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Molecular identification, genotyping, and drug susceptibility of the basidiomycetous yeast pathogen *Trichosporon* isolated from Turkish patients

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Deep-seated infections due to *Trichosporon* species are emerging mycoses that have a very poor prognosis in patients with persistent neutropenia. This study elucidated the mycological characteristics of *Trichosporon* strains obtained from deep-seated infections in Turkish patients and identified by DNA sequence analysis of intergenic spacer (IGS) region 1 of the rDNA locus. In addition, we genotyped the major causative agent, *T. asahii*, and evaluated the *in vitro* drug susceptibility of the isolates. While 87 (81.3%) of the 107 isolates were *T. asahii*, the remaining 20 were *T. faecale* (14.0%), *T. asteroids* (0.9%), *T. coremiiforme* (0.9%), *T. japonicum*, (0.9%), *T. lactis* (0.9%), and a new species (0.9%). In addition to the eight known *T. asahii* genotypes, one novel genotype was identified. The distribution of the *T. asahii* genotypes in this study were genotype 1 (79.3%), followed by 5 (8.0%), 3 (6.9%), 6 (3.4%), 4 (1.1%), and 9 (1.1%). Turkish isolates showed low susceptibility to amphotericin B, 5-flucytosine, and fluconazole. Although relatively low minimum inhibitory concentrations (MICs) were found with all drugs, voriconazole appeared to be the most active. The MICs of the non-*Trichosporon asahiiTrichosporon* species were similar to those of the *T. asahii* strains. Our findings suggest that *Trichosporon* species isolated from Turkish patients are more diverse than those reported from other countries.

Keywords *Trichosporon asahii*, genotype, drug susceptibility, Turkey

Introduction

Deep-seated infections due to *Trichosporon* species are increasing in neutropenic patients undergoing chemotherapy or following organ transplantation and such infections have a very poor prognosis [1–4]. Recently, trichosporonosis has been recognized as a breakthrough infection following treatment with candin derivatives, such as micafungin (MCFG) or caspofungin [5,6]. Similar to candin derivatives, amphotericin B has limited activity against *Trichosporon* species [7] and strains resistant to multiple azole agents have been recovered from patients [8,9].

Currently, 38 species are recognized in the genus *Trichosporon* of which several are considered human pathogens, including *T. asahii* in deep-seated infections and *T. asteroids*, *T. ovoides*, and *T. inkin* in superficial infections [10]. Other species that are rarely isolated from clinical specimens include *T. cutaneum*, *T. mucoides*, *T. japonicum*, and *T. loubieri* [11–14]. Research on deep-seated trichosporonosis has focused on *T. asahii*, which is

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the major causative agent in many countries. This microorganism has several genotypes based on the intergenic spacer (IGS) 1 region located between the 26S and 5S rRNA genes. Because the DNA sequence of this region shows geographic specificity, genotyping of the IGS 1 region can be used as a tool in global epidemiological studies [15].

In hospital laboratories, *Trichosporon* strains are routinely identified through their assimilation of carbon and nitrogen compounds with commercial kits, such as the ID32C kit (bioMérieux, Marcy l'Etoile, France), and morphological characteristics, including the production of arthroconidia on cornmeal agar. However, these methods may occasionally result in misidentification of isolates, particularly at the species level. For molecular identification, analysis of the internal transcribed spacer (ITS) region or 26S rDNA (large subunit) in the rRNA gene is widely used for fungal identification. However, intergenic spacer (IGS) regions show more diversity in their DNA sequences than the ITS regions or 26S rDNA, suggesting that IGS sequence analysis may be superior to analysis of ITS sequences for differentiating closely related species, including several of the genus *Trichosporon* (Fig. 1). Although deep-seated trichosporonosis is an emerging mycosis with high mortality, information on its causative agents is still limited.

This study elucidated the mycological characteristics of *Trichosporon* strains isolated from Turkish patients. Characterization was based on the molecular identification of the IGS 1 region using DNA sequence analysis, genotyping of the major causative agent, *T. asahii*, and drug susceptibility of the isolates.

