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Antimicrob Agents Chemother. 2005 Oct;49(10):4026-34.

https://doi.org/10.1128/AAC.49.10.4026-4034.2005

Running title: Identification of clinically relevant *Trichosporon* spp.

Susceptibility Patterns and Molecular Identification of *Trichosporon* Species

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Keywords: *Trichosporon* spp, Internal Transcribed Spacer Regions, Intergenic Transcribed Spacer Region, Amphotericin B resistance

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ABSTRACT

The physiological patterns, the sequence polymorphisms of the internal transcriber spacer (ITS) and intergenic spacer regions (IGS) of the ribosomal DNA, and the antifungal susceptibility profile were evaluated for their ability and specificity in the identification of 49 clinical isolates of *Trichosporon* spp. Morphological and biochemical methodology was unable to identify seven species and other were misidentified frequently. ITS sequencing was also unable to differentiate several species. However IGS1 sequencing identified unambiguously all *Trichosporon* isolates.

Following the results of DNA-based identification, *T. asahii* was the species most frequently isolated from deep sites (15/25 strains, 60%). In the main, other *Trichosporon* species were recovered from cutaneous samples. The majority of *T. asahii, T. faecale* and *T. coremiiforme* clinical isolates exhibited resistance *in vitro* to amphotericin B, with geometric means (GM) of MICs > 4 µg/ml. The other species of *Trichosporon* did not show high MICs of amphotericin B and GMs were < 1µg/ml. Azole agents were active *in vitro* against the majority of clinical strains. The most potent compound *in vitro* was voriconazole, with GM $\leq$ 0.14 µg/ml.

The sequencing of IGS identified correctly *Trichosporon* isolates, however, this technique is not available in many clinical laboratories and strains should be dispatched to reference centers where these complex methods are available. Therefore it seems more practical to perform antifungal susceptibility testing to all isolates belonging to *Trichosporon* spp. since correct identification could take several weeks delaying the indication of an antifungal agent, which exhibits activity against the infectious strain.

INTRODUCTION

The number of species of yeasts pathogenic for humans reported in the literature has greatly increased in recent decades. This increase is a consequence of the rise of hosts presenting factors, which predispose them to fungal infections such as cytotoxic chemotherapy, neutropenia, broad spectrum antibiotic treatment, steroids and invasive catheterization. In these situations almost all yeasts are potential pathogens (10,32).

Trichosporon species are causative agents of cutaneous infections and are involved in systemic, localized or disseminated mycoses, particularly in patients with underlying hematological malignancy, AIDS, burns and solid tumors (3,12,21). Likewise, non-immunosuppressed patients have suffered from *Trichosporon* infections associated with ophthalmologic surgery, infections of prosthetic devices, intravenous drug abuse, and peritoneal dialysis (16,20).

It was thought that there was only one species of *Trichosporon* and that it was most appropriately called *Trichosporon beigelii*. However, the genus *Trichosporon* has recently undergone extensive taxonomic reevaluation and a rearrangement of the genus has been proposed. Several morphologic and biochemical patterns were recognized among clinical and environmental isolates, and ultraestructural and DNA studies confirmed those findings, being *T. beigelii* split in a number of distinct species (13,14,17). So far, the genus *Trichosporon* includes seven species associated with human infections: *Trichosporon asahii*, *Trichosporon asteroides*, *Trichosporon cutaneum*, *Trichosporon inkin*, *Trichosporon jirovecii*, *Trichosporon mucoides*, and *Trichosporon ovoides*. *T. asahii* appears to be much more common in cases of systemic mycosis while other *Trichosporon* species are involved in superficial skin lesions (2). In addition, differences per species in their antifungal susceptibility profile have been reported as well (23).

The identification of these fungal pathogens by conventional methods is often difficult and inconclusive frequently. DNA-based molecular procedures can provide alternative and more useful methods for characterization and identification of *Trichosporon* spp. (26,28,30). In this study, the morphologic and biochemical patterns, the sequence polymorphisms of the internal transcriber spacer 1 and 2 regions (ITS1 and ITS2), the intergenic spacer 1 (IGS1) of the ribosomal DNA, and the antifungal susceptibility profile were evaluated for their ability and specificity in the identification of a collection of 49 clinical isolates of *Trichosporon* spp.

