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Full Length Research Paper

Identification of *Candida glabrata* and *Candida parapsilosis* strains by polymerase chain reaction assay using RPS0 gene fragment

Emira Noumi[1,2]*, Mejdi Snoussi[1,3], Maria del Pilar Vercher[2], Eulogio Valentin[2], Lucas Del Castillo[2] and Amina Bakhrouf[1]

[1]Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Département de Microbiologie, Faculté de Pharmacie, Monastir, Tunisia.
[2]Departamento de Microbiología y Ecología, Facultad de Farmacia, Universidad de Valencia, Burjassot, Valencia, Spain.
[3]Laboratoire de Traitement et de Recyclage des Eaux. Centre de Recherches et des Technologies des eaux, Technopôle de Borj-Cédria, BP 901, 2050 Hammam-Lif, Tunisia.

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Two *Candida* species were identified by the amplification of the RPS0 gene intron fragment. For this, two pairs of primers were used in PCR analysis performed with genomic DNA of clinical isolates of Candida. The primers designed are highly specific for their respective species and produce amplicons of the expected sizes and fail to amplify any DNA fragment from the other species tested. For *Candida glabrata*, the size of the amplicon was 406 pb and 150 bp for *C. parapsilosis*. The designed primers were able to amplify all *C. glabrata* isolates. One of three *C. parapsilosis* strains was confirmed as *C. orthopsilosis*, when we used the designed oligonucleotides. The used primers cannot amplify the other *Candida* species such as *C. albicans*. These results indicate that sequences of intron genes can be useful to specifically identify Candida strains by PCR. This molecular identification will be considered as an early identification of *Candida* species responsible for all candidiasis.

Key words: *Candida glabrata*, *C. parapsilosis*, *C. orthopsilosis*, PCR- identification, RPS0 intron.

INTRODUCTION

Candidiasis constitutes the majority of fungal infections with an increased incidence (Baquero et al., 2002). *Candida albicans* is the most common pathogenic *Candida* species. Last year, a shift in the spectrum of *Candida* species has been observed, with an increase of other non *Candida albicans* species (Viscoli and Castagnola, 1999) such as *Candida glabrata* and *Candida parapsilosis* (Malani et al., 2001).

C. parapsilosis has emerged as an important nosocomial pathogen. Due to its variable genetic composition, two new species named *Candida metapsilosis* and *Candida orthopsilosis*, have been recently identified replacing the existing designation of *C. parapsilosis* groups II and III (Tavanti et al., 2005). The identification of the two new species is currently performed with the aid of DNA-based techniques (Tavanti et al., 2005). DNA-based techniques of *C. parapsilosis* strain collections revealed that *C. orthopsilosis* and C. metapsilosis constitute 10% of all infections previously attributed to *C. parapsilosis* (Gomez- Lopez, 2008; Lochart, 2008) . These three separate species, *C. parapsilosis*, *C . orthopsilosis* and *C. metapsilosis* have been identified using multigenic sequence analysis and internal transcribed spacer sequencing (Tavanti et al., 2005).

For all Candida strains and in the absence of pathogenic signs or symptoms, the diagnosis of candidiasis is usually based on the isolation and identification of Candida by conventional morphological and carbohydrates assimilation tests which take many days (Warren, 1995; Velegraki et al., 1999). Furthermore,

*Corresponding author. E-mail : emira_noumi@yahoo.fr. Tel: +216 71325044//+216 79325750. Fax: +216 79325802.

Table 1. Origin and designation of strains of *C. glabrata* and *C. parapsilosis* tested for identification by *RPS0* intron amplification.

