



# Molecular identification and susceptibility testing of *Trichosporon* isolates from a Brazilian hospital

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**Summary** In this study the molecular identification and susceptibility profile of 21 clinical isolates, from a Brazilian hospital, belonging to six different species of *Trichosporon* were described. *Trichosporon asahii* was the predominant species and corresponded to 43% of isolates followed by *Trichosporon faecale* (24%) and *Trichosporon inkin* (14%). Eighty three percent of the strains isolated from deep sites were identified as *T. asahii*, while only 17% belong to a non-*T. asahii* species (*Trichosporon inkin*). In general, the MICs were high and independent of the species of *Trichosporon* as well as the clinical origin of strain. Amphotericin B, 5-fluorocytosine and fluconazole were the less active against *Trichosporon* spp. and high MIC values of voriconazole, posaconazole and ravuconazole were observed. Fifty-six percent (5/9) of *T. asahii* strains were isolated from deep sites, whereas 8% (1/12) of non-*T. asahii* strains were isolated from those sites. A total of 89% of *T. asahii* isolates exhibited resistance to amphotericin B in vitro.

**Key words** *Trichosporon asahii*, *Trichosporon inkin*, Antifungal susceptibility, Molecular identification

## Identificación molecular y sensibilidad a los antifúngicos de cepas de *Trichosporon* aisladas en un hospital de Brasil

**Resumen** En este artículo se describe la identificación molecular y el perfil de sensibilidad de 21 cepas clínicas pertenecientes a seis especies diferentes del género *Trichosporon*. La especie predominante fue *Trichosporon asahii*, que supuso el 43% de los aislamientos, seguido de *Trichosporon faecale* (24%) y *Trichosporon inkin* (14%). El 83% de las cepas aisladas de lugares anatómicos profundos fueron identificadas como *T. asahii*, mientras que sólo un 17% pertenecían a especies no-*T. asahii*. En general, los valores de CMI fueron altos, independientemente de la especie de *Trichosporon* y el origen clínico de la cepa. Los antifúngicos menos activos frente a *Trichosporon* fueron anfotericina B, fluorocitosina y fluconazol, aunque también se observaron valores altos de CMI a voriconazol, posaconazol y ravuconazol. El cincuenta y seis por ciento (5/9) de las cepas de *T. asahii* fueron aisladas de muestras profundas, mientras que el 8% (1/12) de las especies no-*T. asahii* fueron aisladas de dichos lugares. El 89% de los aislamientos de *T. asahii* mostraron resistencia in vitro a la anfotericina B.

**Palabras clave** *Trichosporon asahii*, *Trichosporon inkin*, Sensibilidad a los antifúngicos, Identificación molecular

The basidiomycetous yeast *Trichosporon* may be found in soil, water and, occasionally as a member of the normal microbiota of the skin, nails and mucosal surfaces of the human respiratory and gastrointestinal tracts [22].

The genus *Trichosporon* has undergone extensive taxonomic reevaluation [12,33,35]. Currently, the genus *Trichosporon* includes 37 species [4]. Seven of these are implicated in infections in humans: *Trichosporon asahii*, *Trichosporon asteroides*, *Trichosporon cutaneum*, *Trichosporon inkin*, *Trichosporon mucoides*, *Trichosporon ovoides* and *Trichosporon loubieri* [19,25]

The fungus has been associated with superficial infections. However, over the past decade, invasive infection by *Trichosporon* spp. has been recognized. For instance, *T. inkin*, a classical agent of white piedra in pubic hair [5], notably, today, it is also isolated from disseminated and unusual infections [6,16,18,29,30]. The *Trichosporon* species have emerged as important life-threatening opportunistic systemic pathogens, mainly in granulocytopenic patients [5,9,10,15,16,20,21]. The mortality rate in

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neutropenic patients receiving amphotericin B or azole therapy is approaching 80% but may reach 100% in patients with persistent neutropenia [3].

Even with molecular approaches, it has been difficult to establish a direct association between these species and distinct clinical presentations within the wide spectrum of currently recognized trichosporonosis. At present, *T. asahii* is considered one of the most common etiologic agents of non-candidal yeast blood stream infections. It has been isolated from disseminated trichosporonosis in neonates [26], in AIDS patients [14], in patients undergoing bone marrow transplantation [9,11,21] and, even in patients who displayed no evidence of underlying disease [8,31,42], but most reports show that *T. asahii* affects patients with hematological malignancies and profound neutropenia [1,5,20,37,40].