MATERIAL AND METHODS

Fungi

Clinical strains. A collection of 49 clinical isolates was included in the study. All strains were recovered from 42 Spanish and Argentinean hospitals through a period of ten years, from 1993 to 2002. The isolates were obtained from a variety of clinical sources as they are displayed in table 1. Twenty-four strains were isolated from superficial sites and 25 from deep sites including eight blood cultures, five respiratory tract samples and three urines. Each isolate was obtained from a different patient. The isolates were sent to Mycology Reference Laboratory of National Center for Microbiology of Spain for identification and susceptibility testing.

Reference strains and sequences obtained from the GenBank database. Table 2 shows the list of *Trichosporon* strains used for comparative analysis of the ITS and IGS sequences.

Outgroup strains. Sequences of ITS and IGS of *Cryptococcus neoformans* (CBS 131 No. Acc. GenBank AJ300916) and *C. neoformans* var. *gattii* (CBS 6956 No. Acc. GenBank AJ300920) were used as outgroups.

Morphological and biochemical identification

The clinical strains were subcultured on 4% malt extract/0.5% yeast extract agar (MEYA). After 10 days at 30ºC, macroscopic and microscopic examinations were done. The isolates were identified by routine physiological tests: fermentation of and growth on carbon sources, growth on nitrogen sources, growth at various temperatures and ability for hydrolyzing urea (2,7,14). Table 3 displays the distinctive characteristics per species of *Trichosporon*. Biochemical and morphological characterizations were done according to data depicted in references 2, 7 and 14. For *T. dermatis* the biochemical characteristics were obtained from reference 31 (31).

PCR and DNA sequencing of ITS and IGS regions

Yeasts were cultured in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, OXOID, Madrid, Spain), for 24-48 h at 150 rpm at 30ºC. One ml of the medium was centrifuged at 13.000 rpm for 10 minutes. The pellet was suspended in 1 ml of cold SE buffer (20 mM citrate-phosphate buffer pH: 5.6, 50 mM EDTA, 0.9 sorbitol) and centrifuged under the same conditions. This process was repeated twice. A small quantity of glass pearls was added together with 50 µl of lysis buffer (50 mM Tris- HCl pH: 7.2, 50 mM EDTA, 3% sodium dodecyl sulphate). The mixture was vortexed at low speed for at least 15 seconds. Lysis buffer (400 µl and 4 µl of beta-mercaptoethanol (final concentration 1%, Sigma-Aldrich Química) were added, and the mix incubated at 65ºC

for 2 hours. The blend was gently mixed every 30 minutes. DNA was then purified by repeated phenol-chloroform-isoamilic (25:24:1) extractions, ethanol precipitation and RNAase treatment. Finally, DNA concentration was estimated comparing the bands obtained from each sample with the bands of known amounts of lambda phage DNA in a 0.8% agarose gel (Pronadisa, Madrid, Spain). DNA was purified using Chroma Spin + TE 200 columns from (Clontech Laboratories, Inc, Becton Dickinson, Madrid, Spain).

DNA segments comprising the region ITS1 and ITS2 were amplified with primers ITS1 (5` TCCGTAGGTGAACCTGCGG 3`) and ITS4 (5`TCCTCCGCTTATTGATATGC 3`) (34). DNA segments comprising the region IGS1 were amplified with primers 26SF (IGS1-26SS) (5` ATCCTTTGCAGACGACTTGA 3`) and 5SR (IGS2-58S) (5` AGCTTGACTTCGCAGATCGG 3`)(26). Reaction mixtures contained 0.5 µM of each primer, 0.2 mM of each dNTP, 5 µl of PCR buffer 10X (Applied Biosystem, Madrid, Spain), 2.5 U *Taq* DNA polimerase (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 94ºC, followed of 35 cycles of 30 sec at 94ºC, 45 sec at 56ºC, and 2 min at 72ºC, and one final cycle of 5 min at 72ºC. The reaction products were analyzed in a 0.8 % agarose gel.

Sequence reactions were done using 4 µl a DNA sequencing kit (BigDye Terminator Cycle Sequencing Ready Reaction, Applied Biosystem), 1 µM of the primers (ITS1, ITS4, 26SF and 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, Madison, USA). The sequence analysis was performed by comparison with the nucleotide sequences of *Trichosporon* reference isolates obtained from the GenBank database (http://www.ncbi.nih.gov/Genbank/) and from sequences of reference strains from the CBS (Centraalbureau voor Schimmelcultures, Delft, The Netherlands). Those strains are displayed in table 2.

