Phenotypic and genotypic identification of *Candida* spp. isolated from hospitalized patients

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Summary

As candidosis incidence continue to rise, quick laboratory identification of *Candida* species is becoming increasingly important for a growing population of patients at-risk. RAPD techniques were used on samples of *Candida* obtained from patients hospitalized at Santa Casa de Misericordia in Belo Horizonte (SCMBH) Brazil, from March 1998 to December 2000 and then compared with the results of phenotypic identification techniques. Two hundred and forty two yeasts were isolated and phenotypically identified as follows: *Candida albicans* (105), *Candida tropicalis* (62), *Candida parapsilosis* (28), *Candida glabrata* (19), *Candida krusei* (8), *Candida guilliermondii* (5) and *Candida* spp. (15). Samples from the three most frequent species isolated were selected randomly in order to compare the phenotypic and genotypic analyses. Genotypic analysis using RAPD primer M13 (F/R) displayed the best results of all test samples. There was both agreement and consistency between phenotypic and genotypic analysis using RAPD, demonstrating that is possible to apply this method for the identification of *Candida* species.

Key words *Candida,* Hospitalized patients, RAPD, Genotypic, Phenotypic

Identificación fenotípica y genotípica de aislamientos de *Candida* spp. de pacientes hospitalizados

Resumen

Debido al continuo incremento de las candidiasis, en especial en pacientes de grupos de riesgo, es cada vez más importante la determinación de la especie implicada a través de pruebas rápidas de laboratorio. Se analizaron para este trabajo aislamientos obtenidos de pacientes hospitalizados en la Santa Casa de Misericordia en Belo Horizonte (Brasil), desde marzo de 1998 hasta diciembre de 2000, comparando las identidades obtenidas por la técnica RAPD y métodos fenotípicos. Se obtuvieron un total de 240 aislamientos, que identificados fenotípicamente arrojaron la siguiente distribución por especies: *Candida albicans* (105), *Candida tropicalis* (62), *Candida parapsilosis* (28), *Candida glabrata* (19), *Candida krusei* (8), *Candida guilliermondii* (5) y *Candida* spp. (15). Para la comparación de los métodos fenotípicos y genotípicos, en lo referente a la eficacia en la identificación de especie, se seleccionaron aleatoriamente cepas de las tres especies más aisladas. El análisis mediante RAPD con el iniciador M13 arrojó los mejores resultados. Las identificaciones obtenidas por RAPD y por los métodos fenotípicos fueron las mismas, lo que sugiere que la técnica de genotipado utilizada puede ser de utilidad para la identificación de especies de *Candida*.

Palabras clave *Candida*, Pacientes hospitalizados, RAPD, Genotípico, Fenotípico

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The genus *Candida* includes several species implicated in human pathology such as C. *albicans*, *C. tropicalis, C. parapsilosis*, *C. glabrata, C. krusei, C. lusitaniae, C. kefyr, C. guilliermondii* and *C. dubliniensis* [27]. The increased incidence of localized and systemic infections caused by *Candida* species during the past decade has been well-documented, and the reasons for this include the growing number of immunocompromised individuals in the population as a result of the human immunodeficiency virus (HIV) pandemic and the increased use of immuno suppressive therapy in cancer and organ transplant patients. In addition, the widespread use of broad-spectrum antibacterial drugs and the increased use of invasive procedures including intubation, drains and catheters are likely to be important contributory factors [3,9,14,16,19,20].

Fast identification of *Candida* species in the clinical laboratory is becoming increasingly important as the incidence of candidosis continues to rise in the growing-at risk patient population [8,9]. Rapid species-specific identification is necessary for timely, targeted therapy and to facilitate hospital infection control measures [6,15,19,32]. For this purpose, newer identification methods have emerged, such as genotyping methods, including restriction fragment length polymorphism and pulsed-field gel electrophoresis [2,13,17,25]. Techniques based on the use of PCR have been demonstrated to be faster and more sensitive in detecting specific sequences of DNA [1,5,7,10]. The most popular PCR-based technique currently in use is RAPD (randomly amplified polymorphic DNA). In this assay, single or in pairs oligonucleotide primers of arbitrary sequence are used to amplify target DNA sequences by PCR. The amplified products form strain-specific fingerprints when electrophoresed in polyacrilamide or agarose gels [31]. This procedure seemed to be efficient in distinguishing different isolates; it has a high discriminatory power, it is easy to perform, do not require radiolabelled probes, and it is applicable to several microorganisms [18,21,22]. This work has the purpose of correlating phenotypic and genotypic identification using RAPD in samples of *Candida* obtained from patients hospitalized at Santa Casa de Misericordia of Belo Horizonte (SCMBH) - Minas Gerais, Brazil.

