Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 58 (2007) 435–440
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Mycology

# Genotype distribution of clinical isolates of *Trichosporon asahii* based on sequencing of intergenic spacer 1

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Received 24 January 2007; accepted 2 March 2007

**Abstract**

The sequence polymorphisms of intergenic transcribed spacer and the antifungal susceptibility profile of 18 *Trichosporon asahii* isolates from Spain, Argentina, and Brazil together with 43 intergenic transcribed spacer 1 sequences deposited in the GenBank were analyzed. Six genotypes were detected instead of 5 genotypes described previously. Genotype 1 was the most common found comprising 57.3% of all strains, followed by genotype 3 (14.7%) and genotype 5 (13.1%). Spanish strains had members in all genotypes except 2, whereas South American isolates were grouped with genotypes 1, 3, and 6. Our results indicate that all genotypes are present in at least 2 countries suggesting a worldwide distribution. On the other hand, genotype 6 was not previously described but was only composed of 2 South American strains isolated from a subcutaneous abscess and skin. All isolates showed amphotericin B MICs $\geq$2 mg/L supporting the in vitro resistance of this specie to this antifungal. Three isolates from South America showed high MICs to all antifungals analyzed. The true epidemiologic usefulness of classifying *T. asahii* in genotypes should be ascertaining analyzing a high number of isolates from many countries.


*Keywords:* *Trichosporon* spp.; Intergenic transcribed spacer region; Genotypes; Antifungal susceptibility testing

## 1. Introduction

*Trichosporon* spp. are causative agents of cutaneous infections and are involved in systemic, localized, or disseminated mycoses, particularly in patients with underlying hematologic malignancy, AIDS, burns, and solid tumors (Gueho et al., 1994; Cawley et al., 2000; Moretti-Branch et al., 2001). The genus *Trichosporon* includes 7 species associated with human infections: *Trichosporon asahii*, *Trichosporon asteroides*, *Trichosporon cutaneum*, *Trichosporon inkin*, *Trichosporon jirovecii*, *Trichosporon mucoides*, and *Trichosporon ovoides* (Rodriguez-Tudela et al., 2005). *T. asahii* appears to be much more common in cases of systemic mycosis, whereas other *Trichosporon* spp. are involved in superficial skin lesions (Barnett et al., 2000) (Rodriguez-Tudela et al., 2005). Recently, Sugita et al., (1998, 1999, 2002) have done an extensive work analyzing the internal transcribed spacers (ITSs) and intergenic transcribed spacer 1 (IGS1) of *Trichosporon* spp. They concluded that IGS1 is superior to ITS for identifying the different species in the *Trichosporon* genera, especially those species phylogenetically closely related. In addition, they found out that IGS1 might be used as an epidemiologic tool because they described 5 different genotypes in *T. asahii*. Some genotypes were specifically associated with Japan (genotypes 1, 2, and 4) and others with the United States (genotypes 3 and 5) (Sugita et al., 2002). Therefore, we decided to study the sequence polymorphisms of IGS1 and the antifungal susceptibility profile of 18 *T. asahii* from Spain, Argentina, and Brazil together with 43 IGS1 sequences deposited on the GenBank to ascertain which genotypes are predominant in these countries.

## 2. Materials and methods

### 2.1. Fungi

#### 2.1.1. Clinical strains

A collection of 18 clinical isolates was included in the study (4 from Argentina, 1 from Brazil, and 13 from Spain). All strains were recovered from Spanish, Argentinean, and

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doi:10.1016/j.diagmicrobio.2007.03.001

Table 1
Clinical sources, country of origin, genotypes, and MICs of *T. asahii* isolates analyzed

