

Identification and differentiation of *Candida* species from pediatric patients by random amplified polymorphic DNA

Identificação e diferenciação de espécies de *Candida* de pacientes pediátricos por amplificação aleatória de DNA polimórfico

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ABSTRACT

Thirty-four *Candida* isolates were analyzed by random amplified polymorphic DNA using the primer OPG-10: 24 *Candida albicans*; 4 *Candida tropicalis*; 2 *Candida parapsilosis*; 2 *Candida dubliniensis*; 1 *Candida glabrata* and 1 *Candida krusei*. The UPGMA-Pearson correlation coefficient was used to calculate the genetic distance between the different *Candida* groupings. Samples were classified as identical (correlation of 100%); highly related samples (90%); moderately related samples (80%) and unrelated samples ($\leq 70\%$). The results showed that the RAPD proposed was capable of classifying the isolates coherently (such that same species were in the same dendrogram), except for two isolates of *Candida parapsilosis* and the positive control (Netherlands, 1973), probably because they are now recognized as three different species. Concerning the only fluconazole-resistant *Candida tropicalis* isolate with a genotype that was different to the others, the data were insufficient to affirm that the only difference was the sensitivity to fluconazole. We concluded that the Random Amplified Polymorphic DNA proposed might be used to confirm *Candida* species identified by microbiological methods.

Key-words: RAPD. Candidiasis. *Candida* spp. Polymorphism. Molecular typing.

RESUMO

Trinta e quatro isolados de *Candida* foram analisados por amplificação aleatória de DNA polimórfico (primer OPG-10): 24 *Candida albicans*, 4 *Candida tropicalis*, 2 *Candida parapsilosis*, 2 *Candida dubliniensis*, 1 *Candida glabrata* e 1 *Candida krusei*. O coeficiente de correlação de Pearson-UPGMA calculou a distância genética entre os diferentes agrupamentos de *Candida*: amostras idênticas (100% de correlação), amostras muito relacionadas (90%), moderadamente relacionadas (80%), e não relacionadas ($\leq 70\%$). Os resultados demonstram que a amplificação aleatória de DNA polimórfico proposta é capaz de classificar os isolados de forma coerente, ficando os de mesma espécie em um mesmo dendograma, com exceção dos dois isolados de *Candida parapsilosis* e o controle positivo (Holanda, 1973), provavelmente por serem atualmente classificadas em três espécies diferentes. Quanto ao único isolado de *Candida tropicalis* resistente ao fluconazol com genótipo diferente dos outros, os dados não são suficientes para afirmar que a única característica distinta fosse a sensibilidade ao fluconazol. Concluímos que a amplificação aleatória de DNA polimórfico proposta poderia ser usada para a confirmação das espécies de *Candida* identificadas nos testes microbiológicos.

Palavras-chaves: Amplificação aleatória de DNA polimórfico. Candidíase. *Candida* spp. Polimorfismo. Tipagem molecular.

Due to the rise in pediatric *Candida* infections and the associated epidemiological issues (increasing numbers of immunocompromised children, prolonged hospital stays, indiscriminate use of broad-spectrum antibiotics, parenteral nutrition and insertion of central venous and arterial catheters)^{4 14 18}, rapid and reliable identification of yeasts at species level is extremely important.

The standard culturing methods and differentiation of *Candida* species are time-consuming, lack sensitivity and may fail to distinguish *Candida non-albicans* species^{1 2 20}. Blood cultures may be negative in up to 50% of autopsy-proven cases of systemic candidiasis, or may only become positive at a late stage in the infection. Positive cultures from urine or mucosal surfaces do not necessarily indicate invasive disease, although this may occur

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before systemic infection. Therefore, blood cultures should be systematically requested in all cases of at-risk patients, regardless of the difficulties in obtaining blood, in order to perform life-sustaining tests and blood cultures on critically ill pediatric patients⁹.

Recently, molecular typing has been used to identify *Candida* species^{5 11 21}. These tests can be accomplished within one working day, whereas microbiological methods are more time-consuming¹⁰. The random amplified polymorphic DNA (RAPD) technique has proven to be useful for typing many microbiological agents, including *Candida* species^{12 22 24}.

The present study proposed the use of a single short primer for confirming the microbiological *Candida* species identification, by means of RAPD. Since *Candida* species have innate pathogenicity as well as variation in antifungal susceptibility, identification at species level might be relevant for clinical management⁹.

MATERIAL AND METHODS

This study was approved by the Ethics Committee of the University of São Paulo School of Medicine, Brazil (CAPPesq protocol number 1213/05).

***Candida* spp isolates.** Forty *Candida* spp samples from newborns or children were isolated and identified by standard microbiological methods¹⁰ at the Mycology Laboratory of the Adolfo Lutz Institute, a reference center in the city of São Paulo, Brazil.

