

GENETIC DIVERSITY AND ANTIFUNGAL SUSCEPTIBILITY TESTING OF *TRICHOSPORON ASAHII* ISOLATED OF INTENSIVE CARE UNITS PATIENTS

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Submitted: December 10, 2007; Returned to authors for corrections: February 08, 2007; Approved: July 05, 2008.

ABSTRACT

Trichosporon asahii is an opportunistic pathogen, associated with a high mortality rate in immunocompromised patients. In this study, ten isolates, recovered from oral cavity and urine of patients in Intensive Care Units (ICU) over six months, were identified by classical and molecular methods, typed by RAPD and tested *in vitro* for susceptibility to fluconazole, itraconazole, 5-flucytosine and amphotericin B. A total agreement between the identification of *Trichosporon* sp by PCR based on sequences of the Internal Transcribed Spacer Regions (ITS) and on the sequences of small-subunit (SSU) ribosomal DNA (rDNA) was found. Randomly amplified of polymorphic DNA (RAPD), with primers P6 and M13, was used to determine the genomic profiles. The dendrogram analysis indicated that almost all strains showed similarity >0.9 among them and all strains were multidrug-resistant. This study brings new results on the identification and genotyping of *T. asahii* isolated from Brazilian ICU patients and information about their antifungal drugs susceptibility.

Key-words: *Trichosporon*, PCR, RAPD, susceptibility, antifungal drugs

INTRODUCTION

In the past few decades, a worldwide increase in the incidence of fungal infections has been observed, as well as the recognition of innocuous yeasts, previously thought incapable of harming the human body, as pathogens (38). The emergence of less common but medically important fungal pathogens has contributed to the rate of morbidity and mortality, especially in the increasingly expanding population of immunocompromised patients. Various groups have stressed the importance of new opportunistic fungal pathogens as causes of life-threatening infections (1,14). Among them, deep-seated trichosporonosis is a lethal opportunistic infection occasionally found in immunocompromised patients, particularly those who are neutropenic due to cytotoxic therapy for hematological malignancies.

Over the past decade, the taxonomy of the genus *Trichosporon* has been subjected to extensive revision on the basis of molecular data, and the previously named *T. beigellii* (or *T. cutaneum*) corresponds, in the most recent classification, to six different species: *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. inkin*, *T. mucoides*, and *T. ovoides* (15,16).

Trichosporon is found in the commensal mycobiota of the skin surface, mainly in the genito crural and perianal areas of homosexuals (47). This yeast is found in the normal skin, nails and mouth of healthy individuals (28,35,58), as well as, in Brazil, *T. asahii* was also recovered from rice and cassava used in fermented beverages (45).

Trichosporon infections are rare but have been associated with a wide spectrum of clinical manifestations, ranging from superficial involvement in immunocompetent individuals such as white piedra (40,47) to severe systemic disease in

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immunocompromised patients with a pattern similar to candidiasis (6,19,29,30,43,58). Rodrigues *et al.* (43) described twenty-two cases of nosocomial infection caused by *T. asahii*, detected during a period of six years (1999-2005) in a Brazil hospital. Recently, a case was reported of systemic *Trichosporon cutaneum* infection in a 3-year-old infant with Wilms' tumor (5). In the same hospital, an oropharyngeal secretion sample was collected from an AIDS patient with oral lesions and *Trichosporon pullulans* was isolated (34).

At the moment, *Trichosporon asahii* is the most clinically important pathogenic yeast in the genus *Trichosporon*, as this species causes both deep-seated infection and summer-type hypersensitivity pneumonitis (9,15,17,28,49,52). Sugita *et al.* (49) have suggested that *T. asahii* is common environmental pathogen, and Martins-Diniz *et al.* (25) have also encountered this genus in both biotic (professional health) and abiotic sources (areas of hospital) with prevalence around 30% among yeast isolated. Pini *et al.* (39) have found massive air contamination with *T. asahii* in the ward where neutropenic patients were staying and the corridor immediately outside.