Materials and methods

Strains examined

We examined 107 *Trichosporon* clinical isolates obtained from the following six university hospitals in Turkey; Hacettepe University, Dokuz Eylul University, Mersin University, Osmangazi University, Gazi University, and Uludag University. Of these, 76, 18, 7, 2, 2, 1, and 1 specimens were recovered from urine, bronchoalveolar lavage fluid (BALF), blood, bile, nephrostomy, pleural fluid, and catheter, respectively. They were tentatively identified as '*T. asahii*' or '*Trichosporon* spp.' using ID32C or API 20C AUX kits (bioMérieux).

DNA sequencing and identification

Fungal DNA was extracted using the method of Makimura *et al*. [16] and the IGS 1 region was sequenced according to the method of Sugita *et al*. [15]. Briefly, the *Trichosporon* IGS 1 region (approximately 500 bp) was amplified by PCR using the oligonucleotide primers 26SF (5´-ATCCTTTGCAGACGACTTGA-3´) and 5SR (5´-AGCTTGACTTCGCAGATCGG-3´). The PCR products were sequenced with 26SF and 5SR using an ABI 3700 DNA sequencer with an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, CA, USA), according to the manufacturer's instructions.

Genotyping T. asahii and molecular phylogenetic analysis

The IGS 1 sequences of the *T. asahii* strains determined in this study were compared with DNA sequences deposited in GenBank. The sequences were aligned using ClustalW [17]. For the neighbor-joining analysis [18], the distances between sequences were calculated using Kimura's two-parameter model [19]. A bootstrap analysis was conducted with 100 replications [20].

Drug susceptibility testing

The minimum inhibitory concentrations (MICs) of amphotericin B (AMPH-B), 5-flucytosine (5-FC), fluconazole (FLCZ), miconazole (MCZ), itraconazole (ITCZ), micafungin (MCFG), and voriconazole (VRCZ) were determined using a colorimetric antifungal susceptibility testing kit (ASTY; Kyokuto Pharmaceutical Industry, Tokyo, Japan), according to the manufacturer's instructions.

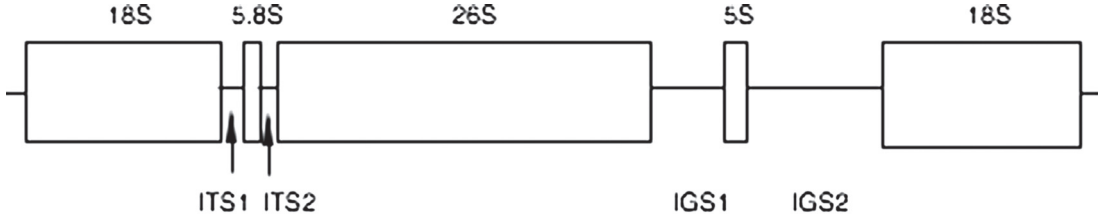

Fig. 1 Structure of the rRNA gene of *Trichosporon* species. The rDNA locus of the type strain, CBS 2479, of *T. asahii* consists of 18S (1787 bp), the ITS 1 region (123 bp), 5.8S (156 bp), the ITS 2 region (175 bp), 26S (3380 bp), the IGS 1 region (485 bp), 5S (118 bp), and the IGS 2 region (1610 bp). The total length of the rDNA locus is 7,834 bp. ITS, internal transcribed spacer; IGS, intergenic spacer region.

The previously determined agreement between the ASTY method and CLSI M27-A2 microdilution procedure was 97.7% in studies of 50 strains of *T. asahii* [21].

Results

DNA-based molecular identification

Of the 107 strains that were tentatively identified as *T. asahii* or *Trichosporon* spp. with the ID32 and API 20C AUX kits, 87 (81.3%) were found to be *T. asahii*. The remaining 20 strains consisted of *T. faecale* (15, 14.0%), *T. asteroids* (1, 0.9%), *T. coremiiforme* (1, 0.9%), *T. japonicum* (1, 0.9%), and *T. lactis* (1, 0.9%). Since the DNA sequence of one (GenBank accession number, AB39006) of the 15 *T. faecale* strains differed from that of the type *T. faecale* (AB066413) by 5.3%, it was tentatively treated as a new genotype 3 of the specie (Fig. 2). The one remaining strain of the 20, B04, could not be identified as any known *Trichosporon* species. The closest species were *T. asteroides* and *T. japonicum*, for which the IGS1 sequence similarities were 89.4 and 89.2%, respectively.

