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Differentiation of *Candida* species obtained from nosocomial candidemia using RAPD-PCR technique

Diferenciação de espécies de *Candida* obtidas de candidemia nosocomial pela técnica de RAPD-PCR

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ABSTRACT

Thirteen strains of the genus Candida *were isolated from catheter, urine and surgical wounds from individual patients of the Santa Casa de Misericórdia, Belo Horizonte, MG, Brazil. Ten strains were characterized as* Candida albicans*, two as* Candida glabrata*, and one as* Candida parapsilosis*. Isolates were evaluated for molecular relatedness by random amplified polymorphic DNA technique using 15 primers. The analysis of the genomic DNA obtained revealed a low intraspecific polymorphism and did not allow for the differentiation between strains of the same species obtained from distinct clinical sources (catheter, urine and surgical wounds). The RAPD profiles generated were able to differentiate among the species of* Candida albicans*,* Candida parapsilosis *and* Candida glabrata *strains isolated in this study.*

Key-words*:* Candida *spp. Random amplified polymorphic DNA. Polymorphism. Nosocomial infection.*

RESUMO

Treze amostras de leveduras do gênero Candida *foram isoladas de catéter, urina e feridas cirúrgicas de pacientes da Santa Casa de Misericórida de Belo Horizonte, MG, Brasil. Dez amostras foram identificadas como* Candida albicans*, duas como* Candida glabrata *e uma como* Candida parapsilosis*. Os isolados foram avaliados quanto ao perfil molecular pela técnica de amplificação aleatória de DNA polimórfico utilizando 15 iniciadores. A análise do DNA genômico obtido revelou um baixo polimorfismo intraespecífico e não permitiu a diferenciação entre amostras da mesma espécie obtidas a partir de diferentes espécimes clínicos (catéter, urina e feridas cirúrgicas). Os perfis de RAPD obtidos foram capazes de diferenciar entre as espécies* Candida albicans*,* Candida parapsilosis *e* Candida glabrata *isoladas neste estudo.*

Palavras-chaves: Candida *spp. Amplificação aleatória de DNA polimórfico. Polimorfismo. Infecção nosocomial.*

The anamorphic yeast of the genus *Candida* contains an assemblage of microorganisms that have been placed into different species primarily on the basis on their physiological, biochemical and morphological characteristics. Typing physiological characteristics include those associated with a variety of compounds that are used as the sole sources of carbon or nitrogen[11]. Diagnostic kits, based on physiological characteristics, have been developed to facilitate the accurate identification of yeast from clinical specimens. However, certain tests results may be borderline, such that the same isolate may show a positive response on one occasion and a negative response on another[17].

Other typing methods, which were found to be suitable for genus *Candida* strain delineation, have relied on biotyping, enzyme profiles, susceptibility to killer toxin, streak morphology, serological agglutination reactions, comparison of the hydrophobic properties of *Candida albicans* and *Candida dubliniensis* and a new monoclonal antibody specific for *Candida albicans* germ tube[6 9 10]. In addition to phenotypic DNA-based studies, other methods have been used, such as DNA-DNA reassociation, DNA fingerprinting, southern blot analysis, electrophoretic karyotypes[4], RFLPs assays[12] and the detection of *C. albicans* DNA in serum by PCR for the diagnosis of invasive candidosis[19 22].

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The random amplified polymorphic DNA (RAPD) technique[23,25] relies on the use of arbitrary primers which are annealed to genomic DNA using low temperature conditions. Priming at a number of closely adjacent complementary sites allows the subsequent amplification of dispersed genomic sequences by *taq DNA polymerase* enzyme. This technique detects genetic polymorphisms and does not depend on prior knowledge of species-specific sequences. Some authors have demonstrated that distinctive RAPD and RFLP patterns can be obtained from *C. albicans*, *C. lusitaniae*, *C. tropicalis*, *C. parapsilosis*, *C. krusei, C. haemulonii* and *C. glabrata* strains[1,3,12,13,16].

In a pilot study, we detected the difference between several clinical isolates of *Candida* species based on RAPD profiles. Here we report on the use of RAPD-PCR assays as a molecular typing to differentiate *Candida* species obtained from distinct clinical sources of isolation.

