilosis* (131 and 505 bp), *C. metapsilosis* (74, 288 and 348 bp), and *C. orthopsilosis* (131, 217 and 88 bp).

Microsatellite PCR Amplification

Microsatellite genotyping was performed using four different species-specific polymorphic microsatellite markers (*CP*1, *CP*4, *CP*6, and *B*5) as previously described [23, 24]. In each panel, one of the amplification primers was labeled with either 6-carboxyfluorescein (FAM), hexachlorofluoroscein (HEX), or 6-carboxytetramethylrhodamine (TAMRA).

PCR products were subjected to electrophoresis using the Applied Biosystems 3730 DNA analyzer. The sizes of the PCR products were determined using GeneScan (version 3.2) analysis software. The alleles have been designated by their size (in base pairs). The clinical isolate (*C. parapsilosis* ATCC 22019) with known genotypes was used as a positive control.

In Vitro Antifungal Susceptibility Testing

In vitro antifungal susceptibility testing of the isolates against fluconazole (FCZ, Pfizer, New York, NY, USA), voriconazole (VRC, Pfizer), posaconazole (POS, Schering-Plough, Kenilworth, NJ, USA), caspofungin (CAS, Merck Research Laboratories, Rahway, NJ, USA), anidulafungin (AFG, Pfizer), and amphotericin B (AmB, Sigma, St Louis, MO, USA) was performed using a broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 [25], and M27-S4 [26] guidelines. FCZ and CAS were dissolved in distilled water; VRC, AmB, POS, and AFG were dissolved in dimethyl sulfoxide. The antifungal agents (the final concentration of which ranged from 0.06 to 64 µg/mL for FCZ, 0.007 to 8 µg/mL for POS, 0.015 to 8 µg/mL for VRC, CAS, and AFG, and 0.03 to 16 µg/mL for AMB) were prepared in RPMI 1640 medium (Sigma) buffered to pH 7.0 using 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and stored at − 70 °C until use. The minimum inhibitory concentrations (MICs) were read after 24 h. Endpoints for azoles and echinocandins were defined as the lowest concentration of drug that resulted in a prominent reduction (approximately 50% inhibition) of visual growth compared with the growth control wells, and MICs of AmB were defined as the lowest concentration of drug that resulted in total inhibition of visual growth. *Candida parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 were used as quality controls, and three independent replicates of each experiment for each strain were performed on different days.

Statistical Analysis

Data were analyzed using GraphPad Prism, Version 7.0, for Mac OS X (GraphPad Software, San Diego, CA, USA). The d MIC distributions of isolates originating from different locations were compared using the Mann–Whitney–Wilcoxon test. A (two-tailed) P value ≤ 0.05 was considered to indicate statistical significance. Allelic and genotyping frequencies were determined using ARLEQUIN (ver 3.5) software [27], and the combined discriminatory power (DP) of the markers was described as calculated by Hunter and Gaston [28].

Results

The PCR-based amplification of the *SADH* gene exhibited by the 170 isolates identified each as *C. parapsilosis* sensu stricto, since each isolate yielded a $\sim$ 720-bp DNA fragment, which was further digested to form two shorter (131- and 505-bp) fragments using the restriction enzyme *Nla*III.

The 24-h geometric MICs of the tested antifungal agents against the *C. parapsilosis* sensu stricto strains, in increasing order, were as follows: POS, 0.10 μg/mL; VRC, 0.21 μg/mL; CAS, 0.38 μg/mL; AmB, 0.61 μg/mL; AFG, 0.68 μg/mL; and FCZ, 2.95 μg/mL. As shown in Table 1, the majority of isolates were susceptible to azole drugs, exhibiting MIC_{90} values of 0.125 and 0.25 μg/mL to POS and VRC, respectively. Only eight (4.5%) isolates showed a resistant phenotype to VRC. All isolates were susceptible to AmB (MIC range 0.25–2 μg/mL). Likewise, the MIC values of the echinocandins CAS and AFG against the isolates were found to range from 0.016 to 1 and 0.016 to 2 μg/mL, respectively, which indicates in vitro activity. The widest MIC range (0.06–32 μg/mL) and the highest resistance rate (9.4%, $n = 16$) were measured for FCZ.