Materials and methods

Sample collection. Samples were obtained from 200 patients suspected of fungal infection, in SCMBH, from various anatomical sites, some associated with invasive procedures, including blood samples, secretions, and urine, collected in the period from March 1998 to December 2001. The clinical specimens corresponded to those sent to the pathology laboratory of CCIH (Comissão de Controle de Infecções Hospitalares) following the guidelines of the ethical comittee of the institution.

Culture and phenotypic analyses. All samples were seeded on Sabouraud Dextrose agar with chloramphenicol. Plates were incubated at 35 °C for 72h. Tests were carried out with pure cultures in 24h and 48h. Each morphological colony isolate was characterized according to standard methods [29]. The yeasts were identified by the keys present in Kurtzman and Fell [8].

DNA extraction and PCR assays. ATCC reference samples *C. albicans* (ATCC 18804); *C. tropicalis* (ATCC 750) and *C. parapsilosis* (ATCC 22019) were used to confirm phenotypic analyses by comparing RAPD banding patterns. DNA was prepared and purified according to modified protocol of Sambrook *et al* [23], after an enzymatic digestion with glucanase (Glucanex - Novo Nordisk, USA). Five oligonucleotides OPA1, OPA2, OPA3, OPA 4 (kit OPERON, Sigma) and M13 F/R (Pharmacia Biotech) containing different GC content were tested for screening of the isolates. The pair of primer M13F/R M13 F (5'-CGACGTTGTAAAACGACGGCCAGT-3') - M13R (5'-CAGGAAACAGCTATGAC-3') showed the best results for all species tested. RAPD analyses were basically carried out as described by Willians *et al*. [33] in a Perkin-Elmer thermocycler. The reaction was archieved in a final volume of 10 µl of a PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl, 350 µm $MgCl_2$) containing 1 picomol of each primer, 0.5 mM of each dNTPs, 0.3U of *Taq* DNA polymerase (GIBCO BRL, USA) Randomly amplified products were analyzed by electrophoresis in 8% polyacrilamide gel for 4 hours at 125V and 30 m A following by silver staining as described by Santos *et al*. [24].

For RAPD data analyses, the relative mobility of shared bands in the total profile obtained in each lane was calculated and transformed in a data matrix. For the analysis of bands obtained by RAPD all the bands were considered that had a size less than or equal to 800 pb since large bands than these were difficult to analyse as they were closely grouped together. The distances obtained for the samples in figures 1, 2 and 3 correspond to the comparative analysis of the polyacrilamide gel. Some bands were very clear and did not appear on the photograph of the gel however they could be seen using transilluminator and was therefore considered in the phenotypic analysis. The phenograms were constructed by UPGMA (Unweighted Pair Group with Arithmetic Mean) method and the robustness of the tree topology was assessed resampling 1000 times by bootstrap analyses contained in the TREECON for Windows computer package program [28].

Results

During a 3-year period (March 1998 to December 2000), 200 patients from different clinics in SCMBH were analyzed. From these patients, 242 yeasts were isolated and phenotypically identified showing the following specific distribution: 105 isolates of *C. albicans*, 62 isolates of *C. tropicalis*, 28 isolates of *C. parapsilosis*, 19 isolates of *C. glabrata*, 8 isolates of *C. krusei*, 5 isolates of *C. guilliermondii* and 15 isolates of *Candida* spp. Samples from the three most frequent species isolated, *C. albicans* (13), *C. tropicalis* (13) and *C. parapsilosis* (11), were arbitrarily chosen to correlate phenotypic and genotypic analyses. Patterns of RAPD analyses and phenograms for each species are demonstrated in Figures 1, 2 and 3. Figure 4 shows the 15 samples whose identification caused some difficulty in the phenotypic analysis were re-identified using the technique of RAPD for comparision with reference ATCC strains of *C. albicans, C. tropicalis* and *C. parapsilosis*. The identification by comparative analysis with the reference samples revealed two which corresponded to *C. tropicalis*, two to *C. parapsilosis* and 11 samples to *C. albicans*.

Discussion

In this work, RAPD was the method of choice to genetically correlate isolates of *Candida* obtained from hospitalized patients because this method has several advantages over conventional methods: only a small amount of DNA is necessary to perform this assay, significant genotypic differences can be revealed as being useful to distinguish isolates from different patients and sources, and it is faster when compared to conventional assays. The results of this work are quite similar to those obtained in reports avaliable in the literature, and shows that there was 100% agreement and consistency between phenotypic analyses and genotypic analyses by RAPD, which demonstrates that this method can be applied to directly identify *Candida* species.