Genotyping the T. asahii isolates

Of the 87 *T. asahii* clinical isolates, the major genotype was 1 (69 strains, 79.3%), followed by genotypes 5 (7 strains, 8.0%), 3 (6 strains, 6.9%), 6 (3 strains, 3.4%), and 4 (1 strains, 1.1%) (Fig. 3). The remaining strain could not be identified as any of the eight known *T. asahii* genotypes, so we designated it as a new genotype 9 (Fig. 2). Phylogenetically, the new genotype was positioned close to genotypes 1, 2, and 8 in the tree. *T. asahii* genotypes isolated from Japanese, American, and Spanish patients were added to Fig. 3 for comparison. The IGS 1 sequences of each genotypic strain ranged in length from 485 to 490 bp.

Drug susceptibility testing

The MICs of seven antifungal agents (AMPH-B, 5-FC, FLCZ, MCZ, ITCZ, VRCZ, and MCFG) were determined using the ASTY colorimetric method. Table 1 shows the MIC_{50}/MIC_{90}, geometric mean (GM), and range of the MICs of the 87 *T. asahii* strains and 20 non-*Trichosporonasahii Trichosporon* strains. All isolates were resistant to MCFG (т16 μg/mL) and the MICs of the *T. asahii* strains were similar to those of the non-*Trichosporonasahii Trichosporon* strains. Greatest inhibition was found with voriconazole, with a GM MIC of 0.158 for *T. asahii* and 0.104 for non-*asahiiTrichosporon*.

No relationship was observed between the MICs of AMPH-B and azole agents. For the 107 strains, those with AMPH-B GM MICs т1 μg/ml and s 1 μg/ml had FLCZ GM MICs 14.333 μg/ml and 12.530 μg/ml, ITCZ GM MICs 1.083 μg/ml and 1.120 μg/ml, and VRCZ GM MICs 0.140 μg/ml and 0.151 μg/ml, respectively.

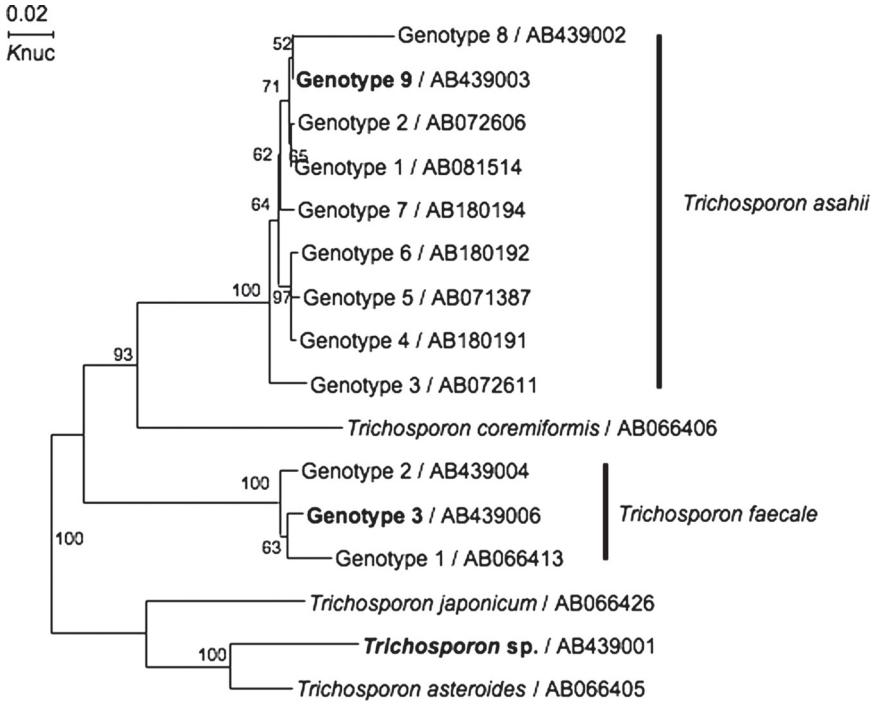

Fig. 2 Molecular phylogenetic trees constructed using the DNA sequence of the IGS 1 region including the new genotype strain from Turkey. The DDBJ/GenBank/EMBL accession numbers are also shown. The numbers indicate the confidence level from 100 replicate bootstrap samplings (frequencies below 50% are not shown). Knuc, Kimura's parameter [19].