Microsatellite genotyping of 170 of the analyzed isolates was achieved using fluorescently labeled primers, and a panel of four different short-nucleotide repeat fragments. Observed heterozygosity rates were 0.98% (168 isolates), 0.91% (155 isolates), 0.88% (151 isolates), and 0.52% (89 isolates) at the *CP*6, *B*5, *CP*4, and *CP*1 locus, respectively (Table 2). The most common genotypes were 253–271 (genotype 10, exhibited by 39.4% of the isolates) at the *CP*6 locus, 241 (genotype 5, 41.7% of isolates) at the *CP*1 locus, 328–353 (genotype 7, 32.4% of isolates) at the *CP*4 locus, and 100–108 (genotype 2, 29.4% of isolates) at the *B*5 locus (Table S1). Using four microsatellite markers (*CP*1, *CP*4, *CP*6, and *B*5), we obtained a DP of 0.99 with a total of 130 genotypes for 170 analyzed isolates (Table 2 and Table S2).

Table 1 Geometric mean of MICs, MIC ranges, and MIC_{50} and MIC_{90} values obtained by testing the susceptibility of 170 independent bloodstream *Candida parapsilosis* sensu stricto isolates to six antifungal agents, according to CLSI M27-A3 and M27-S4 guidelines

	AmB	FCZ	VRC	POS	AFG	CAS
Geometric mean	0.61	2.95	0.21	0.10	0.68	0.38
Range	0.25–2	0.063–32	0.016–8	0.008–0.5	0.016–2	0.016–1
MIC_{50}	0.5	1	0.016	0.063	1	0.5
MIC_{90}	1	4	0.125	0.25	1	0.5
Susceptible (%)	NA	85.7	91	NA	100	100
Intermediate (%)	NA	5.2	4.5	NA	–	–
Resistant (%)	NA	9.1	4.5	NA	–	–

MIC minimum inhibitory concentration, *MIC_{50}* 50% minimum inhibitory concentration, *MIC_{90}* 90% minimum inhibitory concentration. *AmB* Amphotericin B, *FCZ* Fluconazole, *VRC* Voriconazole, *POS* Posaconazole, *AFG* Anidulafungin, *CAS* Caspofungin. *S* susceptible, *I* intermediate, *R* resistant, *NA* not applicable. Interpretive MIC breakpoints were evaluated as follows: FCZ, AFG, and CAS MIC values ≤ 2, $= 4$, and ≥ 8 μg/mL; VRC MIC values ≤ 0.12, $= 0.25$–0.5 and ≥ 1 μg/mL were susceptible, intermediate, and resistant, respectively (CLSI M27-S4). Interpretive MIC breakpoints for POS and AmB have not been established in CLSI. The MICs were read after 24 h

Table 2 Characteristics of the four microsatellites markers

Locus	No. of alleles	Size range (bp)	Allele frequency	No. of genotypes	Obs-Het %	DP
*CP*1	7	224–261	0.009–0.468	8	52	0.74
*CP*4	18	260–389	0.006–0.276	17	88	0.84
*CP*6	31	225–313	0.003–0.224	25	98	0.79
*B*5	17	98–148	0.006–0.244	17	77	0.85
Total						0.99

DP discrimination power; *bp* base pair; *Obs-Het* observed heterozygosity

Discussion

C. parapsilosis sensu stricto is a human commensal mycobiome that belongs to the *C. parapsilosis* species group, along with two other closely related taxa, *C. orthopsilosis* and *C. metapsilosis* [14, 15]. Numerous studies have previously reported the isolation of *C. parapsilosis* sensu stricto from "man-made" environments, such as hospitals and domestic laundry appliances [29], and furthermore showed that colonization of health workers' hands with *C. parapsilosis* sensu stricto is significantly associated with an increased incidence of patient bloodstream infections [30]. Thus, effective surveillance and infection-prevention programs are essential to limit the impact of *C. parapsilosis* sensu stricto on human health.

The present study investigated the genetic diversity and antifungal susceptibility profiles exhibited by a large collection of bloodstream *C. parapsilosis* strains obtained between 2006 and 2014 in Izmir, Turkey. No increased rate of resistance to the tested antifungal agents was observed among the isolates; furthermore, no correlation was observed between the genotypes of *C. parapsilosis* isolates and their exhibited antifungal resistance patterns.

Consistent with several previous reports, POS showed potent in vitro activity against all isolates [31, 32]. In contrast, the present study showed that 9.4% (n = 16) and 4.7% (n = 8) of the isolates were resistant to FCZ and VRC, respectively; furthermore, five of the FCZ-resistant isolates were also resistant to VRC. These data are consistent with those previously reported by Tan et al. [33], who demonstrated that bloodstream isolates from the Asia–Pacific region exhibited a low (2.2%) rate of resistance to FCZ and no resistance to VRC. Similarly, an international collection of 197 *C. parapsilosis* isolates was previously shown to exhibit FCZ and VRC resistance at a rate of 2.5 and 0%, respectively [32]. In contrast, Govender et al. [31] previously reported that 53 and 23% of 531 bloodstream *C. parapsilosis* isolates collected in South Africa exhibited resistance against FCZ and VRC, respectively, and further showed that 44% of the FCZ-resistant isolates were also resistant to VRC. Thus, the results of the present and previous studies suggest that the resistance of *C. parapsilosis* isolates to azole treatment varies among different patient populations.