In this study we have investigated twenty-one clinical isolates of *Trichosporon* identified by molecular methods and discussed the susceptibility in vitro of these species to ten different antifungal drugs.

## Material and Methods

**Identification by genus.** Strains were obtained from patients of the Hospital das Clinicas of Universidade Federal do Espirito Santo, Brazil (Table 1). The samples were sent to Brazilian Laboratory for Mycological Diagnosis and all isolates were previously identified at the genus level on the basis of urease enzyme production, absence of fermentation of carbohydrates and the most typical microscopic features of this genus, the presence of arthroconidia on cornmeal Tween 80 agar, associated to the production of pseudohyphae, hyphae and blastoconidia [13].

### Molecular identification by species:

**Amplification of the Internal Transcribed Spacer (ITS) and Intergenic Transcribed Spacer (IGS1) regions:** Yeast cultures, DNA extraction and purification were done

by using methods described elsewhere [32]. The DNA segments comprising the region ITS1 and ITS2 were amplified with primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3') [41], and DNA segments comprising the region IGS1 were amplified with primers 26SF (IGS1-26SS) (5' ATCCTTTGCAGACGACTTGA3') and 5SR (IGS2-5,8S) (5' AGCTTGACTTCGCAGATCGG 3') [36]. Reaction mixtures contained 0.5 µM of each primer, 0.2 mM of each dNTP, 5 µl of PCR buffer 10X (including 25 mM of magnesium chloride) (Applied Biosystem, Spain), 2.5 U Taq DNA polymerase (Amplitaq, Applied Biosystem) and 25 ng of DNA in a final volume of 50 µl. The samples were amplified in a GeneAmp PCR System 9700 (Applied Biosystem) using the following cycling parameters: one initial cycle of 2 min at 94 °C, followed of 35 cycles of 30 s at 94 °C, 45 s at 56 °C, and 2 min at 72 °C, and one final cycle of 5 min at 72 °C. The PCR products obtained from both ITS and IGS1 ribosomal DNA regions were analyzed in a 0.8% agarose gel and then sequenced.

**Sequencing of ITS and IGS1 regions of the ribosomal DNA:** Sequence reactions were done using 2 µl from a DNA sequencing kit (BigDye Terminator Cycle Sequencing Ready Reaction, Applied Biosystem), 1 µM of the primers (ITS1, ITS4, 26SF or 5SR) and 3 µl of the PCR product in a final volume of 10 µl. Sequences were assembled and edited using the SeqMan II and EditSeq software packages (DNASTar, Inc. Lasergene, USA). The sequence analyses were performed by comparing them with the nucleotide sequences of *Trichosporon* reference isolates obtained from the database of the Department of Mycology of the Spanish National Center for Microbiology, and further analysis was done with the help of InfoQuest FP software, version 4.50 (Bio-Rad, Spain). Phylogenetic analyses were conducted using maximum parsimony clustering. Phylogram stability was assessed via parsimony bootstrapping with 2,000 simulations. Phylograms were outgroup rooted with *Trichosporon cutaneum* (CBS 2466).