Confirmation of microbiological identification of *Candida* isolates. A previously described nested PCR method was used to confirm microbiological species identification of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata* and *Candida krusei* isolates¹³. Briefly, in the first round of amplification, gender-specific primers were designed within the internal transcribed spacer (ITS) region and were called ITS1 and ITS4. During the second round of amplification, species-specific primers were designed to amplify only one *Candida* species: CALB1 and CALB2 for *Candida albicans*; CTR1 and CTR2 for *Candida tropicalis*; CPA2 and CPA3 for *Candida parapsilosis*; CGL1 and CGL2 for *Candida glabrata*. *Candida krusei*-specific amplification was performed with the primers CKR2 and CKR3³. The first-round amplification for *Candida dubliniensis* was performed using the fungus-specific universal primers ITS-3 and ITS-4, designed within the 5.8S rDNA region, and in the second round within the 28S rDNA region, yielding a 343 bp fragment¹⁷.

The nested ITS-PCR revealed that 34 of the 40 (85%) isolates were correctly identified at species level, and were thereafter included in the study. The characteristics of the 34 isolates are described in Table 1.

Reference strains (prototype species used as controls). ATCC *Candida* strains of the following species (*albicans*, *tropicalis*, *glabrata*, *krusei* and *dubliniensis*) were used as prototypes (controls) in order to define the DNA banding patterns obtained by RAPD corresponding to each species. The ATCC numbers were: 36082 *Candida albicans* (sensitive to fluconazole); 204276 *Candida albicans* (resistant to fluconazole); 204318 *Candida tropicalis* (sensitive to fluconazole); 200918 *Candida glabrata*; 200917 *Candida krusei*; and MYA-577 *Candida dubliniensis*.

Table 1 - Characteristics of the 34 isolates analyzed by microbiological methods, nested ITS-PCR and random amplified polymorphic DNA.

Isolate	Biological sample	<i>Candida</i> species	Fluconazole
1-16	blood	<i>albicans</i>	S
17-20	blood	<i>albicans</i>	R
21-23	oropharynx	<i>albicans</i>	R
24	urine	<i>albicans</i>	R
25-27	blood	<i>tropicalis</i>	S
28	blood	<i>tropicalis</i>	R
29-30	blood	<i>parapsilosis</i>	S
31	blood	<i>dubliniensis</i>	S
32	blood	<i>dubliniensis</i>	R
33	oropharynx	<i>glabrata</i>	S
34	blood	<i>krusei</i>	R

S: fluconazole-sensitive, R: fluconazole-resistant.

The *Candida parapsilosis* strain used as a control was kindly sent by the Central Bureau of Fungal Cultures (*Centraalbureau voor Schimmelcultures*) of the Royal Netherlands Academy of Arts and Sciences, to the Mycology Laboratory of the Tropical Medicine Institute (*Instituto de Medicina Tropical*) of São Paulo, Brazil, in 1973.

DNA extraction of *Candida* spp isolates. The DNA extraction of *Candida* isolates was performed in accordance with a protocol described elsewhere²⁵. Briefly, 500µl of the fungal suspension were spun at 15,300g for 5 min in a refrigerated centrifuge (Eppendorf, 5417R) and the pellet was mixed with 600µl of a solution containing 50mM Tris, 10mM EDTA, 250U/ml lyticase (L2524, Sigma) and 10% β-mercaptoethanol. The samples were then kept at 37°C for two and a half hours to promote cell wall digestion. Following this, 0.2% SDS and 200µg/ml proteinase K (Sigma) were added to the samples, which were kept at 65°C for 45 min in order to obtain nuclear lysis and protein digestion. The samples were then centrifuged at 15,300g for 5 min. The supernatants were mixed with isopropanol (Merck) (v/v) and these samples were kept overnight at -20°C. After centrifugation again at 15,300g for 5 min, the pellet containing DNA was rehydrated with 500µl of 70% ethanol (Merck) and the final DNA pellet was resuspended in 1M Tris-EDTA buffer.

Random amplified polymorphic DNA. The assays were performed in a total volume of 50µl, containing 5ng of genomic DNA from each *Candida* isolate or strain (ATCC prototype), 0.4µM of the OP-G10 primer 5'-AGGCCCGTCT-3' (IDT, USA); 600 µM of each dNTP (Amersham Biosciences); 3.5mM of MgCl₂; and 5U of Taq DNA polymerase (Promega). The amplification consisted of 40 cycles: 1 min at 95°C, 2 min at 25°C and 2 min at 72°C, followed by a final extension of 5 min at 72°C (PT-150, Minicycler, MJ Research). Ten microliters of each PCR product were analyzed by horizontal electrophoresis (Horizon 58, Gibco) in 1.5% ethidium bromide-stained agarose gels (Sigma) prepared in 1X TAE buffer.