Trichosporon infection is associated with high morbidity and mortality and is difficult to treat, frequently involving resistance to 5-fluorocytosine, azoles and amphotericin B (10,41). Walsh *et al.* (57) noted that blood isolates were more resistant to amphotericin B than skin isolates. The optimal antifungal therapy for these infections is unclear. There is growing evidence that azole drugs have good activity against *Trichosporon* spp. (3,11,36) and that combined administration of fluconazole and amphotericin B may be superior to either drug used alone against invasive infection (2,18). Meyer *et al.* (28) report a case of chronic disseminated *T. asahii* infection in a child with leukemia, where a cure was achieved after treatment with itraconazole.

The therapies are only effective if the disease is detected at an early stage, and, therefore, early diagnosis is an important factor in the successful management of patients with disseminated trichosporonosis. Unfortunately, difficulties in the identification of these microorganisms lead to delays in treatment and post-mortem diagnosis (30,31).

Species identification and molecular typing have become critical elements of nosocomial fungal outbreak investigations. The studies should include the origins of nosocomial infection, genetic comparison between invasive or noninvasive isolates, and comparison of the genotypes and susceptibilities of the isolates (53).

Many investigations of the intraspecies diversity and epidemiology of pathogenic fungi using random amplified polymorphic DNA (RAPD) analysis, hybridization with specific probes such as CA3, and multilocus enzyme electrophoresis (MLEE) have been reported, but there are only a few reports for *Trichosporon* species (12,48).

Sugita *et al.* (51) have sequenced the internal transcribed spacer (ITS) region and describe a rapid PCR-based approach

for detection of all species of emerging yeasts of the *Trichosporon* genus. The PCR product length alone identified all the isolates, the primers TRF (forward) and TRR (reverse) being chosen to align with regions were not conserved in other medically important yeasts (50). Their data indicate that the PCR detection system is useful for identifying these yeasts.

In Brazil there are few survey on these yeasts (30,34,43). In this study we examined the genetic diversity and biochemical characteristics of *T.asahii* strains recovered from clinical specimens (oral cavity and urine) from patients in Intensive Care Units (ICU), as well as the correlation between the drug susceptibility profile and genetic changes detected by RAPD (primer M13 and 6).

MATERIALS AND METHODS

Yeast isolates

All isolates were recovered from clinical specimens (two from oral cavity and eight from urine) from patients in Intensive Care Units (ICU), collected from April to September 2001. The isolates were subcultured on Sabouraud glucose agar (SGA). Isolates were identified by API 20C AUX (BioMérieux, France) strip in accordance with the manufacturer's instructions and by evaluation of their micromorphological features, on cornmeal Tween 80-agar. The interpretation of the data was based on Kurtzman and Fell (21).

Oligonucleotide primers and PCR amplification

The internal transcribed spacer (ITS) regions were amplified with primers ITS₁ (5' - TCC GTAGGT GAA CCT GCG G - 3') and ITS₄ (5' - TCC TCC GCT TAT TGA TAT GC - 3'), which were designed from conserved regions to amplify the 18S rDNA, 25S and 5,8S regions of ribosomal DNA (51). The primers, TRF (forward) (5' - AGA GGC CTA CCA TGG TAT CA - 3') and TRR (reverse) (5' - TAA GAC CCA ATA GAG CCC TA - 3'), were chosen because they would specifically amplify only *Trichosporon* species and to align with regions which were not conserved in other medically important yeasts (50). These PCR primers correspond to nucleotides 154 to 173 and 354 to 335 of *Saccharomyces cerevisiae* SSU rDNA. For both PCRs, the amplification reactions were performed in 25 µL sterile distilled water containing 1 µM of each primer, 3.0 µL of template DNA solution (1.5 µg/mL), 1.5 mM MgCl₂, 2.5U of *Taq* polymerase, 200 µM of each deoxynucleoside triphosphate (dNTP). The reaction mixtures were amplified in a Perkin-Elmer 9700 thermal cycler with following programs: for TRF/TRR, denaturation at 94°C for 3 min was followed by 30 cycles comprising denaturation at 94°C for 30 s, annealing at 56°C for 15 s and amplification at 72°C for 15 s, with a final extension period at 72°C for 10 min, for the ITS region, 94°C for 3 min followed by 40 cycles of 94°C for 1 min., 57°C for 1 min. and 72°C for 2.5 min., with a final 10 min period at 72°C. After thermal cycling, 5 µL of the amplified

products were run on 2.0% agarose gel in 1 × TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8) buffer for 150 min at 150 V, stained with 0.5 µg/mL ethidium bromide (GIBCO), and visualized under UV light, with the aid of Image Master VDS (PHARMACIA BIOTECH), and photographed.