**Table 1.** *Trichosporon* species identification and MICs of ten antifungal drugs.

Strain ID	Source	Species (Genotype)	AMB	5FC	FLZ	ITZ	VRZ	RVZ	POS	CPF	MCF	AND
6504	Urine	<i>T. asahii</i> (1)	4	4	8	0.12	0.12	0.12	0.12	16	16	16
6501	Urine	<i>T. asahii</i> (1)	8	64	8	0.5	0.25	0.5	0.25	16	16	16
6503	Urine	<i>T. asahii</i> (1)	4	32	32	1	1	4	0.5	16	16	16
6509	Nail	<i>T. asahii</i> (1)	0.25	64	8	0.25	0.25	0.25	0.5	16	16	16
6513	Blood	<i>T. asahii</i> (1)	8	8	8	0.5	0.25	0.5	0.5	16	16	16
6508	Nail	<i>T. asahii</i> (3)	4	32	8	1	0.5	1	0.5	16	16	16
6502	Blood	<i>T. asahii</i> (5)	16	32	16	0.25	0.25	0.5	0.5	16	16	16
6510	Nail	<i>T. asahii</i> (5)	8	64	4	0.5	0.25	0.5	0.5	16	16	16
649	Skin	<i>T. asahii</i> (6)	32	16	128	16	16	16	16	16	16	16
6507	Nail	<i>T. faecale</i>	1	8	8	0.25	0.25	0.5	0.5	16	16	16
6506	Skin	<i>T. faecale</i>	16	4	64	8	8	8	4	16	16	16
6505	Nail	<i>T. faecale</i>	16	16	8	0.25	0.5	0.12	0.5	16	16	16
6515	Nail	<i>T. faecale</i>	16	64	32	1	0.5	1	0.5	16	16	16
998	Nails	<i>T. faecale</i>	4	16	128	16	16	16	16	16	16	16
6512	Scalp	<i>T. inkin</i>	16	64	64	1	8	8	8	16	16	16
6516	Skin	<i>T. inkin</i>	0.5	64	1	0.25	0.01	0.25	0.5	16	16	16
297	Blood	<i>T. inkin</i>	1	128	4	0.25	0.06	0.06	0.06	16	16	16
6514	Skin	<i>T. coremiiforme</i>	1	32	4	0.12	0.06	0.12	0.12	16	16	16
997	Nails	<i>T. coremiiforme</i>	32	8	128	16	16	16	16	16	16	16
58	Vaginal mucosa	<i>T. asteroides</i>	2	128	32	0.50	0.25	0.25	0.25	16	16	16
67	Vaginal mucosa	<i>T. japonicum</i>	2	128	32	0.50	0.25	0.25	0.25	16	16	16

AMB: amphotericin B; 5FC: flucytosine; FLZ: fluconazole; ITZ: itraconazole; VRZ: voriconazole; RVZ: ravuconazole; POS: posaconazole; CPF: caspofungin; MCF: micafungin; AND: anidulafungin.

