

MOLECULAR BIOLOGICAL TECHNIQUES USED FOR THE IDENTIFICATION OF *CANDIDA SPP.*

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Abstract

Early identification of *Candida* isolates is necessary for an effective antifungal therapy. Conventional identification methods are often difficult and time-consuming. Molecular biological techniques are representing an useful alternative approach. Yeast clinical isolates were obtained from 50 independent patients admitted to the hospital during the year 2008. In the present study yeast identification was based on gene restriction profiles ARNr 5.8S by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) and on random amplification profiles, RAPD (Random Amplification of Polymorphic DNA).

Rezumat

Identificarea rapidă a tulpinilor de *Candida* la nivel de specie este necesară pentru un tratament antifungic eficient. Metodele convenționale de identificare sunt adesea dificil de realizat și durează mult timp. Tehnicile moleculare de identificare reprezintă metode alternative de abordare. Tulpinile de *Candida* au fost izolate de la 50 pacienți spitalizați în anul 2008. În această lucrare sunt prezentate rezultatele identificării unor tulpini de *Candida spp.* prin tehnici moleculare, bazate pe profilele de restricție a genei pentru ARNr 5.8S RCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) dar și pe profilele de amplificare randomizată RAPD (Random Amplification of Polymorphic DNA).

Keywords: *Candida spp.*, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Random Amplification of Polymorphic DNA (RAPD)

Introduction

Since the early 1980s, fungi have emerged as major causes of human diseases, especially among the immuno-compromised and those

hospitalized with serious underlying diseases [5]. *Candida albicans* is still the most frequent cause of fungal infections [5, 7]. The identification of yeast pathogens with this increasing diversity by conventional methods may be difficult and sometimes inconclusive [1]. Commercially available yeast identification systems, such as the Vitek Yeast Biochemical Card (bioMérieux Vitek), API 20C (bioMérieux), and API ID32C (bioMérieux), are convenient to use. However, an incubation period of 24 to 48 h is normally required before biochemical reactions can be interpreted [4]. While these commercial products are effective for the identification of commonly encountered yeasts, their application is rather limited for the identification of less frequently recovered taxa [3]. Susceptibility testing is increasingly used to guide the management of candidiasis, especially in situations in which there is a failure in the response to initial antifungal therapy. Reference methods for yeasts (CLSI M27-A3) [2] are now available. The assessment of the microorganisms by molecular techniques is able to provide in a short time, phylogenetic and taxonomic important data that led to the establishment of phylogenetic trees and reviewing the way of grouping all organisms [6].

Materials and methods

A total of 50 strains were isolated, of which tracheal secretions (25), urine (5), blood (2), abdominal drainage (3) and vaginal secretion (15 strains). The two strains isolated from the blood, noted *Candida* no. 1 and *Candida* no. 2, were identified by PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) and RAPD (Random Amplification of Polymorphic DNA). For DNA isolation, *Candida strains* were grown on YPG (yeast extract peptone) medium for 20 hours. To destabilize the cell walls, the suspension was treated with 4 mL β -mercaptoetanol and incubated for 30 minutes at 37°C and yeast protoplasts were obtained by the treatment with lyticase with a final concentration of 3 mg/mL and incubated for 90 min. at 37°C. Total cell lysis was achieved using 10% SDS (sodium dodecyl sulphate) solution to a final concentration of 1%. It was used for deproteinisation 2.5M KCl solution, proteinase K to a final concentration of 200 mg/mL and was incubated 30 minutes at 37°C. At last, in the final stage of deproteinisation it was added an equal mixture volume of chloroform and isoamyllic acid (vol:vol 24:1) and stirred for 10 minutes at room temperature. At the end of the DNA isolation protocol, RNA molecules were destroyed by the treatment with RNAase which was added up to a final concentration of 100 mg/mL. Nuclear DNA precipitation was achieved by adding 0.5-1 mL of isopropanol (at room temperature) and

which was re-suspended in 30 mL TE (TRIS-EDTA) solution. Checking DNA extracts was achieved by their migration in agarose gel 0.8% and the purity of DNA extracted could be determined by spectrophotometry, considering the absorbance at different wavelengths (A_{260} , A_{230} and A_{280}) and equivalent 1 DO(optical density) $_{260nm}$ = 50 μ g DNA/mL for DNA double stranded. ITS1 (internal spaces of transcription) and ITS4 (internal spaces of transcription) were used to amplify gene rRNA 5.8S, using the following sequences:

- ITS (internal spaces of transcription) 1-5 'TCCGTAGGTGAACCTGCGG 3', and
- ITS (internal spaces of transcription) 4-5 'TCCTCCGCTTATTGATATGC 3'.