RAPD analysis

Genomic DNA was extracted and purified by a slight modification of the method of Lasker *et al.* (22). RAPD was carried out with primer 6 (5'-d[CCC GTC AGC A]-3'), (Amersham Pharmacia Biotech) and M13 (5'-d[GAG GGT GGC GGT TCT]-3'), in a total volume of 25 µL, using the Ready-To-Go RAPD Kit in accordance with the manufacturer's instructions. The following cycle conditions were used for primer 6: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and amplification at 72°C for 2 min, with a final extension at 72°C for 10 min. The cycle used for primer M13 was: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 1 min, and amplification at 72°C for 20 s with a final extension at 72°C for 6 min. Amplification products were separated by electrophoresis on 2% agarose gels in 1X TBE buffer (0.1M Tris, 0.09 M boric acid, 0.001 M EDTA [pH 8.4] at 150V for 2.5 h, stained with 0.5 mg of ethidium bromide per mL of deionized water for 30 min., and then visualized under UV light and photographed. Patterns were analyzed both by visual comparison and by the Image Master VDS Software (Pharmacia Biotech). The computer program GelCompar II version 2.0 was used to determine the genetic relationship of the isolates. Similarity coefficients were calculated by the Dice Algorithm and cluster analysis was performed by an UPGMA algorithm (46).

In vitro antifungal susceptibility testing

The susceptibility testing followed the Clinical and Laboratory Standards Institute (CLSI) recommendations for microdilution procedures, but included modifications described previously (7,44). Briefly, in the susceptibility test, RPMI 1640 supplemented with 2% glucose was inoculated with 2x10⁵ CFU/mL, in the flat-bottomed wells of microplate cultures trays. 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 35°C for 48 h. *Candida parapsilosis* (ATCC 22019) was used as the control strain. The antifungal agents used in the study were: amphotericin B (AMB) (Sigma Aldrich Quimica S.A.), 5-fluorocytosine (5FC) (Sigma Aldrich), fluconazole (FCZ) (Pfizer S.A.) and itraconazole (ITZ) (Janssen S.A.). AMB, FCZ, and ITZ were dissolved in 100% dimethyl sulfoxide (Sigma Aldrich) and 5FC in sterile distilled water. All drug stock solutions were frozen at -70°C as 100x stocks until used. The MICs were determined spectrophotometrically as follows: after 48 h. incubation, the

optical density of each well of the microtiter plate was read with a microplate reader (Bio-Rad) at 490 nm. For AMB, the MIC endpoint was defined as the lowest drug concentration exhibiting a reduction in growth of 90% or more, compared with the control. For 5FC and the azole drugs, the MIC endpoint was defined as the concentration producing 50% inhibition (13). The breakpoints MICs was based on CLSI M27-A2 (7) reference microdilution method described for *Candida* sp. Experiments were repeated in duplicate, and MICs with one dilution higher were found and accepted as described by CLSI (7).

RESULTS

Identification

All isolates presented membranous and finely cerebriform, white to cream-colored colonies. Initial microscopic examination of portions of these colonies, as well as slide cultures, demonstrated round to oval, budding yeast-like cells and true hyphae forming cylindrical arthroconidia. They grew on SGA at 30 and 35°C but not at 45°C. The overall micro- and macroscopic appearance was consistent with that for members of the genus *Trichosporon* and morphological, physiological, and biochemical features were as described for *T. asahii*.

In vitro antifungal susceptibility testing

The MICs of the four antimicrobial agents for the quality control organisms were consistently within 2 or 3 dilutions of each other. No differences were observed between MICs obtained by the CLSI reference procedure and those achieved by the modified method. The MICs of four antifungal drugs for the *T. asahii* isolates, obtained by the CLSI broth microdilution method, are presented in Table 1. As indicated, the isolates had reduced susceptibility *in vitro* to all drugs, showing 4-6 times higher MICs to itraconazole and 3-4 times to 5-flucytosine compared of MICs breakpoint values. FCZ exhibited the best activity *in vitro* against the majority of the isolates (90%), with MIC of 16 µg/mL and only one isolate with 32 µg/mL. Three isolates have MIC value for AMB above 2 µg/mL.

