COMPARISON BETWEEN RANDOM AMPLIFIED POLYMORPHIC DNA AND CLASSIC BIOCHEMICAL AND MORPHOLOGICAL TESTS FOR *TRICHOSPORON* SPECIES IDENTIFICATION

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Abstract: *Trichosporon* species are microorganisms implicated in systemic infection in immunocompromissed individuals. Quick and reliable identification of clinical isolates can contribute to an efficient therapy of the patients. The present study aimed to evaluate the efficacy of the classic methods of identification in comparison with the identification using the ITS region and the RAPD method. Thirty four isolates were tested. The agreement in the species identification by RAPD with primer OPA2 and classical test reached 88 % for *T. asahii*, 60% for *T. asteroids* and 100% for *T. ovoides*. Considering the total of species identified, the agreement reached 82.3%. Regarding the genus identification by PCR of ITS region, only two samples could not be identified as *Trichosporon* spp. Therefore, classic biochemical and morphologic tests of identification, as well as RAPD method provide reliable, low cost alternatives for *Trichosporon* identification.
Key words: *Trichosporon* spp., RAPD, microbial identification, Biochemical tests.
Area of knowlegment: Microbiology

INTRODUCTION

Trichosporon species are soil inhabitants and common colonizers of human skin and gastrintestinal tract (Depree et al. 1993, Guého et al. 1998). The genus includes approximately 30 species, at least six of which can cause trichosporonosis. *T asahii* and *T. mucoides* are associated with deep infection while *T. ovoides*, *T. asteroides, T. cutaneun* and *T. inkin* are involved in superficial mycoses (Ichikawa et al. 2004, Kendirli et al, 2006). Since 1970, *Trichosporon* has been implicated in severe disseminated infections associated with various immunocompromised states, as well as localized cutaneous disease (Gueho, 1994, Bonaventura, 2006, Chowdhary et al., 2007). It is important to notice that the correct therapy for trichosporonosis is influenced by the identification of *Trichosporon* species, since differences in the antifungal susceptibility pattern have been reported (Paphitou et al., 2002, Rodriguez-Tudela et al 2005). Thus, considering the increase in the mortality rates caused by trichosporonosis, rapid and reliable identification of clinical isolates has great importance.

According to Rodriguez-Tudela (2005), the identification of *Trichosporon* species fungal pathogens by conventional methods is often difficult and relatively time consuming. In this way, several molecular methods have been employed to identify fungal species. Sugita et al. (1999) constructed an accurate identification system for the genus *Trichosporon*, including the six medically relevant species, based on comparative sequence analysis of the internal transcribed spacer (ITS) regions. The ITS region was amplified with primers ITS1 and ITS4, which were derived from conserved regions of the small-subunit (SSU) and Large-subunit (LSU) rRNA genes, respectively. However, the identification by using the ITS region includes a subsequent sequencing analyses of the PCR product obtained. Besides, the ITS region is homologous across *Trichosporon* species. Rodrigues-Tudela (2005), tested identification methodologies and affirmed that ITS sequencing was not able to differentiate several species, which were grouped into clades. In this way, differentiation requires the analysis of regions with greater divergence. Other molecular based typing methods have emerged for *Trichosporon* spp. identification (Sugita et al, 2002). The sequence analyses of the rRNA IGS1 (Intergenic Spacer 1) provided a more powerful method to distinguish between phylogenetically close related species (Sugita 2002). In a comparative study between ITS and IGS1 methodologies, Rodrigez-Tudela (2005) found better results with the intergenic sequencing technique.

Although ITS, and, especially IGS sequencing methods are rapid and reliable alternatives for correct *Trichosporon* spp. identification, these techniques are not available in most clinical laboratories. Furthermore, if a small laboratory has to send samples to reference centers, where complex methods of identification could be employed, the analysis could take several weeks.

One of the most popular and simple methods for DNA fingerprinting is Random Amplified Polymorphic DNA (RAPD) (Soll 2000). The technique consists in the use of random

primers of approximately 10 bases, in order to amplify DNA fragments through the genome. Since a pattern of bands is generated when the amplicons are submitted to electrophoresis in agarose gel, this pattern could be used to presumptively identify different species. The aim of the present study was to evaluate the efficacy of the classic methods of identification in comparison with RAPD method. The present proposal is available to most microbiology laboratories, being an alternative procedure with low cost and high precision.