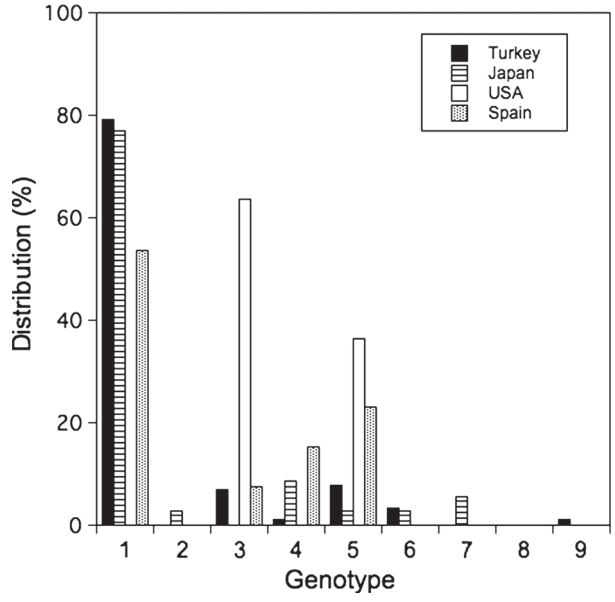

Fig. 3 The distribution of each genotype of *Trichosporon asahii*. Information on the distribution of Japanese, American, and Spanish *T. asahii* isolates is included for comparison [15,28].

Discussion

The D1/D2 region of the large subunit (LSU) and ITS region of the RNA gene have been widely used in the molecular identification of pathogenic fungi, and an enormous number of DNA sequences for these regions are deposited in GenBank [22,23]. In general, when there is a ô1% difference in the D1/D2 LSU or ITS regions between two strains, there is a high degree of probability that they are distinct species [24]. However, several species in the genus *Trichosporon* do not conform to this standard. For example, *T. montevideense* and *T. domesticum*, which are the causative agents of summer-type hypersensitive pneumonitis, share identical nucleotide sequences in their ITS regions, but differ at two positions in D1/D2 LSU. Likewise, the nucleotide differences in the ITS regions and D1/D2 LSU among *T. asahii*, *T. coremiiforme*, and *T. faecale* are very small, i.e., only one or two base pairs. Sugita *et al.* [15] first introduced the usefulness of the IGS region for identifying closely related *Trichosporon* species, as this region is more diverse than ITS or LSU. There is an approximately 20% difference among *T. asahii*, *T. coremiiforme*, and *T. faecale* and a 7% difference between *T. domesticum* and *T. montevideense* in the IGS1 region. In the genus *Trichosporon*, the nucleotide difference in the IGS1 region of two strains is generally s 50% when the nucleotide difference in the ITS regions of the same two strains is only 2%. Recently, many researchers have adopted the IGS region for the identification of pathogenic yeasts [25–30].

Table 1 Antifungal susceptibility of clinical isolates of *T. asahii* and non-*asahii Trichosporon* species

	MIC (ug/mL)								
	AMPH-B			5-FC			FLCZ		
Species	MIC50/MIC90	GM	Range	MIC50/MIC90	GM	Range	MIC50/MIC90	GM	Range
T. asahii (87)[a]	1/2	1.138	0.125–4	16/16	12.93	0.125–32	8/16	13.66	4–64
Non-asahii spp. (20)	0.5/1	0.597	0.125–2	16/16	14.91	4–32	8/8	7.455	0.5–2
T. faecale (15)		0.570	0.125–2		17.5	8–32		7.75	4–64
T. japonicum (1)		–	0.5		–	8		–	2
T. asteroides (1)		–	1		–	8		–	16
T. coremiforme (1)		–	0.5		–	4		–	2
T. lactis (1)		–	0.5		–	8		–	8
New Trichosporon sp. (1)		–	1			8			16