DNA fragment

Both primers for the ITS region and TRR/TRF amplified all DNA isolates, producing distinct DNA fragments of approximately 530 bp and 170 pb, respectively, as shown in Fig. 1.

RAPD analysis

In this study, each of the 10 isolates was analyzed with two individual primers, primer M13 and 6, by the RAPD method. All amplifications were repeated twice at least. The most intense bands were reproducible, even with different extracts of DNA from the same isolate. However, differences were observed in some low-intensity bands. Therefore, in comparative analyses,

Table 1. Isolation sites and antifungal susceptibility test results for *T. asahii* isolates recovered from patients in an Intensive Care Unit.

Isolate	MIC (mg/liter) ^a			
	FLZ	ITZ	5FC	AMB
20 urine	16	8	32	2
22 urine	32	4	32	2
33 oral cavity	16	2	32	4
37 oral cavity	16	2	16	2
88 urine	16	2	16	4
91 urine	16	4	32	2
121 urine	16	2	16	2
131 urine	16	2	16	4
132 urine	16	2	16	2
134 urine	16	2	16	2
ATCC 22019 (type strain)	2-8	0,06-0,25	0,12-0,5	0,25-1
Susceptibility breakpoints ^b	≤ 8	≤ 0.125	≤ 4	>2 ^c

^aAbbreviations: FLZ, fluconazole, ITZ, itraconazole, 5FC, 5-fluorocytosine, AMB, amphotericin B; ^bThe breakpoints are based on NCCLS M27-A2 reference microdilution method described by *Candida* sp.; ^cEspinell-Ingroff (8).

only high-intensity bands were used. Each primer generated between six and nine bands for an individual isolate.

Among the 10 isolates analyzed by primer 6, various RAPD profiles were observed with molecular weights of bands between 170 and 1100 bp. All visible and definite bands were included in the analysis (Fig. 2). Dendrogram analysis obtained with this random primer showed a high homogeneity among the isolates from different body sites, with five RAPD profiles. Only one group (A) was generated with a coefficient of similarity greater than 85%. The largest (A1) contained seven isolates from urine, one grouped in a cluster (5 isolates) and two isolates with high degree (>90%). The subgroup A2 grouped in a cluster contained two isolates from oral cavity and urine. Subgroups A1 and A2 were related by similarity coefficient above 85%, suggesting these strains are closely related to each other. The isolate 33 from oral cavity has a low similarity coefficient of 70% in relation to the other groups, suggesting different strains.

For the primer M13 (Fig. 3), four different profiles were observed. One group was generated by a coefficient of similarity higher than 80%. Two clusters were formed in the subgroup A1, the first with five isolates from urine and the second containing three isolates, two from urine and one from oral cavity. In this last cluster, the three isolates suggest colonization of different sites with the same strain. The subgroup A2 and the isolate 22

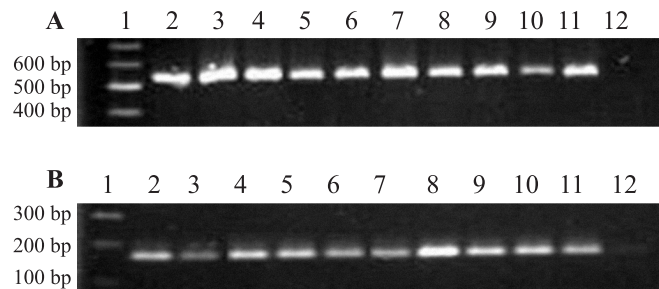


Figure 1. Agarose gel electrophoresis of PCR products of *T. asahii* isolates recovered from clinical specimens (oral cavity and urine) from patients in Intensive Care Units (ICU) using primers ITS1/ITS4 (A) and TRR/TRF (B). Lane 1- molecular size marker; lane 2- 20 urine; lane 3- 22 urine; lane 4- 33 oral cavity; lane 5- 37 oral cavity; lane 6- 88 urine; lane 7- 91 urine; lane 8- 121 urine; lane 9-131 urine; lane 10- 132 urine; lane 11- 134 urine; lane 12- negative control.

from urine also presented a high degree of similarity with subgroup A1.