MATERIAL AND METHODS

Samples

Thirty-four samples belonging to the collections of the laboratory of Mycology of the Department of Microbiology of Federal University of Minas Gerais, Brazil, were used in the present work. The samples were obtained from different anatomic sites: Ten samples were obtained from urine, five from the oral mucosa, six from the oropharinx, four samples from blood, four from nail and three from skin. One sample was obtained from each one of the following sites: hair, bronchoalveolar lavage and fingers. Samples were maintained in Sabouraud dextrose agar (SDA) amended with chloramphenicol 300 mg/ l at 4˚ C.

Classic biochemical, physiological and morphological identification

The classic biochemical, morphological and physiological tests were applied for identification of the yeasts according to Guéro et al (1998) and Kurtzman and Fell (1998). CBS (Centralbureau Voor Schimmelcultures, Baarn, the Netherlands) reference samples were used in all tests (*T. asahii* CBS – 2479, *T. coremiiforme* CBS - 2482, *T. asteroides* CBS - 3481, *T. inkin* CBS - 5585, *T. mucoides* CBS - 7625 e *T. ovoides* CBS – 7556).

All samples were inoculated in SDA amended with choramphenicol 300 mg/ l and incubated at 37˚ C for 72 h in order to detect the presence of *T. cutaneum*, the only one of the genus that is not capable to growth at this temperature. The species *T. coremiiforme* is rarely isolated from humans but it was included with the aim to increase the probability of identification.

For the morphological analysis, a giant colony was obtained in SDA amended with chloramphenicol. Samples were incubated at 37˚ C for 30 days. The following macro-morphological features were observed in the colonies: size, margin, elevation, color, superficies, opacity, smell, and consistence. Microcultive was performed in malt extract agar incubated at 25˚ C for 10 days to observe the presence of blastosporum, arthroconidiospores, pseudo-mycelium, true mycelium and apressorium.

The biochemical profile of the yeasts was characterized by carbon source and nitrogen source assimilation. The carbon sources tested were galactosis, saccarosis, lactosis, maltosis, dextrose, glucose rhamnose, melibiose, melizithose, eritrithol, L-arabinosis, D-arabinosis, D-manithol, galactitol, myo-inosithol, sorbithol, raffinose, ribithol and galactiol. Species were identified according to a key stated by Ghéro et al. (1992). Peptone and potassium nitrate were used as nitrogen sources (Beijerinck et al. 1989). For urease detection, yeasts were inoculated in Christensen urea agar (Becton Dickinso, Cockeysville, Michingan Md) and incubated at 37˚ C for 7 days.

Identification of *Trichosporon* species by RAPD assay

Yeast cells were cultivated in 2 ml of Sabouraud dextrose broth (SDB) medium for 16 h at 30º C with agitation (220 rpm), reaching a density of 2 x 10^8 cells/ml. DNA was extracted employing the protocol described by Wach et al. (1994). DNA concentration and purity were determined by optical density at 260nm and ratio 260 nm/ 280nm, respectively (Genequant, GE Heathcare).

The oligonucleotides M3 (5'GAGACAATGA 3'), M2 (5' CTTGATTGCC 3'), B14 (5' GATCAAGTCC 3'), SOY5 (5'AGGTCACTGA 3'), OPA4 (5'AATCGGGCTG 3') and OPA2 (5'TGCCGAGCTG 3') were employed for RAPD reactions. Briefly, a reaction was prepared in a final volume of 25 µl containing 2.5 µl of 10 x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3.5 mM $MgCl_2$, 4 µl of dNTP mix (1.25 mM each dNTP), 0.5 µl of primer, 0.5 µl of Taq polymerase (5 U/ µl - Promega), 1µl of DNA (60 ng/ µl) and 13.0 µl of ultra-pure sterilized water. Cycle conditions were 94 ºC for 1 min, 35 ºC for 1 min, 72 ºC for 2 min, 35 cycles and a final extension at 72º C for 10 min, in a PTC- 100™ DNA thermocycler (Programmable Peltier – Effect Cycling Thermal Controller – MJ Research, Inc.).

RESULTS

The primers M2, M3, B14, SOY5, OPA4 and OPA 2 were tested for identification of the yeasts by the analysis of the position of the fragments generated. Best results were obtained with primer OPA2. The RAPD technique using the primer OPA 2 with the CBS reference samples created six fragments with 250 to 1000 bp in *T. asahii*, nine fragments with 3000 to 1400 bp in T. *asteroides*, four fragments with 100 to 1000 bp in *T. coreiformii*, six fragments with 100 to 1400 bp in *T. inkin,* and eleven fragments with 150 to 1400 bp in *T. mucoides* and *T. ovoides* (figure 1).