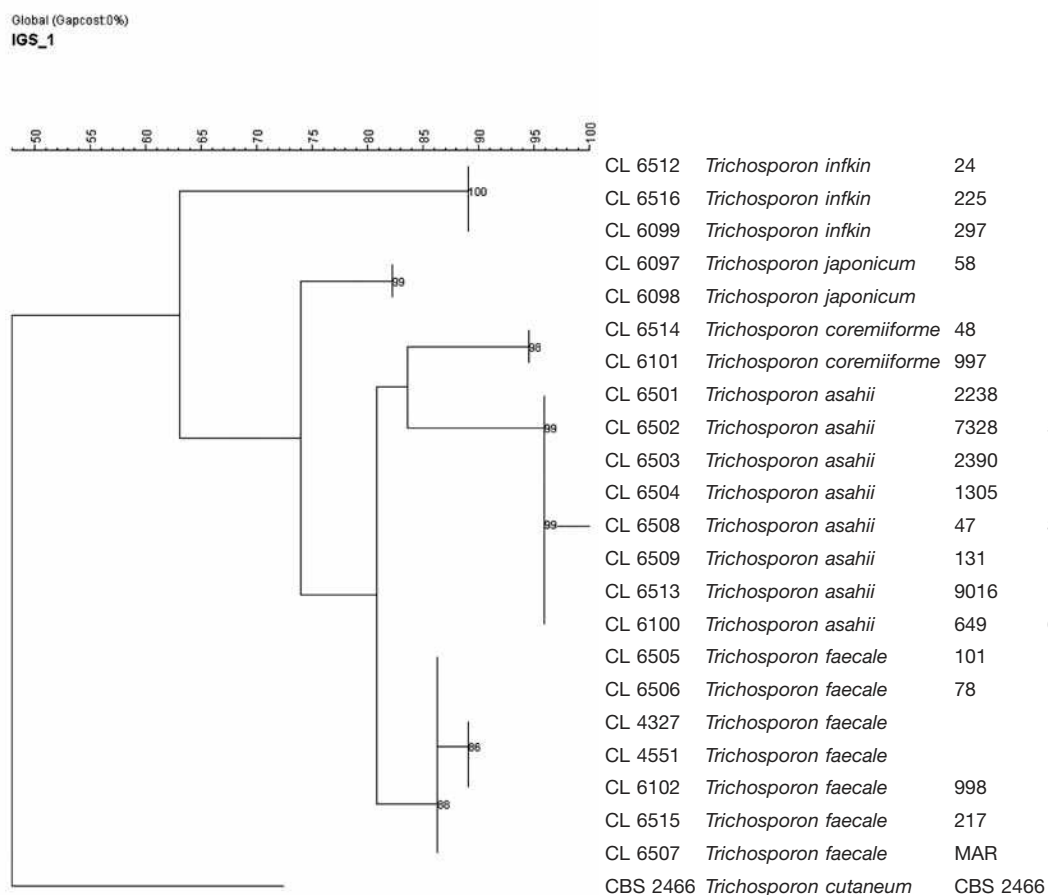


Figure. Rooted phylogenetic tree of *Trichosporon* species by using parsimony cluster analysis and 2,000 bootstrap simulations based on aligned IGS1 sequences.

**Susceptibility testing.** The susceptibility testing strictly followed the recommendations proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST, discussion document 7.1) [7]. These recommendations are based on NCCLS procedures described in M27-A2 document [23] but with some modifications to allow for automation of the susceptibility method and to shorten the incubation period from 48 to 24 h. All microplates were wrapped with film sealer to prevent the medium from evaporating, attached to an electrically driven wheel inside the incubator, agitated at 350 rpm and incubated at 30 °C. *Candida parapsilosis* ATCC22019 and *Candida krusei* ATCC 6258 were used as quality control strains. The antifungal agents used in the study were as follows: Amphotericin B (AMB, Sigma Aldrich Quimica S.A., Spain), flucytosine (5FC, Sigma Aldrich Quimica), fluconazole (FLZ, Pfizer S.A, Spain), itraconazole (ITZ, Janssen S.A., Spain), voriconazole (VRZ, Pfizer S.A.), posaconazole (POS, Schering-Plough, USA), ravuconazole (RVZ, Bristol-Myers Squibb, USA), caspofungin (CPF, Merck & Co., Inc., USA), micafungin (MCF, Astellas Pharma Inc, Japan) and anidulafungin (AND, Pfizer S.A.). For amphotericin B the Minimum Inhibitory Concentration (MIC) endpoint was defined as the lowest drug concentration exhibiting reduction in growth of 90% or more compared with that of the control growth. For azole drugs and echinocandins the MIC endpoint was defined as 50% of inhibition.

## Results

The PCR amplification generated fragments of 600 kb (ITS) and 700 kb (IGS1) from rDNA, corresponding to *Trichosporon* spp. Sequencing of these amplifications allowed the identification of six species of *Trichosporon* spp. and detection of four genotypes of *T. asahii* (Figure). This species predominated and corresponded to 43% of *Trichosporon* isolates, followed by *T. faecale* (24%) and *T. inkin* (14%). Eighty three percent (5/6) of the strains isolated from deep sites (urine and blood) were identified as *T. asahii*, while only 17% (1/6) belong to a non-*T. asahii* species (*T. inkin*) (Table 1).

The susceptibility results are shown in table 1. Standard methods and MIC breakpoints for this genus are still not included in CLSI (former NCCLS) and EUCAST guidelines; therefore, in this study, the MIC values for *Trichosporon* species were based on the breakpoints previously established for *Candida* spp.: MIC values ( $\mu\text{g mL}^{-1}$ ) related with antifungal drug resistance were  $\geq 2$  for AMB,  $\geq 32$  for 5FC,  $\geq 1$  for ITZ,  $\geq 4$  for VRZ, RVZ and POS,  $\geq 2$  for echinocandins and  $\geq 8$  for FLZ [34] In general, the MIC values were high, independent of the species of *Trichosporon* or source (Table 1) and all species were insensitive to echinocandins in vitro.

AMB and 5FC were less active against *Trichosporon* spp. and with the exception of FLZ, the isolates tended to be more susceptible to triazole drugs. Overall, VRZ and POS demonstrated comparable and good activity, exhibi-

ting lower percentages of MICs related to resistant phenotypes (Table 2). When analyzed separately, *T. asahii* exhibited higher level of resistance to AMB and FLZ (89%) than other species (67% and 75%), whereas the two new triazole drugs VRZ and POS seem to be more effective in vitro for *T. asahii* species (11%) than for non-*T. asahii* (33%) (Table 2).