	MIC (ug/mL)								
	MCZ			ITCZ			VRCZ		
Species	MIC50/MIC90	GM	Range	MIC50/MIC90	GM	Range	MIC50/MIC90	GM	Range
T. asahii (87)[a]	1/2	1.152	0.5–4	1/2	0.985	0.25–2	0.125/0.25	0.154	0.03–0.25
non-asahii spp. (20)	1/1	0.977	0.5–2	1/1	0.955	0.5–2	0.125/0.125	0.103	0.03–0.25
T. faecale (15)		1.031	0.5–2		0.937	0.5–1		0.105	0.06–0.25
T. japonicum (1)		–	0.5		–	0.5		–	0.06
T. asteroides (1)		–	1		–	2		–	0.125
T. coremiforme (1)		–	0.5		–	0.5		–	0.03
T. lactis (1)		–	1		–	1		–	0.125
New Trichosporon sp. (1)			1			2			–0.25

[a]Number of strains examined

Interestingly, approximately 20% of the isolates in our study were *T. coremiiforme* and *T. faecale*, although *T. asahii* (major agent) and *T. mucoides* (minor agent) are usually the causative agents of deep-seated trichosporonosis. *T. coremiiforme* and *T. faecale* are considered non-pathogenic yeasts, and are mainly isolated from the environment, e.g., soil. Rodriguez-Tudela *et al*. [28] also identified *Trichosporon* clinical isolates from Spanish and Argentinean patients using IGS sequence analysis. The number of strains examined was limited, but of 25 strains obtained from deep-seated sites, 14 were *T. asahii* and 10 were non-*Trichosporon asahii Trichosporon* species, including *T. coremiiforme*, *T. dermatis*, *T. faecale*, *T. inkin*, *T. jirovecii*, *T. japonicum*, and *T. ovoides*. In Turkey, the first case of *T. japonicum* infection in a child following bone-marrow transplant was reported in 2008 [14].

Strain B04 could not be identified as any known *Trichosporon* species. The IGS1 sequence similarity between B04 and the closest phylogenetic species, *T. asteroides* and *T. japonicum*, was approximately 10%. As yet, there is no consensus on taxonomic criteria for the IGS region among yeast scinetists, although Sugita *et al*. [15] reported that con-specific strains in the genus *Trichosporon* had approximately 95% DNA sequence similarity. According to their proposal, strain B04 is considered to be a new species in the genus *Trichosporon*, although further taxonomic investigation is needed. In this paper, we treat strain B04 as a *Trichosporon* spp. Our study also demonstrates that molecular identification using IGS sequence analysis is a powerful tool for identifying members of the *Trichosporon* genus.

T. asahii, the major pathogen causing trichosporonosis, has eight genotypes in the IGS1 region. Sugita *et al*. [15] first showed that IGS genotyping could be used as a tool for global epidemiological studies. The distribution of each *T. asahii* genotype in Japan, the United States, and Spain is also shown in Fig. 3. The genotype distribution pattern of Turkish isolates is similar to that of Japanese isolates, i.e., genotype 1 strains constituted approximately 80% of all isolates. The major genotypes among American patients are 3 and 5, whereas 1 and 5 are the major types in Spain, although numbers are limited. In this study, we identified a new genotype strain (type 9) from a urine sample. Previously, our research group isolated a genotype 8 strain from a Turkish patient. Of the *T. faecale* strains, a genotype 2 strain was also previously isolated from a superficial site of a Turkish patient. In this study, we isolated a new genotype strain (type 3) for *T. faecale* from a urine sample. The first case report of infection due to *T. faecale* was in Germany [31]. Thus, although *T. faecale* is considered to be non-pathogenic, it may cause infection in rare instances.

The MICs of the antifungal agents examined in this study for Turkish *Trichosporon* isolates were similar to previous reports [29,32,33]. Namely, the microorganism showed low susceptibility to AMPH-B, 5-FC, and FLCZ. VRCZ was the most active compound. Regarding the drug susceptibility of non-*Trichosporon asahii Trichosporon* isolates, the MICs are similar to those of *T. asahii*, based on a few reports.

In conclusion, we elucidated the characteristics of Turkish *Trichosporon* clinical isolates by examining 107 isolates obtained from six laboratories. Turkish *Trichosporon* appears to be more diverse than in other countries.

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