DISCUSSION

The total agreement between the identification of *Trichosporon* sp by PCR based on sequences of the Internal Transcribed Spacer Regions (ITS) and on the sequences of small-subunit (SSU) ribosomal DNA (rDNA) aligned by the primers TRF/TRR indicates that *T. asahii* can be readily identified by these two methods, as shown by Sugita *et al.* (51).

T. asahii has been isolated from various types of clinical specimens from immunocompromised patients, including blood, skin biopsy, and urine specimens (9,17,19,29,32,43,52), but few were isolated from patients without immunological disorders (20). Factors that enhance mucosal colonization and subsequent invasion of *Trichosporon* species include broad-spectrum antibiotic treatment and scratches in mucosal barriers (6,24,56).

Despite the increasing frequency and severity of trichosporonosis, data on the antifungal susceptibility of *Trichosporon* spp. are limited and recommendations for in vitro testing of this fungus are not included in the guidelines of the National Committee for Clinical Laboratory Standards (27). There are few data available regarding *T. asahii* susceptibility to antifungal drugs in Brazil. The emergence of *T. asahii* isolates with reduced susceptibility to common antifungal drugs is a matter for concern. In our study, all isolates had reduced susceptibility to AMB, 5-FC and azoles. ITZ and FCZ exhibited MICs of 2 to 8 and 16 to 32 µg/ml, respectively. The first one corresponds to resistant isolates (≤ 1) and for the FCZ intermediate susceptibility (16-32 µg mL⁻¹) based on CLSI breakpoints for *Candida* sp (7). For AMB, the controversy is

greater, because we did not have MICs values that discriminated susceptible and resistant yeasts strains. Recently, Espinel-Ingroff (8) discussed the MIC value of 2 µg mL⁻¹ as the breakpoint to filamentous fungus. Wolf *et al.* (58) reported the

isolation of multidrug-resistant *T. asahii* from infected patients who exhibited risk factors and had developed either superficial infections or invasive infections while in ICU. Thus, the pathogenesis of *Trichosporon* infection in the ICU setting may