MATERIAL AND METHODS

***Candida* isolates.** Thirteen strains of genus *Candida* were isolated from catheter, urine, and surgical wounds from patients hospitalized in the Santa Casa de Misericórdia, Belo Horizonte, Minas Gerais State, Brazil (Table 1). The strains were identified by germ tube and chlamydoconidia formation and subjected to confirmation using methods described by van der Walt and Yarrow[20] and Kurtzman and Fell[7]. Ten of these strains were characterized as *C. albicans*, two as *C. glabrata* and one as *C. parapsilosis*. The one reference strain of *C. albicans* (The London School of Hyg. & Trop. Med. 3153) was evaluated for molecular relatedness using RAPD technique.

Table 1 - Sources of strain isolation.

Species	Sources of isolation	Isolate number	Lanes	
			Figure 1	Figure 2
Candida albicans	catheter	14	1	1
	catheter	68	2	2
	catheter	94	3	3
	catheter	123	5	5
	surgical wounds	115	7	7
	surgical wounds	157	8	8
	surgical wounds	166	10	10
	urine	72	11	11
	urine	153	12	12
	urine	164	13	NC
Candida parapsilosis	catheter	107	4	4
Candida glabrata	surgical wounds	44	6	6
	surgical wounds	162	9	9

NC: negative control

Acquisition of genomic DNA. The strains were grown on Sabouraud Dextrose Agar (SDA; Difco) for 48 hours at 28°C as preinoculum. After this period, 3 x 10^8 cells.ml^{-1} were inoculated on 50ml of SDA for 48 hours at 28°C. The cellular biomasses were separated by centrifugation at 10,000 x g and resuspended in 5ml of 0.1M natrium citrate/1.1M sorbitol buffer (pH 5.5) containing glucanase enzyme at 5mg.ml^{-1} and incubated at 33°C for 3 hours in a shaking water bath. The protoplasts obtained were transferred to 5ml of lysing buffer (0.04M Tris HCl, pH 8.0; 0.20M NaCl, SDS and 0.01M Na_2EDTA) and washed three times with 5ml of phenol-chloroform and then precipitated with absolute EtOH and 0.3M NaCl. The precipitate obtained was centrifuged and washed twice with 70% ethanol, dried and resuspended in 100ml of 0.10mM Tris HCl (pH 7.5). DNA aliquots were diluted to 50ng.ml^{-1} for the RAPD reaction.

RAPD profiles. The RAPD reactions were conducted in a 30µl volume containing buffer 1X (Promega), 0.2mM each of dATP, dGTP, dCTP and dTTP (Promega), 50ng of genomic-DNA, 2mM of MgCl2 (Promega), 160nM of primer (Operon) and 1 unit of *Taq thermostable DNA polymerase* (Promega). The amplification parameters consisted of 35 cycles of denaturation at 95°C for 45 sec, primer annealing at 36°C for 2 min, and extension at 72°C for 2 min. In the first cycle, the denaturing step was 5 min and in the final cycle the final extension step was 7 min. The reactions were performed using decamer primers of the OPERON kit (OPA 01, 02, 03, 07, 08, 09 and 10), and the arbitrary primers SOY, RP1-4, RP-2, RP4-2[8]. Amplification using the ribosomal primers NS1, NS2, ITS1 and ITS-4[24] was performed increasing the melting temperature to 45°C. The primer sequences are shown in Table 2.

Table 2 - Primers included in this study.

Primers-Operon/Design	10-mer in length - 5' to 3'	19/21-mer in length - 5' to 3'
OPA-01	CAGGCCCTTC	
OPA-02	TGCCGCGCTG	
OPA-03	AGTCAGCCAC	
OPA-07	GAAACGGGTG	
OPA-08	GTGACGTAGG	
OPA-09	GGGTAACGCC	
OPA-10	GTGATCGCAG	
RP1-4	TAGGATCAGA	
RP-2	AAGGATCAGA	
RP4-2	CACATGCTTC	
NS-1		GTAGTCATATGCTTGTCTG
NS-2		GGCTGCTGGCACCAGACTTGC
ITS-1		TCCGTAGGTGAACCTGCGG
ITS-4		TCCTCCGCTTATTGATATGC
SOY	AGGTCACTGA	

Fingerprints were produced by electrophoresis of 10µl aliquots of reaction in 1.5% agarose gels run in TBE (0.45M Tris borate, 0.001M EDTA) buffer at 120V for 90 minutes. The gels were stained with 1µg.ml^{-1} ethidium bromide, photographed under UV light using a Polaroid camera (Model DS-34) with a black and white film (Type 667, Polaroid Corp). For each experiment, the base pair sizes were measured from size markers included in every gel (DNA lambda/*Hind* III or 100-bp ladder, Gibco-BRL).