Statistical analysis. The RAPD results were used to build up a binary matrix by means of the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA; GelWorks-1 AD Bioimaging System, Upland, CA, USA), to generate dendrograms representing clusters of similarities. The degree of similarity was then calculated by applying the Dice coefficient test, which was able to analyze the

matches and mismatches between pairs of DNA profiles according to the presence or absence of bands. In the second part of the statistical analysis, the similarity correlation coefficient was calculated using the same software coupled to Pearson's correlation coefficient test, and this was used to determine the genetic relatedness of strains or isolates analyzed in pairs (two by two)^{8,16}. In accordance with the proposal by Dassanayake & Samaranyake⁷, the strains were classified in terms of the similarity correlation coefficient, as follows: 1.0 or 100% = identical samples; 0.9 or 90% = highly related samples; 0.8 or 80% = moderately related samples; and ≤ 0.7 or 70% = unrelated samples.

RESULTS

The RAPD banding patterns using the primer OP-G10 for the different *Candida* isolates and corresponding prototypes (controls) are shown in Figure 1.

The UPGMA-Dice similarity coefficient test generated dendrograms representing 19 clusters of similarity, with degrees of similarity ranging from 70 to 100% (data not shown).

The second part of the statistical analysis used the UPGMA-Pearson correlation coefficient test to determine the genetic distance (or relatedness) between pairs of *Candida* clusters (dendrograms) (Figure 2):

Using these criteria, Figure 2 yielded the following results.

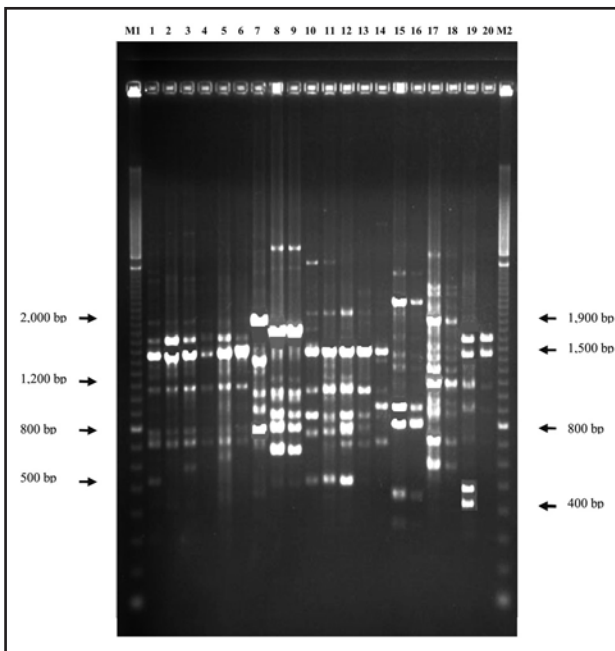


Figure 1 - Random amplified polymorphic DNA banding pattern with the primer OP-G10.

Lanes M1 and M2, molecular weight marker 100 bp (Amersham); lane 1 *Candida albicans* S control; **lanes 2 to 4** *Candida albicans* S; **lane 5** *Candida albicans* R control; **lane 6** *Candida albicans* R; **lane 7** *Candida parapsilosis* S control; **lanes 8 and 9** *Candida parapsilosis* S; **lane 10** *Candida tropicalis* S control; **lanes 11 to 13** *Candida tropicalis* S; **lane 14** *Candida tropicalis* R; **lane 15** *Candida glabrata* S control; **lane 16** *Candida glabrata* S; **lane 17** *Candida krusei* R control; **lane 18** *Candida krusei* R; **lane 19** *Candida dubliniensis* S control; **lane 20** *Candida dubliniensis* S.

S: fluconazole-sensitive, R: fluconazole-resistant.

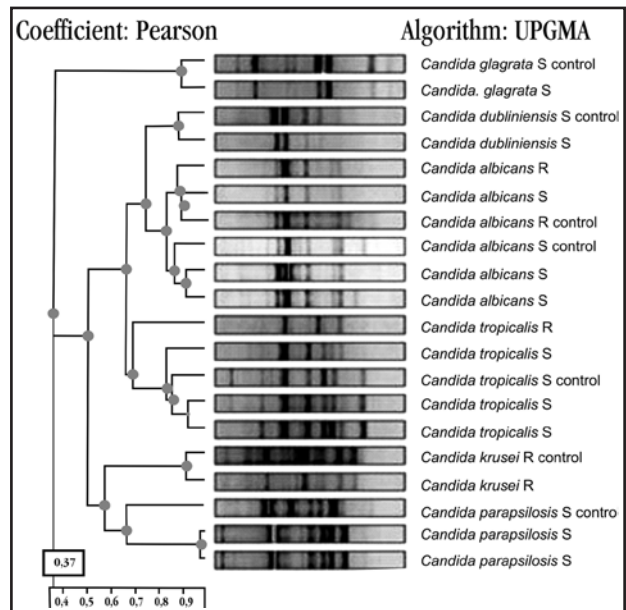


Figure 2 - The dendrograms representing the clusters of similarity were classified according to the degree of genetic relatedness: 1.0 or 100% for identical samples; 0.9 or 90% for highly related samples; 0.8 or 80% for moderately related samples; and ≤ 0.7 or 70% for unrelated samples.