PCR was performed in a final volume of 25 mL and contained: 10 ng genomic DNA, the mixture of nucleotides (dGTP, dATP, dTTP, dCTP), 0.8 mM final concentration, each of the two primers at a concentration of 0.2 μ M, 2.5 mL Taq DNA polymerase buffer, 1.25 U Taq DNA-dependent DNA polymerase. After mixing the components, the following version of amplification was applied : *a*) denaturation of 3 ' mold at 95°C; *b*) 30 cycles of denaturation 1' to 95°C, 1' primer attachment at 50°C polymerization 2' at 72°C; *c*) Polymerization 10 ' at 72°C. At the end of the amplification, agarose gel (1.5% concentration) electrophoresis was performed in TBE (Tris base /boric acid/ EDTA) buffer with ethidium bromide 0.5 μ g/ μ L. The applied voltage is 3V/cm. To check the contamination of the reaction mixtures it was carried out a blank with no DNA. In order to obtain restriction profiles of rDNA 5.8S amplicons, the total reaction volume was 15 μ L, in which were added 5 μ L amplicon (obtained by PCR), restriction buffer and 10U of enzyme for each reaction part. The reaction was incubated overnight at 37°C. Checking restriction products by eletrophoresis was achieved in agarose gel (2.5% concentration), ethidium bromide 0.25 μ g/mL, 2 V/cm. For assessing the size of restriction fragments obtained, it was loaded a 100 bp (base pairs) molecular weight marker (Promega). For the evaluation of the random amplification profile (RAPD) of the studied strains, OPA 02 primer was used. The amplification volume was 30 μ L and the reaction components were: 50 ng DNA, 0.2 nm primer, 1U Taq polymerase (Promega) and nucleotide mix at 0.2 Mm concentration for each type of nucleotide in part. Amplification parameters included 35 cycles with the following steps: denaturation at 95°C for 45 seconds, attaching primers at 36°C for 2 minutes and elongation at 72°C for 2 minutes. In addition, the first stage of denaturation of DNA was performed at 95°C for 5 minutes and the final elongation was covered in 10 minutes at the same temperature (72°C). At the end of the amplification, the analysis of 10 μ L amplification products

was performed by electrophoresis in agarose gel (2.5% concentration). A 50 base pairs molecular weight marker (Promega) was loaded to determine the molecular weight of DNA fragments.

Results and discussion

Lately, the large number of infections caused by *Candida* strains has increased interest in the identification and characterization of these organisms to obtain valuable information that will lead eventually to the development of strategies to combat these microorganisms. During this study, two *Candida* strains isolated from invasive infections (blood cultures) were subjected to a protocol for isolation and purification of chromosomal DNA in place after that described by Vassu [9], taking into account both the optimization of cell wall lysis and deproteinisation steps of the cell. The integrity of chromosomal DNA was checked by agarose gel electrophoresis revealing its integrity and the efficiency of therapy with RN-ase A. Following electrophoresis and spectrophotometric analysis it was found that the extracts were: *a)* in an optimum concentration for subsequent molecular analysis; *b)* did not show protein contamination according to the A_{260}/A_{280} ratios, being within the permissible limit (1.8 - 2), and also no polysaccharide contamination ($A_{260}/A_{230} > 2$) (Figure 1)

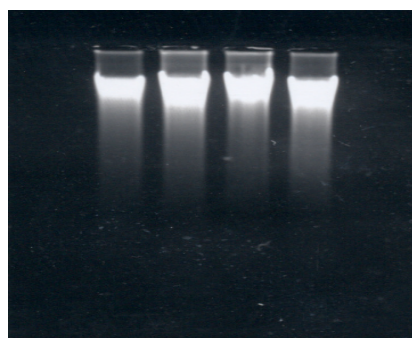


Figure 1

Agarose gel electrophoresis of genomic DNA

1). *C. albicans* ATCC 10231; 2). *Candida* strain no. 1;
3). *Candida* strain no. 2; 4). *C. albicans* ATCC 10231

Molecular studies on 5.8S rRNA gene were carried out to obtain additional information for a precise taxonomic classification. *Candida* gene regions 18S, 5.8S and 28S that encode ribosomal RNA evolved slowly and are relatively conserved in terms of sequences. Between regions encoders there are internal spaces of transcription 1 and 2 (ITS1 and ITS2); which have evolved much faster and can vary from one species to another and

even from one genre to another. Moreover these genes are found in a number of copies higher than other genes (40-80 copies) being more sensitive in the amplification process. These genes were chosen for further molecular analysis due to the above issues. In this respect DNA strains studied had undergone a process of *in vitro* amplification using ITS1 (internal spaces of transcription) and ITS4 (internal spaces of transcription) primers. The amplicons dimensions were checked by electrophoresis and by using two molecular weight markers, namely 100 base pairs ladder and 50 base pairs ladder (Promega) and amplicons were found to have the expected size (about 550 base pairs) (Figure 2).