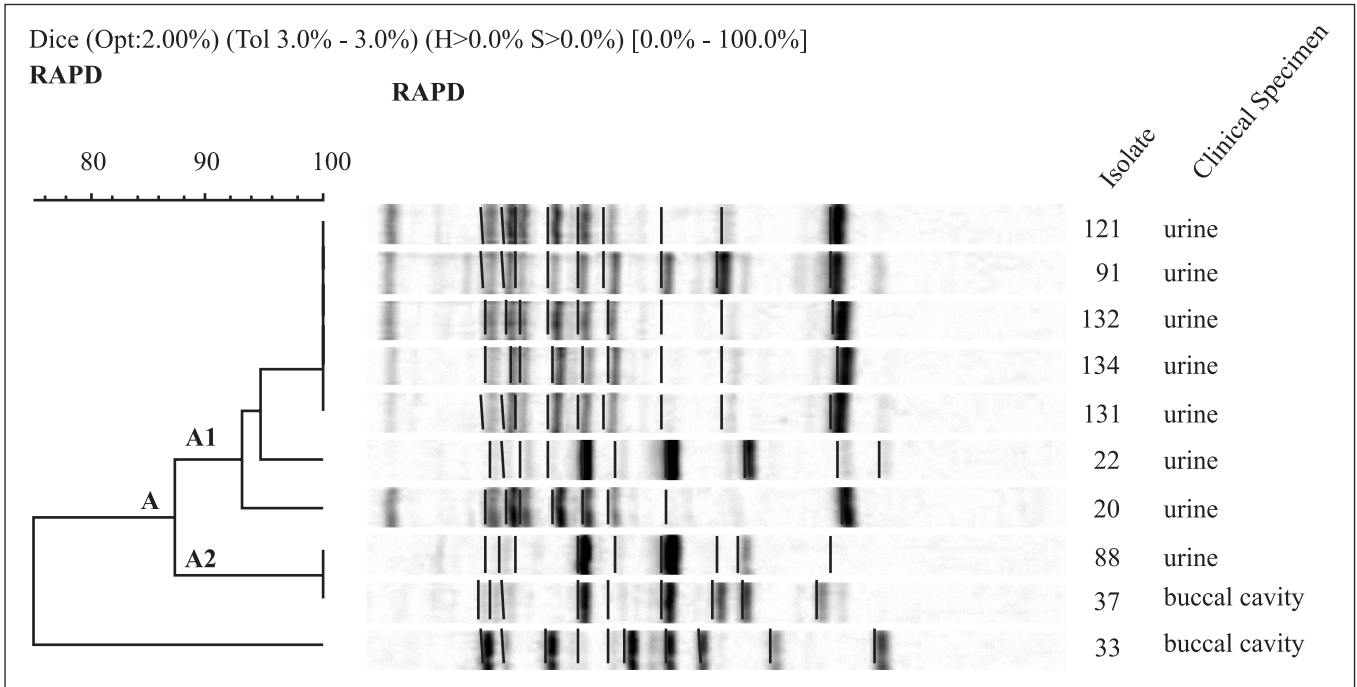


Figure 2. Dendrogram of *T. asahii* obtained from RAPD molecular patterns with primer 6.

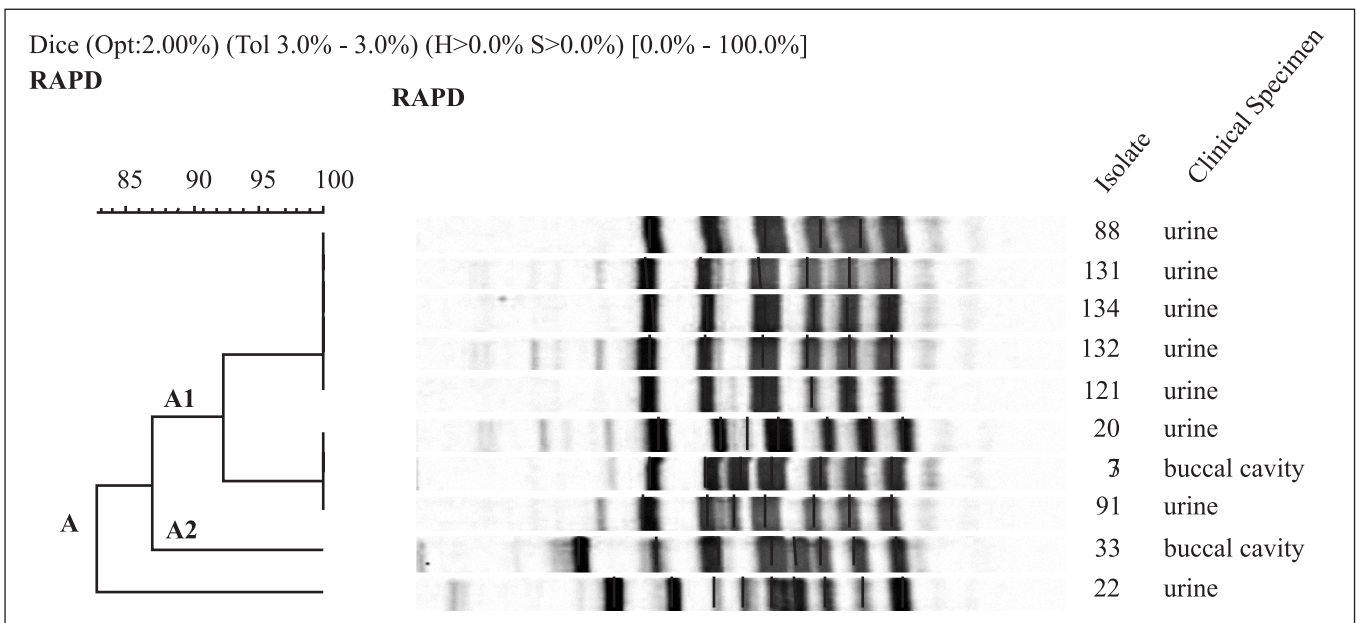


Figure 3. Dendrogram of *T. asahii* obtained from RAPD molecular patterns with primer M13.