Species	Strain	Origin
C. glabrata	E_{69}	Vaginal
C. glabrata	P_2	Vaginal
C. glabrata	P_5	Vaginal
C. glabrata	P_8	Vaginal
C. glabrata	P_9	Vaginal
C. glabrata	P_{11}	Vaginal
C. glabrata	P_{12}	Vaginal
C. glabrata	P_{13}	Vaginal
C. glabrata	$P_{14'}$	Vaginal
C. glabrata	$P_{15'}$	Vaginal
C. glabrata	P_{17}	Vaginal
C. glabrata	$P_{19'}$	Vaginal
C. glabrata	P_{21}	Vaginal
C. glabrata	P_{25}	Vaginal
C. glabrata	P_{26}	Otitis pus
C. glabrata	$P_{26'}$	Otitis pus
C. glabrata	E_{68}	Urine
C. glabrata	8	Oral
C. glabrata	15_T	Oral
C. parapsilosis	H_{11}	Vaginal
C. parapsilosis	H_{12}	Vaginal
C. parapsilosis ATCC 22019	R_2	Type strain
C. orthopsilosis J981226	R	Type strain

clinical yeast isolates are sometimes misidentified when automated biochemical systems are used (Dooley et al., 1994). Thus, rapid and accurate identification methods of pathogenic fungi at the species level would prove very helpful in clinical terms. Thus, it is necessary to develop a simple and rapid system that can identify the majority of *Candida* species. The conventional antibody detection tests for the direct detection of *Candida* species antigens has been shown to have potential as an early diagnostic test (Lemieux et al., 1990). Also, CHROMagar Candida is another conventional method of the identification. The sensitivity of CHROMagar identification of Candida was 66.7% to 100%, and specificity was 95.7% to 100% (Willinger and Manafi, 1999).

In recent years, several molecular biology-based methods have been developed to diagnose Candida infections and for specific identification of Candida to the species level (Xiang et al., 2007). PCR methods are particularly promising because of their simplicity, specificity and sensitivity. One of some sensitive and specific method to rapidly and simultaneously identify the most common pathogenic Candida yeast species is based on the use of primers targeted to the yeast RPS0 gene (intron or exon) to obtain a DNA fragment specific for each yeast species by PCR assay. The RPS0 gene codes for a protein which is a component of the translational machinery and is extremely conserved among species (Baquero et al., 2002, Garcia et al., 2010). Its homology extends to the whole DNA coding sequence, allowing the design of degenerate primers of this gene for amplification purposes, even if the sequence is unknown. However, more yeast species and almost all the fungal species, contain one or more introns that completely differ in size and sequence and enable the design of specific primers for the identification of the species.

In this study, we used the oligonucleotide primers deduced from the *C. glabrata* and *C. parapsilosis* RPS0 intron to specifically amplify, by PCR assay, a DNA fragment in these two species. The RPS0 gene is the same gene Ca YST1 amplified in *C. albicans* strains with difference in intron sequence.

MATERIALS AND METHODS

Clinical strains, media and growth conditions

A total of twenty two strains (19 *C. glabrata* and three *C. parapsilosis*) were subject of this study. Two *C. glabrata* isolates (8 and 15T) were collected from patients admitted to the dental hospital of Monastir (Tunisia) and suffering from denture stomatitis. The other 17 *C. glabrata* isolates were obtained from women attending the service of genecology of Farhat Hached hospital (Sousse, Tunisia). Fourteen of these *C. glabrata* strains were vaginal isolates, two of them were isolated from otitis pus and one strain was isolated from urine. Two *C. parapsilosis* strains (H11 and H12) were isolated from oral cavity of patients; the other strain was *C. parapsilosis* ATCC 22019 reference strain (R2) (Table 1).

The *C. orthopsilosis* J981226 strain was kindly provided by Dr. Odds (University of Aberdeen, Scotland) and served as a positive control. All samples were cultured on Sabouraud chloramphenicol agar (Bio-rad, France) for 48 h at 30°C. All clinical isolates were identified by standard microbiological methods: macroscopic test of culture on Sabouraud chloramphenicol agar, microscopic test and carbohydrates assimilation test by using the ID 32 C system (bio-Mérieux, Marcy l'Étoile, France).

Biochemical identification of Candida strains

All the strains have been identified by assimilation tests using the ID32 C strips (bio-Mérieux) according to the manufacturer's specification. Identification is produced using identification software (Gutierrez et al., 1994). Strains were stored at 4°C on Sabouraud dextrose broth (Bio-rad, France) supplemented with glycerol at 10% (v/v).