RESULTS

Using 15 primers, a total of 114 different reproducible RAPD markers were generated from the strain genomes. A large number of bands were typically generated for each

amplified sample. The RAPD profiles using the OPA kit, RP1-4, RP-2, RP4-2 and SOY primers were able to characterize interspecific polymorphisms among the *Candida* species. The OPA 02 and NS2 primers gave representative profiles for the species clusters (Figure 1).

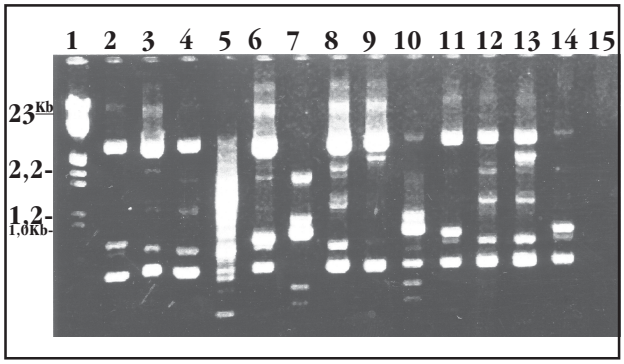

Figure 1 - Genetic variation within* Candida albicans, Candida glabrata *and* Candida parapsilosis *determined by RAPD analysis using primer NS2; lane 14, positive control; lane 15, molecular weight lambda* EcoRI/ Hind *III (lanes are according to Table1).

Among the *C. albicans* isolates no polymorphic traits were detected, even though the isolates were obtained from different yeast reservoirs (catheter, surgical wounds and urine). Similar RAPD profiles of *C. albicans* and *C. parapsilosis* strains were generated using the primers ITS-1 and ITS-4. However, this set of primers generated a different RAPD profile when *C. glabrata* DNA was used as a template (Figure 2). For all the primers tested, the RAPD profile of *C. albicans* type strain was differentiated from other strains of this same species by some polymorphic amplicons (Figure 1).

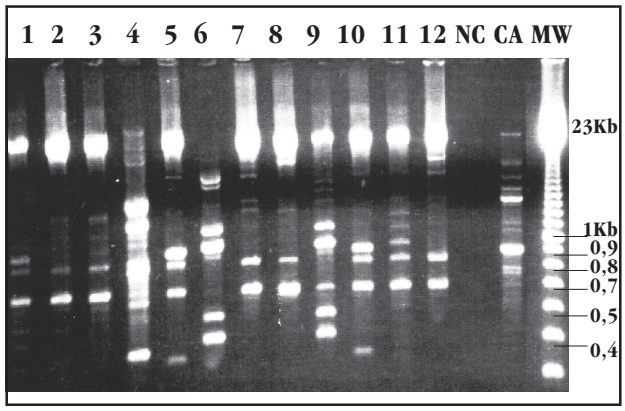

Figure 2 - Genetic variation within* Candida albicans, Candida glabrata *and* Candida parapsilosis *determined by RAPD analysis using primer OPA2; NC, negative control; CA,* Candida albicans *reference strain; MW, molecular weight: ladder 100bp (lanes are according to Table 1).

The phenogram grouped the strains of *Candida albicans*, *Candida parapsilosis* and *C. glabrata* by the similarity in the genotypic characteristics obtained from the RAPD reactions[5]. The type strain of *C. tropicalis* was positioned on the phenogram separately, as shown Figure 3, where the clustering of most of the *C. albicans* strains was relatively differentiated.

The two isolates 44 and 162, belonging to the species *C. glabrata*, were originally misidentified as *C. albicans*. These results suggest that their original presumptive identification by germ tube and chlamydoconidia formation was unreliable. Therefore, the new identities were correctly confirmed by additional biochemical and physiological tests realized in this work, using previously established techniques[7,20].