Phylogenetic Analyses

All phylogenetic analyses were conducted with Fingerprinting II informatix software version 3.0 (BIORAD laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed via parsimony bootstrapping with 1000 simulations. Phylograms were outgroup rooted with *Cryptococcus neoformans* (CBS 132) and *C. neoformans* var. *gattii* (CBS 6956).

Antifungal susceptibility testing

The susceptibility testing followed strictly the recommendations proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing for testing fermentative yeasts (AFST-EUCAST, discussion document 7.1) (24). These recommendation are based on NCCLS procedure described in M27-A2 document (22), but including some modifications in order to allow for automation of the susceptibility method and to permit the incubation period to be shortened from 48 to 24 h. Briefly, the susceptibility testing included RPMI supplemented with 2% glucose as assay medium, inoculum size of 10^5 CFU/ml, flat-bottomed trays, and spectrophotometric reading. In order to improve the growth a minor modification was include (25). That was, 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 30ºC for 48 hours. *Candida parapsilosis* ATCC22019 and *Candida krusei* ATCC6258 were used as quality control strains.

The antifungal agents used in the study were as follows: Amphotericin B (Sigma Aldrich Quimica S.A., Madrid, Spain), 5-flucytosine (Sigma Aldrich Quimica), fluconazole (Pfizer S.A, Madrid, Spain), itraconazole (Janssen S.A., Madrid, Spain) and voriconazole (Pfizer S.A.,). The MICs were determined at 24 and 48 h. MICs were obtained measuring the absorbance at 530 nm with a MRXII reader (Dynatech, Cultek, Madrid, Spain). For AMB the MIC endpoints were defined as the lowest drug concentration exhibiting reduction in growth of 90% or more compared with that of the control growth. For flucytosine and azole drugs the MIC endpoint was defined as 50% of inhibition.

RESULTS

Morphological and biochemical identification

Table 1 includes the identification of clinical isolates tested obtained by morphological and biochemical methods. Table 3 shows the differential morphological and biochemical characteristics of *Trichosporon* species. Sixteen strains were identified as *T. mucoides,* fifteen as *T. asahii*, eleven as *T. inkin,* four as *T. cutaneum*, and three as *T. ovoides*.

Molecular identification

The genetic relatedness of 49 clinical strains (table 1) together with reference strains and nucleotide sequence data obtained form GeneBank included in table 2 were analyzed. Aligned complete sequences of the IGS1, and ITS1 and ITS2 were analyzed phylogenetically using two strains of *C. neoformans* (CBS 132 and 6956 respectively) to root the trees.

Maximum parsimony analysis of ITS sequences did not resolve adequately *Trichosporon* species as figure 1 shows. Three clades were identified that can be consulted in table 1. Clade I had a maximum of 8 base conversions and clade III a maximum of 3 base conversion. As an exception, in clade II, *T. sporotrichoides. T. brasiccae and, T. veenhuisii* were resolved. However, there is only one sequence for each species and therefore any conclusion cannot be obtained. The rest of species including in this clade have a maximum of 5 base conversions.

Maximum parsimony analysis of IGS sequences resolved adequately all *Trichosporon* species. Figure 2 shows a rooted cladogram with all clinical isolates and the respective reference strains or sequences from GeneBank included in this study. Those species without clinical isolates are not included in the cladogram, although they were unambiguously differentiated. Therefore, each clinical isolate was considered to belong to the species that maximum parsimony analysis of IGS sequences indicated. Table 1 contains the final identification of clinical strains. Thus, the 49 clinical isolates were identified as follows: fifteen *T. asahii,* eight *T. dermatis*, seven *T. inkin,* five *T. ovoides*, four *T. faecale*, two *T. coremiiforme*, two *T. cutaneum*, two *T. jirovecii,* two *T. japonicum*, one *T. montevideense* and, one *T. domesticum*. Notably, all *T. asahii* were isolated from deep samples while other species were more frequently associated with superficial sources (100% vs 29.4%; table 1). As exceptions,

one *T. faecale* isolated from a catheter, two *T. coremiiforme* from urine and a subcutaneous abscess, two *T. inkin* from a bone biopsy and a subcutaneous abscess, one *T. ovoides* from peritoneal fluid, one *T. jirovecii* from urine, one *T. japonicum* from pleural fluid and two *T. dermatis* from subcutaneous abscess and blood culture respectively. The last two species *T. japonicum* and *T. dermatis* have not been associated with human samples before but in this study were detected twice and seven times, respectively.