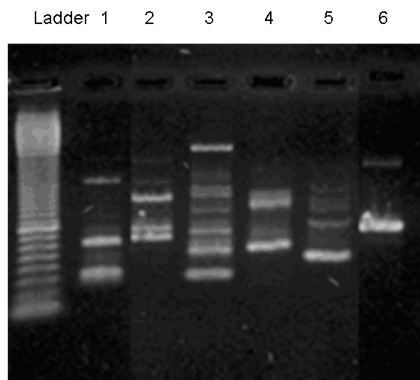

Figure 1- Agarose gel electrophoresis of RAPD products from samples of *Trichosporon* obtained with primer OPA2. Line 1, *T. asahii* (CBS 2479); Line 2, *T. asteroides* (CBS 3481); Lane 3, T-03; Line 4, *T. mucoides* (CBS 7625); Line 5, *T. ovoides* (CBS 7556); Line 6, *T. inkin* (CBS 5585) and 100bp ladder (from top to bottom in bp, 2072 and a 1500 to 100 bp at 100 bp intervals).

Clinical isolates were also characterized by RAPD with primer OPA 2. The identification analyses were made considering the CBS reference sample with a maximum of 3 different bands in number or fragment size (Power 1996). Twenty-six samples were classified as *T. asahii*, five as *T. asteroids*, two as *T. ovoides* and one as *T. mucoide*.

The agreement in the species identification by RAPD and classical test was 88.0 % for *T. asahii*, 60.0 % for *T. asteroids* and 100.0 % for *T. ovoides*. Considering the total of species identified, the agreement reached 82.3 %.

DISCUSSION

Infections caused by opportunistic fungi have increased in previous decades due primarily to the increasing population of immunocompromised patients. In addition, infections caused by less common species such as *Pichia, Rhodotorula, Trichosporon*, and *Saccharomyces* spp. have been widely reported. Nevertheless, the efficacy in the identification of these fungi has not been observed. Considering the facts, it becomes necessary to establish a guideline for identification, diagnosis and treatment of fungi and fungal infections (Perfect and Shell 1996). Indeed, Commercial methods have been introduced in mycology laboratories with the purpose of achieving quicker results, but the results are contradictory when considering less common fungal pathogens. The importance of the tests employed in the classic method is evident (Hazen 1995). Espinel-Ingroff et al. (1998) attempted for the sensibility and specificity of the classic method in comparison with the commercial systems.

In the present study, only three samples could be identified uniquely through the carbon and nitrogen assimilation tests. However, when complementary tests were added to micro and macro morphology, 82.3% of the samples (28) could be identified. The enhancement in identification proves that the use of several combined techniques is acceptable for the identification of this yeast in small laboratories. The association of techniques must be considered as a relevant option in order to improve the quality of microbiological analyses to several groups and clinical laboratories with limited resources.

Rodrigues-Tudela (2005) evaluated the identification of *Trichosporon* species by classic biochemical and morphological tests and by molecular methodologies. These authors considered that either classic identification or ITS sequencing were unsatisfactory for *Trichosporon* identification. They also affirmed that IGS1 sequencing promotes correct identification but since the technique is complex and could be time consuming, the more practical alternative is to perform antifungal susceptibility testing of all *Trichosporon* spp. isolates.

RAPD technique was able to identify all *Trichosporon* species tested, including those that could not be identified by classic tests. The identification was more efficient than that obtained with classic tests and the agreement between the methodologies was adequate. Ahamad et al. (2005) used different RAPD primers to evaluate genetic diversity among *Trichosporon* strains and could also reach great differentiation. A set of primers is usually necessary for RAPD genotyping. However, the present analyses aimed to establish a simple and available method for species identification. Since OPA2 reached the greater number of bands with distinction between species, this primer is recommended for *Trichosporon* species identification.

Small laboratories can not always count with specific identification kits or primers for all species of isolates. The advantage of RAPD is the versatility of a set of primers, which can be used for the identification of other fungal species. Therefore, RAPD method provides an easy, reliable, low cost alternative for identification of *Trichosporon* spp. Furthermore, it is also important to notice that although time consuming, classic methods of *Trichosporon* identification are as efficient as molecular techniques. These methodologies can be used in laboratories that do not possess the apparatus to perform PCR based tests.

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