Consistent with several studies reported previously [32–34], all isolates in the present study exhibited susceptible MIC to CAS and AFG. Tosun et al. [35] tested the resistance of 38 *C. parapsilosis* isolates collected in Turkey against five antifungal agents and resultantly found that while all isolates were sensitive to treatment with VRC, CAS, and AFG, only a single isolate was dose dependently susceptible to FCZ treatment. Similarly, in agreement with previous studies [31, 32], we found the rate of resistance to AmB to be very low among the analyzed isolates. Thus, the results of the present study support that AmB remains the antifungal drug of choice for the treatment of infections caused by this species [36].

The results of the present study revealed significant genetic diversity among the analyzed *C. parapsilosis* isolates, based on the identification of different allelic combinations at four microsatellite loci (*CP*1, *CP*4, *CP*6, and *B*5) (Table 2). The conducted multi-locus' analysis identified that the largest number (25) of allelic genotypes occurred at the *CP*6 locus, followed by the *B5, CP*4, and *CP*1 locus, where 17, 17, and 8 genotypes were observed, respectively. However, our data suggest the presence of identical genotypes among the isolates, which appeared to be epidemiologically related. Consistent with our results, a

previously conducted microsatellite-based genotyping of *C. parapsilosis* sensu stricto isolates revealed the dominance and persistence of a particular epidemiological clone among neonatal intensive care unit patients [37]. For example, Lasker et al. [38] were the first to discriminate *C. parapsilosis* isolates and used seven microsatellite markers to genotype 42 isolates with a DP of 97%. They resultantly identified identical microsatellite profiles that were exhibited by *C. parapsilosis* isolates collected from two separate patients and thereby revealed contamination that affected medical devices and solutions, including indwelling catheters [38]. Similarly, Delfino et al. [30] previously showed that while most (15/21; 71.4%) *C. parapsilosis* isolates obtained from the hands of healthcare workers exhibited new and unrelated genotypes, many (16/21; 76.2%) bloodstream isolates were closely related.

In another study, Sabino et al. [24] genotyped 233 independent bloodstream *C. parapsilosis* sensu stricto isolates using the four microsatellite markers employed in the present study and resultantly showed that the analyzed isolates exhibited 20–42 alleles and 39–92 genotypes at the tested loci. This allowed them to conduct a multiplex analysis of an overall total of 192 genotypes to thereby achieve a combined DP of 99% across the four microsatellite loci. Likewise, a previous study used five microsatellite markers to discriminate between 40 *C. parapsilosis* isolates that were collected from an unselected population that comprised patients treated at three neonatal intensive care units in the USA, achieving a DP of 99.7% [39].

Thus, the present and various previously conducted studies clearly show that microsatellite typing is a useful technique to clarify the genetic relatedness of *C. parapsilosis* sensu stricto isolates. Data generated using this technique also offer insights into both the routes of hospital transmission and molecular epidemiology of *C. parapsilosis* [12, 23]. Of note, among different allelic genotypes used in the present study, the genotyping profiles obtained with dinucleotide repeat loci such as the *B*5 locus may contain many stutter peaks, which may lead to misinterpretation or ambiguous profiles [40, 41].

Several other methods, including random amplification of polymorphic DNA (RAPD) [42], electrophoretic karyotype [43], and multi-locus sequence-typing [15] analyses, have also been used successfully to type and distinguish between *C. parapsilosis* isolates. In a previous study conducted in Turkey, 56 *C. parapsilosis* isolates recovered from bloodstream and urine cultures were genotyped via RAPD analysis, and three genotypes were resultantly detected. Notably, while all bloodstream isolates were shown to exhibit genotype II, the analyzed urine-derived isolates exhibited all three (I–III) genotypes [44].

In conclusion, the 170 *C. parapsilosis* sensu stricto isolates analyzed in the present study exhibited the greatest in vitro susceptibility to treatment with POS, CAS, and AmB among tested systemic azole, echinocandin and polyene agents, respectively, and their susceptibility was not dependent upon their respective sources of isolation. The isolates exhibited a diverse range of genotypes at the analyzed loci, and several identical genotypes were observed, supporting the efficacy of the infection-prevention programs implemented by the Ege University Hospital to exclude common *C. parapsilosis* sensu stricto infection sources.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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