Discrepancies among molecular and morphological and biochemical identification

Table 4 shows the discrepancies among molecular and morphological and biochemical identification. Morphological and biochemical methods were unable to identify the following species: *T. dermatis, T. faecale, T. coremiiforme, T. jirovecii, T. japonicum, T. montevideense* and *T. domesticum* (table 4). In addition *T. asahii, T. ovoides* and *T. cutaneum* were misidentified most of the times as other *Trichosporon* species (table 4). As an exception, *T. inkin* was correctly identified by classical methodology 6 out 7 times (Table 4).

Antifungal susceptibility testing

Susceptibility results are showed in table 4. Table displays geometric means (GM) and ranges of MICs, per antifungal agent and grouped per *Trichosporon* spp. and per identification procedure. According to DNA-based identification, it should be stressed that the majority of *T. asahii, T. faecale* and *T. coremiiforme* clinical isolates exhibited resistance *in vitro* to amphotericin B, with GM of MICs >4.0 µg/ml. Only one isolate of *T. faecale* had an amphotericin B MIC of 0.25 µg/ml while the rest had MICs $\geq$ 2 µg/ml. The other species of *Trichosporon* did not show high MICs of amphotericin B and GMs were < 1.0 µg/ml. In addition, all eleven species exhibited high MICs of flucytosine, and most isolates were resistant *in vitro*, with MICs over 16.0 µg/ml. On the contrary, azole agents were active *in vitro* against the majority of clinical strains, independently of species of *Trichosporon* analyzed. The most potent compound *in vitro* was voriconazole, with GM $\leq$ 0.14 µg/ml.

Four strains of *T. asahii* (4/15, 26.6%) and three isolates of *T. dermatis* (3/8, 37.5%) exhibited MICs of fluconazole as high as 32.0-64.0 µg/ml. Five of those strains (two *T. asahii* and all *T. dermatis*) showed higher MICs of itraconazole ($\geq$ 2.0 µg/ml) and voriconazole ($\geq$ 8.0 µg/ml) as well.

There were differences in antifungal susceptibility were found when MICs were analyzed per isolation site (deep vs. superficial isolates). Thus, the strains isolated form deep samples had higher MICs to all antifungals except for flucytosine. The most significant difference was found among the MICs of amphotericin B. Hence, the GM of amphotericin B of strains isolated from deep samples were 2.7 μg/ml and from superficial sources 0.3 μg/ml.

DISCUSSION

The identification of yeasts is based largely on physiological and biochemical characteristics. The tests most used for routine identification purposes are fermentation of and growth on carbon sources, growth on nitrogen sources, requirements of vitamins, growth at various temperatures, hydrolysis of urea and resistance to antibiotics (2,14). The combined used of biochemical characteristics and direct observation of fungal structures under a light microscope permits the identification of species causing the majority of human infections. However, the number of new species of opportunistic yeasts causing mycoses has increased during the past two or three decades, making the identification by conventional methods more difficult, even having expertise in Mycology (19). In addition, conventional identification by the evaluation of morphological and biochemical characteristics can be laborious, leading to inconclusive or presumptive identifications, particularly for infrequent pathogenic species.

In order to overcome these drawbacks, several groups have designed molecular procedures for identifying yeasts. One of the main advantages of molecular methods is their sensitivity and specificity, being fully discriminative even for species closely related (7). The majority of molecular methods are PCR-based techniques using either specific probes or universal fungal primers, normally directed to conserved regions of ribosomal DNA, particularly towards the ITS regions (4-6). The identification using ITS regions must include a subsequent analysis such as sequencing or restriction fragment length polymorphism analysis. This approach has been put into practice in clinical laboratories for rapid detection and identification of yeasts found in positive blood cultures, being evaluated as a promising diagnostic tool (11,18).