## Discussion

Previous reports have questioned the phenotypic methods for identification of the *Trichosporon* species. Additionally, current morphological and biochemical methodologies are unable to differentiate all *Trichosporon* species thus leaving molecular techniques as the most reliable method of species identification [17,33,35].

DNA-based identification was previously approached by the diversity of sequence polymorphisms of the Internal Transcriber Spacer 1 and 2 regions (ITS1 and ITS2) of rDNA genes. However, it was demonstrated that some species showed less than 1% dissimilarity in the ITS region and these sequence analyses are sometimes incapable of distinguishing between phylogenetically related species. Alternatively, authors have characterized and identified *Trichosporon* species by evaluating the Intergenic Spacer 1 (IGS 1) sequences, which present a greater discriminatory power than the ITS [32,36].

In this study, sequencing of ITS regions was unable to identify the species. The cladogram generated with ITS sequences could not distinguish at the species level, classifying all species in only one clade, whereas IGS1 sequences were more discriminatory, grouping different species in distinct clades. Undoubtedly, the sequencing of IGS1 regions is the most useful tool for *Trichosporon* identification (Figure).

The species *T. asahii* has been divided into six genotypes [33]. Isolates from Japan have mostly been classified as genotype 1 and in lower frequencies as genotypes 2 and 4 whereas strains from the United States were either genotypes 3 or 5 [24,36]. In this study, we have shown a predominance of genotype 1. Interestingly, genotype 6 has been found only in South America [33].

Despite the increased frequency and severity of deep-seated trichosporonosis, data on the antifungal susceptibilities of *Trichosporon* spp. is limited. After the taxonomic reevaluation, comparisons with most of the published reports are not possible since MIC variation may be species or strain dependent [2,27,39]. In this study, the in vitro activity of ten antifungal drugs were tested against *Trichosporon* spp. and overall azole drugs showed to be more effective in vitro than AMB, which is in agreement with other studies [17,27,28,38]. It was also observed that in vitro susceptibilities to azole drugs differ depending on the particular species. The *T. asahii* species appears to be more sensitive to azole drugs, especially VRZ and POS,

**Table 2.** Percentages of MICs values of antifungal drugs corresponding to phenotype of resistance.

Antifungal agents	<i>T. asahii</i> (n = 9)	non- <i>T. asahii</i> (n = 12)	Total (n = 21)
Amphotericin B	89% (8/9)	67% (8/12)	76% (16/21)
Flucytosine	67% (6/9)	58% (7/12)	62% (13/21)
Fluconazole	89% (8/9)	75% (9/12)	81% (17/21)
Itraconazole	33% (3/9)	42% (5/12)	38% (8/21)
Voriconazole	11% (1/9)	33% (4/12)	24% (5/21)
Ravuconazole	22% (2/9)	33% (4/12)	28% (6/21)
Posaconazole	11% (1/9)	33% (4/12)	24% (5/21)
Caspofungin	100% (9/9)	100% (12/12)	100% (21/21)
Micafungin	100% (9/9)	100% (12/12)	100% (21/21)
Anidulafungin	100% (9/9)	100% (12/12)	100% (21/21)

than the non-*T. asahii* species. The high percentage of *T. asahii* resistance to AMB and FLZ raises concern since these two drugs are more commonly used in Brazil for treatment of deep-seated mycosis and this species tends to be more frequent in this clinical form of fungal infection. In addition, our data showed that when MICs of FLZ achieve values  $\geq 64$   $\mu\text{g/ml}$  there is a pattern of cross-resistance to all azole drugs, including VRZ, RVZ and POS. Most multiresistant strains belong to non-*T. asahii* species, and no strain isolated from deep sites was multiresistant: Isolate 6512 (*T. inkin*, from scalp) was resistant to all agents, while isolates 649 (*T. asahii*, skin), 6506 (*T. faecale*, skin), 998 (*T. faecale*, nails), and 997 (*T. coremiformii*, nails) were resistant to all but one agent (5FC). (Table 1).

The speciation within this genus could facilitate clinical practice in distinguishing between *T. asahii* and less pathogenic species as well as determining their respective susceptibility profiles. However, different antifungal susceptibilities may exist within species, even within genotypes, and a possible approach to overcoming this current lack of standardized data would be to perform susceptibility testing in vitro for all *Trichosporon* isolates.

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