Several authors used RAPD to distinguish or compare isolates obtained from different patients or inanimate sources [26,30]. Lin and Lehmann [11], using different methods such as RAPD, RFLP, PFGE and isoenzymes (MLEE) showed genetic and epidemiological correlation among the isolates from surgical wounds, hands and nasopharynx of nurses in the first described outbreak caused by *C. tropicalis*. Comparison among these methodologies shows that genetic similarities obtained from RAPD were >94,4%, and the authors suggest that this method can be successfully used to analyze hospital outbreaks. Francesco *et al*. [4], comparing four typing methods for clinical isolates of *C. glabrata* showed that methods based on electrophoretic karyotyping (EK) and RAPD were those that demonstrated the greatest discriminatory power among isolates. In all isolates identified as a certain species by phenotypic analysis, RAPD patterns demonstrated compatible profiles for that same species, when compared to the reference ATCC strain as seen in figures 1, 2 and 3. In figure 1, all isolates were very similar to each other and to the reference ATCC strain, so it was not possible to establish a correlation among the date of isolation, the unit or source of the specimen with the genetic distance obtained for RAPD. Figure 2 showed that isolates 14N and 23N are very closely grouped, they came from the Nursery, although with dates of isolation separated by 3 months. One interesting fact is that isolate 14N was obtained from an inanimate source (pacifier/nursery) suggesting nosocomial transmission. Several authors demonstrated that if separate patients are found to be infected with the same strain, this suggests cross-infection or acquisition of the strain from the same environmental sources [19,30]. Isolates 28N and 32N are also closely grouped and both were collected from the same source (urine), at the same date of isolation and unit, suggesting they are a single strain colonizing different patients.

In figure 3, although isolates 5N and 22N have different characteristics such as the unit, sources and date of isolation, they have identical RAPD patterns and were grouped in the same branch of the tree. Samples 6N and 5N were isolated at the same date but they presented a larger distance to each other when compared to sample 22N. Samples 70N and 81N have in common the same date of isolation, but they came from different clinics. The other five samples did not present very close genetic distances, compared with those of the first five samples. Figure 4 shows isolates not identified by phenotypic analysis but directly examined using RAPD and with comparisons to ATCC patterns. Difficulties were encountered in the identification of these 11 isolates as C. albicans, due to the fact that none of them was capable of producing germ tubes or chlamydospores, although presenting different patterns of nutrient uptake to those which are used in identification keys.

These results are supported by other authors [10,19,22,25] which show the efficacy of this technique in identifying samples of *Candida* spp. Molecular techniques such as RAPD are a valuable aid in the identification of species principally by comparision of band profiles of reference samples to the profiles obtained from the samples under analysis. When techniques such as RAPD are employed the results obtained are more reliable since observation of composition of the genomic DNA bands enables these doubts to be more easily resolved. The ideal would be to use phenotypic techniques (whose cost is less and it is possible to identify a greater number of samples in each experiment) in conjunction with genotypic techniques such as RAPD that allows comparison between the isolates obtained (specially, if the majority of them belong the same species).

This work correlates these two identification methods: conventional phenotypic analyses and genotypic analyses using RAPD. Many interesting features were observed when comparing different isolate characteristics by phenogram grouping. In conclusion, RAPD analysis is not only faster and simple to use, but additionally useful given its consistency with conventional methods.

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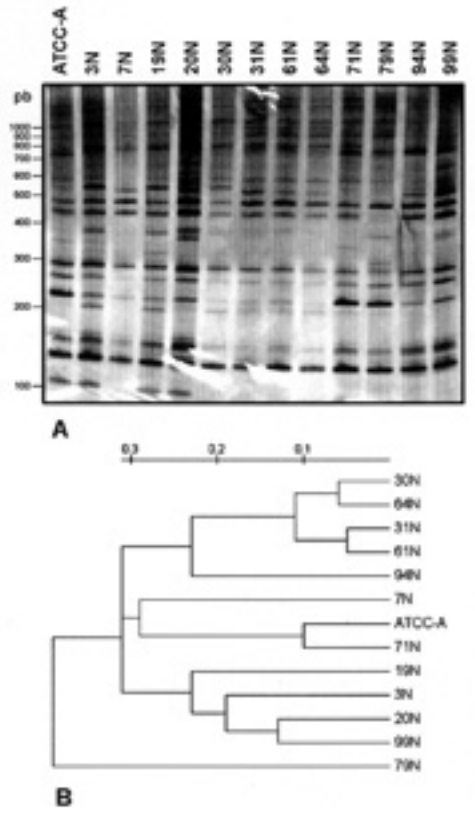

Figure 1. (A) Polyacrylamide gel showing RAPD profiles generate by primers M13 F/R using DNA isolated from different samples of *C. albicans*. (B) UPGMA phenogram of *C. albicans* samples based on pairwise band sharing. The numbers of samples refer to those listed in table 1.