Sugita et al have developed procedures based on ITS regions for identification of *Trichosporon* spp. These procedures are capable of characterizing the majority of species of *Trichosporon*, including those pathogenic for humans (26,28,30). The results obtained with these techniques have led to reclassify both clinical and environmental isolates of these species, design a nested PCR assay to detect DNA in sera for the diagnosis of deep-seated trichosporonosis, describe the first bloodstream infection due *T. asteroides* and associate *T. ovoides* with hypersensitivity pneumonitis (15,27-29). Recently, DNA-based procedures for identification of *Trichosporon* have been stepped up by the sequence analysis of the ribosomal DNA intergenic spacer 1 regions (IGS) which

provides a more powerful and alternative method to distinguish between phylogenetically closely related species (26).

The data presented in this work suggest that morphological and biochemical methodology is not an useful and reliable method for *Trichosporon* identification. This methodology was unable to identify seven species and others were misidentified frequently (table 4). On the other hand, sequencing of DNA fragments seems to be a better approach for identification to species level of this genus. However, ITS sequencing was also unable to differentiate several species as unrooted cladogram of figure 1 shows. Therefore, three clades were established. Clade I comprised *T. cutaneum, T. dermatis, T. jirovecii*, *T. moniliforme* and *T. mucoides;* clade II, *T. brasiccae*, *T. domesticum*, *T. dulcitum*, *T. gracile*, *T. laibachii*, *T. loubieri*, *T. montevideense*, *T. sporotrichoides* and *T. veenhuisii*, and clade III, *T. asahii, T. asteroides, T. coremiiforme, T. faecale, T. inkin, T. japonicum* and *T. ovoides*. Furthermore, looking at sequences (data not shown) there are very few differences among the ITS sequences of each clade and therefore it is hazardous to assume the identification to level species based on this DNA fragment.

IGS sequences identified unambiguously all *Trichosporon* isolates as Figure 2 shows. For some species as *T. jirovecii, T. dermatis. T. inkin, T. faecale* and, *T. asahii,* polymorphisms were detected in IGS sequences. Further experiments with more strains are required to ascertain if those polymorphisms can be used for genotyping. However, there are preliminary evidences of the existence of five genotypes when IGS sequences of *T. asahii* were analyzed (26). In this work, *T. asahii* was also divided in 5 types supporting the results obtained by Sugita et al (26).

A second area to consider is the antifungal susceptibility testing of *Trichosporon* spp. A 1990 study defined *T. beigelii* as an emerging pathogen resistant to this antifungal compound (33). After the taxonomic reevaluation, it has been proven that *T. asahii* is more resistant *in vitro* to amphotericin B than to triazole compounds (1,23). These data *in vitro* has been corroborated by results *in vivo* and successful outcomes with treatments with voriconazole have been documented in some cases of deep infections due to this species that did not respond to amphotericin B therapy (8,9,35). On the contrary, the non-*T. asahii* species appear more resistant to triazole

agents than amphotericin B (23), although these data have not been proven *in vivo*. Our results confirmed that *T. asahii* is resistant *in vitro* to amphotericin B because all isolates exhibited amphotericin B MICs $\geq$ 2.0 µg/ml. In addition, *T. faecale* and *T. coremiiforme* seem to be also *in vitro* resistant to amphotericin B because all except one of the isolates included in this study had MICs > 2.0 µg/ml. These two species are considered by some authors varieties of *T. asahii* (14). The other *Trichosporon* species were susceptible *in vitro* to the polyene. With regard to azole agents, resistance *in vitro* to fluconazole was found in a minority of *T. asahii* and *T. dermatis* isolates, being the remaining isolates susceptible *in vitro*, although a number of fluconazole MICs were of 4.0-8.0 µg/ml . Itraconazole and voriconazole were the most potent agents *in vitro* against all *Trichosporon* spp., particularly voriconazole with GM of MICs under 0.14 µg/ml, confirming that these compounds can be useful in treating these infections.

This work shows that biochemical and morphological identification and ITS sequencing are not reliable techniques for *Trichosporon* spp. identification as they were unable to distinguish between species. Correct characterization of these species can be significant at therapeutic level in view of their distinct antifungal susceptibility profile, particularly for *T. asahii,* which is highly resistant to amphotericin B. The sequencing of IGS identified correctly *Trichosporon* isolates, however, this technique is not available in many clinical laboratories and strains should be dispatched to reference centers where these complex methods are available. Therefore it seems more practical to perform antifungal susceptibility testing to all isolates belonging to *Trichosporon* spp. since correct identification could take several weeks delaying the indication of an antifungal agent, which exhibits activity against the infectious strain.

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