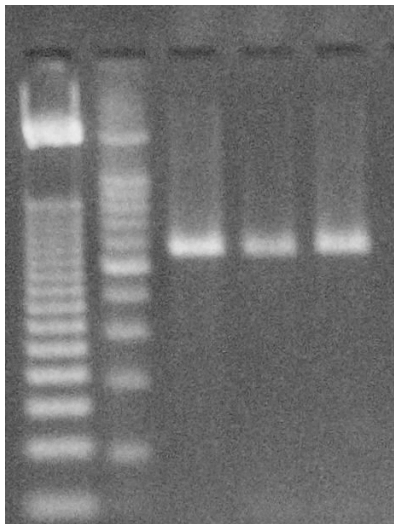


Figure 2

Gel electrophoresis of rRNA 5.8 S amplicons of the analyzed strains

- 1). 50 base pairs molecular weight marker; 2). 100 base pairs molecular weight marker; 3). *C. albicans* ATCC 10231; 4). *Candida* strain no. 1; 5). *Candida* strain no. 2

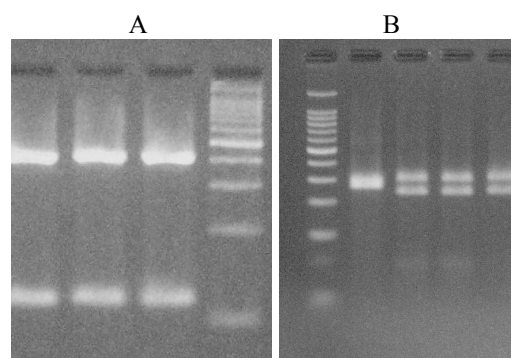
In the recent years, techniques based on PCR fingerprint, especially ARDRA (amplified rDNA restriction analysis) proved more useful. ARDRA technique essentially has the following advantages: address changes in the sequence of 5.8S rRNA gene which can provide with high accuracy the taxonomy information and molecular phylogeny, do not require sequencing of 5.8S rDNA amplicons sequencing using technical variants less laborious (digestion with restriction endonucleases) [8]. ARDRA was found to be the highest among the discriminatory power techniques. Experiments aimed to compare our ARDRA patterns with reference strains *C. albicans* ATCC 10231. After getting the amplicons by

PCR, they were subjected to digestion (in separate reactions) with *Hae III* (from *Haemophilus aegyptius*) and *Cfo I* (from *Clostridium formicoaceticum*) restriction endonucleases. The analysis of gels and comparison of restriction fragments could determine the restriction patterns of the 2 strains of *Candida*, calculating the size of obtained restriction fragments. For checking, the amount of restriction fragments was calculated for each reaction in part, in all cases achieving a value of about 550 base pairs. After obtaining the pattern of restriction sites we found that both strains showed identical restriction patterns with the species *C. albicans* ATCC 10231. (Table I and figure 3).

Table I

Molecular weight of restriction fragment of rDNA 5.8S after treatment with *Cfo I* (from *Clostridium formicoaceticum*), and *Hae III* (from *Haemophilus aegyptius*) endonucleases

Strains	<i>Cfo I</i> (bp)	<i>Hae III</i> (bp)
<i>C. albicans</i> ATCC 10231	290+260	100+450
<i>C. albicans</i> no.1	290+260	100+450
<i>C. albicans</i> no.2	290+260	100+450

**Figure 3**

Agarose gel electrophoresis of restriction fragments obtained with *Hae III* (A) and *Cfo I* (B) endonucleases

A – 1) *C. albicans* ATCC 10231; 2) *Candida* strain no1

3) *Candida* strain no2; 4) 100 base pairs molecular weight marker

B – 1) 50 base pairs molecular weight marker; 2) *C. albicans* amplicon;

3) *C. Albicans* ATCC 10231; 4) *Candida* strain no. 1; 5) *Candida* strain no. 2

Obtaining amplification patterns by RAPD technique - For identification of the studied yeast strains it was used the RAPD technique, which allows the analysis of "genetic fingerprints" obtained and comparing them with those obtained for the standard strains. In this technique there

were used OPA 02 primers, with short sequence of nucleotides. The RAPD technique is used to analyze the genomic variability of isolates. This technique is capable of producing amplification profiles that allow the identification of intra and inter-specific polymorphisms of the studied isolates. The pattern of amplification of our analyzed strain was similar to the reference strain *Candida albicans* ATCC 10231 and different from other reference strains used in the study. (Figure 4)

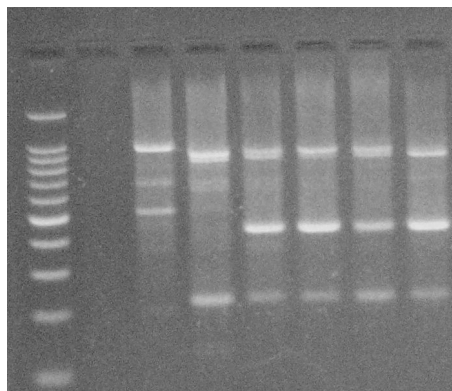


Figure 4

RAPD (Random Amplification of Polymorphic DNA) pattern obtained with the OPA 02 primer

- 1) 100 base pairs molecular weight marker; 2) free; 3) *C.tropicalis*; 4) *C. krusei*; 5) *C. albicans* ATCC 10231; 6) *Candida* strain no. 1; 7) *Candida* strain no. 2; 8) *C. albicans* ATCC 10231;

Conclusions

The RAPD (Random Amplification of Polymorphic DNA) assay with the OPA-02 primer is very specific and sensitive for the identification of important pathogenic *Candida* species. The assays here described allow the relatively rapid identification of *Candida* species and offer alternatives to conventional morphologically and physiologically based identification procedures and their associated problems.

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