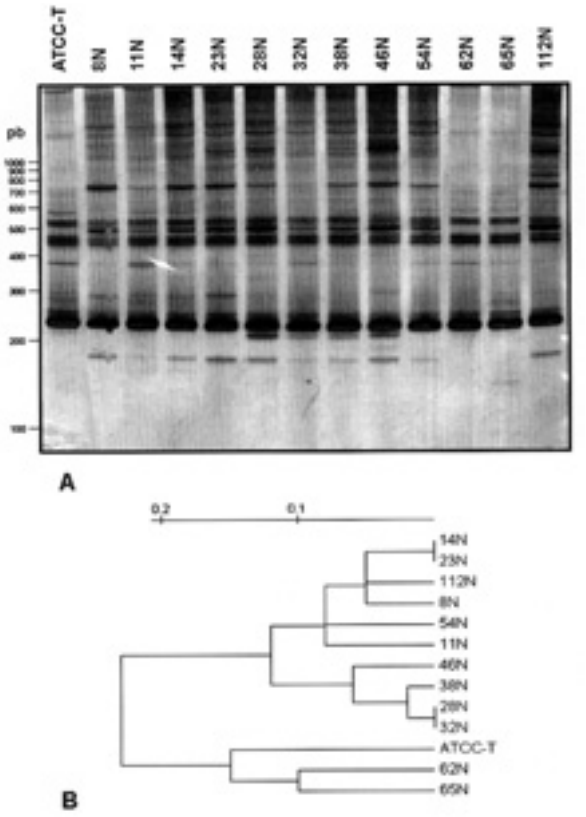

Figure 2. (A) Polyacrylamide gel showing RAPD profiles generate by primers M13 F/R using DNA isolated from different samples of *C. tropicalis*. (B) UPGMA phenogram of *C. tropicalis* samples based on pairwise band sharing. The numbers of samples refer to those listed in table 1.

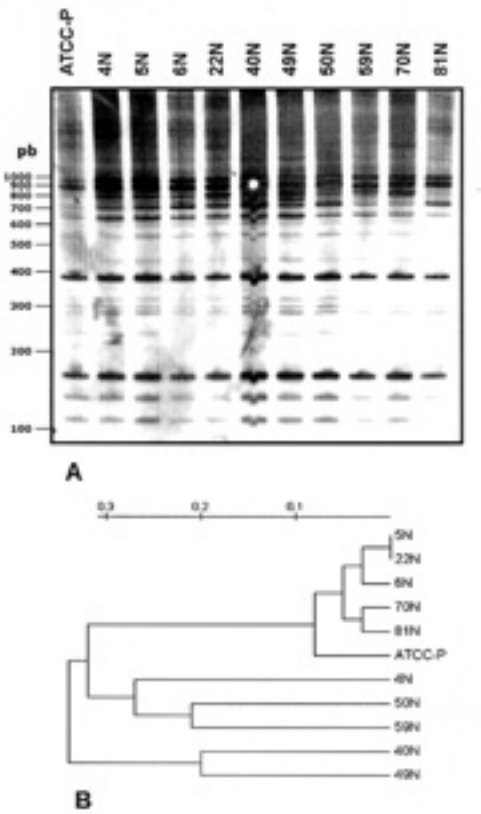

Figure 3. (A) Polyacrylamide gel showing RAPD profiles generate by primers M13 F/R using DNA isolated from different samples of *C. parapsilosis*. (B) UPGMA phenogram of *C. parapsilosis* samples based on pairwise band sharing. The numbers of samples refer to those listed in table 1.

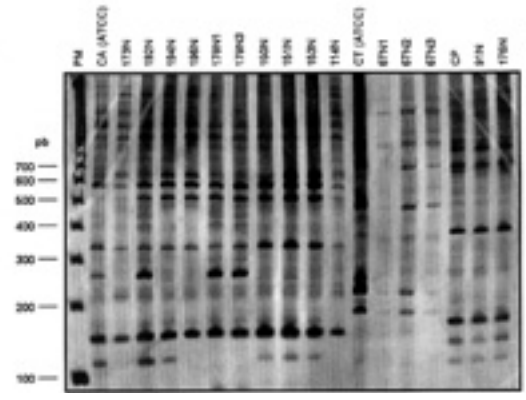

Figure 4. Polyacrylamide gel showing RAPD profiles generate by primers M13 F/R using DNA isolated from 15 non-identified samples of *Candida* by phenotyphic analysis.

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