be similar to that of the more commonly observed *Candida* infection. Paphitou *et al.* (36) results confirmed the previous observation of Perparim *et al.* (37) as well as of other authors (26,33,54,55) that the azoles, in general, appear to be more active *in vitro* against this pathogen than to AMB. Recently, a 47-year-old man with diagnosed acute myeloblastic leukemia and non-insulin-dependent diabetes mellitus developed *T. asahii* fungemia and the *in vitro* antifungal susceptibilities showed high caspofungin and amphotericin B MICs values (16 µg/ml, and > 32 µg/ml, respectively) while fluconazole, itraconazole, and voriconazole exhibited low MICs (4 µg/ml, 0.5 µg/ml, and 0.015 µg/ml, respectively) (4). In another patient, a neutropenic individual with acute myeloid leukaemia experienced a breakthrough infection of *T. asahii* despite posaconazole treatment (42). Although there are no established breakpoints to define the antifungal susceptibility of *Trichosporon* spp, our study suggests the FCZ may be used to treat patients with trichosporonosis. McGinnis *et al.* (26), as well as in our study, also showed that *T. asahii* strains exhibited reduced susceptibility to AMB (MICs, 1 to 4 mg/liter), but they were susceptible to ITZ and FCZ (MICs=0.125 to 1 and 0.5 to 4 mg/liter, respectively). On the other hand, the FCZ MICs for two *T. asahii* isolates recovered from esophageal biopsy specimens of two patients were found to be relatively high and these isolates were also resistant to nystatin. Lemes *et al.* (23) verified that the isolates of *T. asahii* were 5FC susceptible, ITZ resistant and some isolates were resistance to FCZ. The *in vitro* resistance to antifungal drugs may not be critical in immunocompetent patients, who may overcome the infection when the precipitating cause (e.g., a catheter) is removed, even if only partial inhibition of fungal growth is achieved through the use of antifungal drugs. However, immunocompromised patients may be dependent on the action of fungicidal drugs, so that infection with multidrug-resistant *Trichosporon* species may be catastrophic in this population.

RAPD analysis with primers 6 and M13 generated a similar dendrogram structure. In general, there was, in both dendrograms, formation of groups containing strains with high similarities (>0.8) among them associated with high MICs. The exception was strain 33 recovered from oral cavity, which showed minor similarity (around 0.7) with primer 6, but high (> 0.85) with primer M13. Moreover, RAPD analysis suggested that the isolates the different body sites were similar, suggesting a common origin. In addition, these patients were hospitalized in ICU between April and December, revealing a possibly clonal source of infection.

In conclusion, a rapid and reliable identification of *T. asahii* was achieved by molecular techniques. In addition, RAPD can be used to differentiate genotypes and reveal the correlation among them, drug susceptibility and the origin of a strain. The higher correlation among genotypes and the higher MICs of these strains offer potential risks of exogenous nosocomial

infection (e.g. to catheter). In addition, increasing use of antifungal drugs in the ICU may lead to the selection and isolation of more resistant species in the future.

ACKNOWLEDGEMENTS

This investigation was financially supported by the Brazilian Organizations, CNPq (Processo n. 400141/1998-1), FCF-UNESP and Instituto Adolfo Lutz.

RESUMO

Diversidade genética e suscetibilidade a antifúngicos de *Trichosporon asahii* isolado de pacientes de Unidades de Terapia Intensiva

Trichosporon asahii é um patógeno oportunista que apresenta altos índices de mortalidade em pacientes imunocomprometidos. No presente trabalho, dez cepas foram isoladas da cavidade bucal e urina de pacientes internados na Unidade de Terapia Intensiva (UTI) por seis meses. Todos os isolados foram identificados por métodos clássicos e moleculares, tipados por RAPD e testados *in vitro* quanto à sensibilidade ao fluconazol, itraconazol, 5-fluorocitosina e anfotericina B. Houve concordância total entre a identificação de *Trichosporon* sp por PCR baseado na seqüência da região ITS (*Internal Transcribed Spacer*) e na seqüência da subunidade menor do DNA ribossômico (rDNA). Os perfis genéticos foram determinados por RAPD utilizando dois iniciadores P6 e M13. A análise do dendrograma mostrou que a maioria das amostras apresentou alta similaridade entre elas (>0.9) e todas foram multidrogas resistentes. Este estudo traz novos resultados em relação à identificação e genotipagem de isolados brasileiros de *T. asahii* em pacientes internados em UTI, bem como dados sobre o perfil de sensibilidade aos antifúngicos.

Palavras-chave: *Trichosporon*, PCR, RAPD, sensibilidade às drogas antifúngicas, drogas antifúngicas.

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