| No. | Strain code | Clinical Source | Country | Genotype | AmB | 5FC | FZ | ITZ | VZ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | CL-4323 | Blood culture | Argentina | 1 | 16.0 | 16.0 | 64.0 | 8.0 | 8.0 |
| 2 | CL-2457 | Bronchial secretion | Spain | 1 | 2.0 | 16.0 | 1.0 | 0.12 | 0.03 |
| 3 | CL-4322 | Blood culture | Argentina | 1 | 16.0 | 64.0 | 64.0 | 8.0 | 8.0 |
| 4 | CL-4296 | Catheter | Argentina | 1 | 2.0 | 64.0 | 32.0 | 0.5 | 0.5 |
| 5 | CL-4099 | Pleural fluid | Spain | 1 | 4.0 | 64.0 | 32.0 | 0.5 | 0.25 |
| 6 | CL-3553 | Blood culture | Spain | 1 | 4.0 | 8.0 | 8.0 | 1.0 | 0.25 |
| 7 | CL-3876 | Sputum | Spain | 1 | 2.0 | 4.0 | 8.0 | 0.25 | 0.03 |
| 8 | CL-3562 | Blood culture | Spain | 1 | 2.0 | 4.0 | 8.0 | 0.5 | 0.25 |
| 9 | CL-5891 | Cerebrospinal fluid | Spain | 1 | 32.0 | 16.0 | 16.0 | 1.0 | 0.25 |
| 10 | CL-6212 | Urine | Spain | 1 | 4.0 | 32.0 | 16.0 | 0.5 | 0.25 |
| 11 | CL-2324 | Urine | Spain | 3 | 4.0 | 32.0 | 0.5 | 0.25 | 0.12 |
| 12 | CL-4032 | Bronchial secretion | Spain | 4 | 8.0 | 16.0 | 8.0 | 0.25 | 0.12 |
| 13 | CL-3316 | Blood culture | Spain | 4 | 16.0 | 16.0 | 8.0 | 1.0 | 0.5 |
| 14 | CL-2846 | Blood culture | Spain | 5 | 4.0 | 8.0 | 8.0 | 0.25 | 0.25 |
| 15 | CL-2847 | Blood culture | Spain | 5 | 4.0 | 8.0 | 4.0 | 0.06 | 0.25 |
| 16 | CL-2848 | Blood culture | Spain | 5 | 8.0 | 4.0 | 8.0 | 0.5 | 0.25 |
| 17 | CL-4295 | Subcutaneous abscess | Argentina | 6 | 16.0 | 4.0 | 1.0 | 0.03 | 0.12 |
| 18 | CL-6100 | Skin | Brazil | 6 | 32.0 | 16.0 | 64.0 | 16.0 | 16.0 |
| Geometric mean of MIC results | | | | | 6.3 | 14.2 | 9.3 | 0.55 | 0.34 |
| Range of MIC | | | | | 2.0–32.0 | 4.0–64.0 | 0.5–64.0 | 0.03–16.0 | 0.03–16.0 |

AmB = amphotericin B; 5FC = flucytosine; FZ = fluconazole; ITZ = itraconazole; VZ = voriconazole.

Brazil hospitals through a period of 12 years, from 1993 to 2005. The isolates were obtained from a variety of clinical sources as they are displayed in Table 1. 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.

### *2.1.2. Reference strains and sequences obtained from the GenBank database*

Fig. 2 shows the list of 43 *Trichosporon* strains and the GenBank accession number used for comparative analysis of the IGS sequences.

### *2.2. Polymerase chain reaction and DNA sequencing of IGS region*

Yeasts were cultured in YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose; OXOID, Madrid, Spain) for 24 to 48 h at 150 rpm at 30 °C. One milliliter of the medium was centrifuged at 13 000 rpm for 10 min. The pellet was suspended in 1 mL of cold SE buffer (20 mmol/L citrate–phosphate buffer, pH 5.6, 50 mmol/L ethylenediaminetetraacetic acid [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 mmol/L Tris–HCl, pH 7.2, 50 mmol/L EDTA, 3% sodium dodecyl sulfate). The mixture was vortexed at low speed for at least 15 s. Lysis buffer (400 and 4 μL of β-mercaptoethanol; final concentration, 1%; Sigma-Aldrich Química, Madrid, Spain) were added, and the mixture was incubated at 65 °C for 2 h. The blend was gently mixed every 30 min. 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, Becton Dickinson, Madrid, Spain).

DNA segments comprising the region IGS1 were amplified with primers 26SF (IGS1-26SS) (5′ ATCCTTTGCAGACGACTTGA 3′ ) and 5SR (IGS2-58S) (5′ AGCTTGACTTCGCAGATCGG 3′ )(Sugita et al., 2002). Reaction mixtures contained 0.5 μmol/L of each primer, 0.2 mmol/L of each dNTP, 5 μL of polymerase chain reaction (PCR) buffer 10× (Applied Biosystem, Madrid, 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: 1 initial cycle of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 56 °C, and 2 min at 72 °C, and 1 final cycle of 5 min at 72 °C. The reaction products were analyzed in a 0.8% agarose gel.

Sequence reactions were done using 4 μL of a DNA sequencing kit (BigDye Terminator Cycle Sequencing Ready Reaction, Applied Biosystem), 1 μmol/L of the primers (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; Lasergene, Madison, WI). 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 Figure 2.