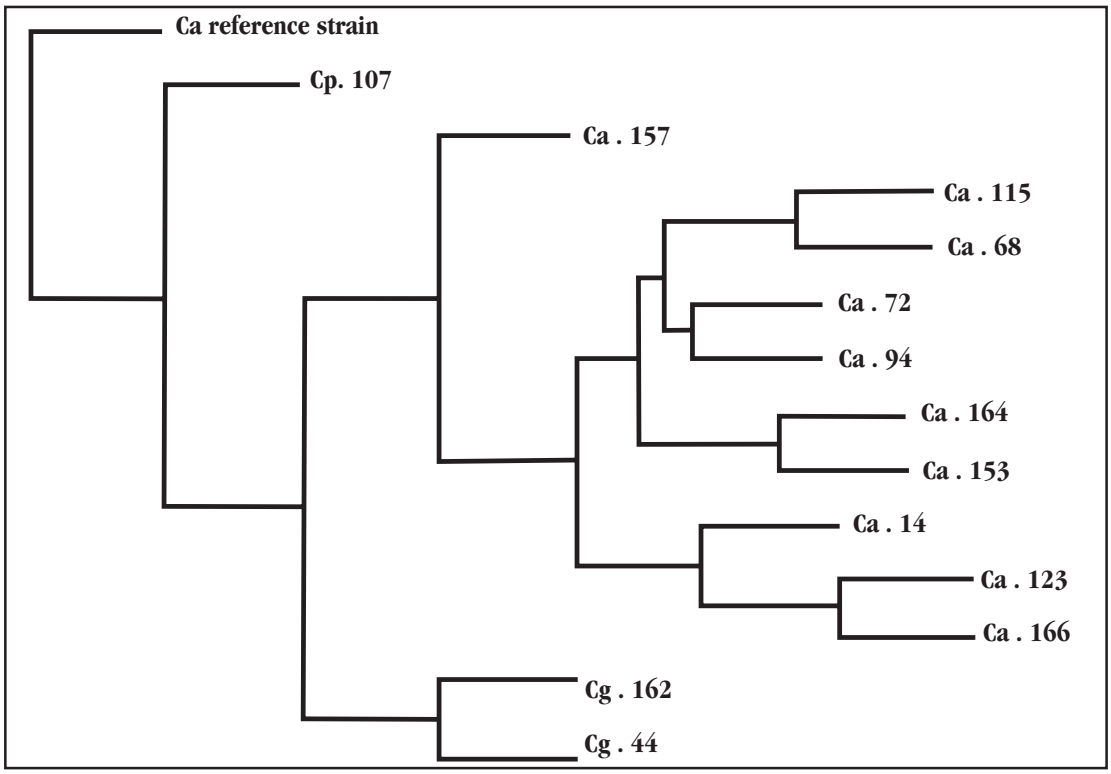

Figure 3 - Phenogram of isolates based on RAPD profiles. The consensual phenogram was constructed using the Wagner parsimony option within the computer program Phylip v.3,572c. Branch lengths and scale bar correspond to the distance values among these* Candida albicans *(Ca),* Candida parapsilosis *(Cp), and* Candida glabrata *(Cg) strains.

DISCUSSION

Candida spp has recently emerged as an important cause of nosocomial infection through cross-transmission, particularly in intensive care units[14]. Fungemia caused by this yeast are reported to be related to previous digestive tract colonization, mucosal surfaces of mammalian bodies, more specifically in human mucosa, or exogenous contamination[16 21]. These emerging threats of fungal infection require standardized methods for strain characterization in order to identify hospital clusters[15]. RAPD assays may be an important tool for *Candida* species identification, increasing the capability of previously established traditional methods. In this work the applicability of this technique was demonstrated, using the primers OPA2 and NS2 to differentiate between *C. albicans, C. glabrata and C. parapsilosis*.

The RAPD profiles generated showed few differences between strains of the same species. This result demonstrates that it is difficult to identify primers able to detect intraspecific polymorphism in these clonal species[26].

The misidentification of strains 44 and 162 demonstrated that the patterns provided with biochemical, physiological and micromorphological tests, widely used for strain identification, can sometimes be unclear and obscure differences in the biochemical tests realized, thus masking the incorrect identification of yeasts from nosocomial candidemia[9].

The clinical value of a single procedure, such as RAPD analysis, for determining both species and biotype is most clear in the area of molecular epidemiology[18]. According to some authors, given the increase in nosocomial infections caused by *Candida* species, there is an urgent need for a rapid and simple procedure which would allow for the analysis of both the outbreaks and the incidence of person-to-person transmission associated with these organisms[2 13 16]. As such, more profound posterior epidemiological analyses are required to more accurately clarify whether there are simply genetic similarities in a characteristic cloned population among these isolates or whether hospital procedures helped disseminate the agents among these patients through cross-infections.

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