- 1.0 or 100% (identical samples):
 - Cpar 1 versus Cpar 2.
- 0.9 or 90% (highly related samples):
 - Cglab versus CglabC+;
 - Cdublin versus CdublinC+;
 - CalbR, Calb3S versus CalbC+R;
 - CalbC+, Calb1S versus Calb2S;
 - Ctrp3S, CtrpC+, Ctrp1S versus Ctrp2S;
 - CkrusC+ versus Ckrus.
- 0.8 or 80% (moderately related samples): the two *Candida dubliniensis* studied (sample and control) versus all the *Candida albicans* studied.
 - ≤ 0.7 or $\leq 70%$ (unrelated samples):
 - all *Candida dubliniensis* and *Candida albicans* versus all *Candida tropicalis*;
 - the only fluconazole-resistant *Candida tropicalis* (CtrpR) versus all other *Candida tropicalis* studied;
 - the *Candida parapsilosis* prototype (CparC+) versus the two *Candida parapsilosis* studied (Cpar1 and Cpar2);
 - the two *Candida krusei* (control and isolate) versus the three *Candida parapsilosis* (CparC+, Cpar1 and Cpar2);
 - all *Candida dubliniensis*, *Candida albicans* and *Candida tropicalis* versus the group of all *Candida krusei* and *Candida parapsilosis*;
 - the lowest degree of genetic relatedness (37%) was found between the two *Candida glabrata* (CglabC+ and the isolate) versus the dendrogram formed by all other isolates and prototypes included in the present study.

DISCUSSION

Forty *Candida* isolates from pediatric patients were identified by microbiological methods, but only 34 (85%) had the species confirmed by the *Candida* nested ITS-PCR^{3 13 17}. We decided to exclude the six isolates that showed discrepancies between microbiological and nested ITS-PCR typing. It is noteworthy that the nested ITS-PCR and RAPD with the primer OP-G10 yielded identical results for all 40 isolates. Unreliability concerning identification of *Candida* species by microbiological techniques has already been reported^{1 22}. To circumvent this problem, a complex diagnostic algorithm based on a three-step procedure integrating morphological criteria, biochemical investigation and sequence analysis of the ITS region in order to achieve unequivocal identification of *Candida* species was proposed in a recent study⁶.

The fact that the two *Candida parapsilosis* included in the study were classified as unrelated to the control (degree of relatedness $\leq 70\%$) indicates that RAPD with the primer OPG-10 was able to discriminate between species. The two *Candida parapsilosis* were recovered from patients recently, while the prototype was sent from the Netherlands a long time ago, in 1973. At that time, the three species *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* were considered to be a single species²³. Moreover, the RAPD proposed was able to discriminate between one of the *Candida tropicalis* studied and the other three *Candida tropicalis* isolates (genetic relatedness $\leq 70\%$). For the moment, it is not possible to determine whether this finding might be attributable to fluconazole sensitivity. Other molecular tests such as DNA sequencing and gene expression assays will have to be performed in order to clarify this finding.

The RAPD used in the present study provided an accurate and rapid technique for identifying and differentiating *Candida* species. However, it had some disadvantages: the accuracy of DNA patterns relies on strict maintenance of reagent concentration, template DNA extraction protocol, amplification parameters and thermal cycler type¹⁵. RAPD cannot be used to determine *Candida* species directly from biological samples without prior culture isolation. Although the DNA fragments generated by RAPD cover the entire fungus genome, they are not associated with any of the four proposed mechanisms that lead, for instance, to triazole compound resistance: failure of drug accumulation, alteration in Erg11p, upregulation of ERG11, and alteration in sterol composition¹⁹.

Apart from corroborating previous studies that reported failure of microbiological techniques to identify *Candida* species^{1 22}, the present study has successfully identified *Candida* isolates. It was able to discriminate between species through the use of a single primer, while other studies have reported success in *Candida* species typing through the use of a set of primers²⁴.

We conclude that RAPD with the primer OP-G10 could be performed after culture isolation, in order to confirm microbiological species identification, thus providing faster and more reliable *Candida* species typing.

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