Genotypic identification of *Candida* spp.

Preparation of DNA

For DNA extraction, yeasts were routinely grown on Sabouraud dextrose agar plates at 28°C for 24 h to 48 h. A single colony was then grown overnight on YPD broth (1% yeast extract, 2% peptone, 2% dextrose) at 28°C, with shaking at 200 rpm. DNA was extracted from cultures by adapting the method described previously to yeast (Del Castillo et al., 1995) as described by Garcia et al. (2010). DNA concentrations and A260/A280 ratios were determined by means of a "Gene Quant Spectrophotometer" (Pharmacia). An A260/A280

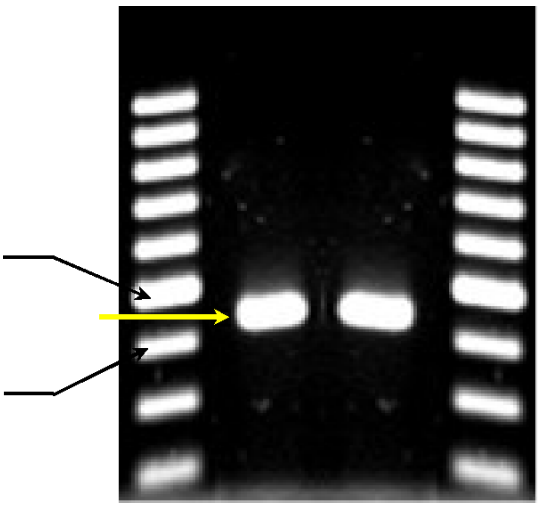

Figure 1. Representative agarose gel electrophoresis (1% agarose) showing the amplification of products obtained for oral (strain 8) and vaginal (strain P21) *C*. *glabrata* isolates. M: 100-pb molecular weight marker (Fermentas).

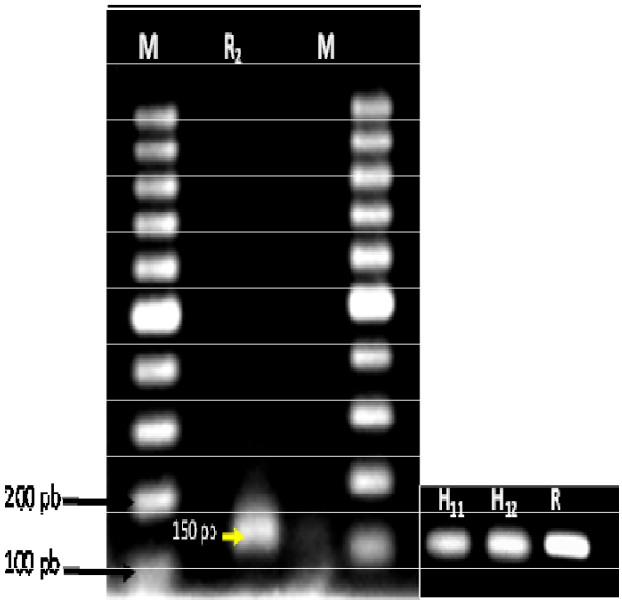

Figure 2. Representative agarose gel electrophoresis (1% agarose) showing the amplification of products obtained from *C. parapsilosis* ATCC 22019 (R2) and *C*. *orthopsilosis* (H11, H12 and J981226 (R)) isolates. M: 100-pb molecular weight marker.

ratio of 1.8 to 2.1 was considered acceptable.

Primers, conditions of PCR amplification and agarose gel electrophoresis

The sequences of synthetic oligonucleotides used as primers were CG1 (5'-acatatgtttgctgaaaaggc-3') and CG2 (5'-acttttttcttagtgttcaggacttc-3') for *C*. *glabrata*, CP1 (5'-agggattgccaatatgccca-3') and CP2 (5'-gtgacattgtgtagatccttgg-3') for *C*. *parapsilosis* (Garcia et al., 2010). The primers used for the identification of *C. orthopsilosis* strains were generously provided by Dr. Lucas Del Castillo (University of Valencia, unpublished data). CO1 (5'-tttcaatatgcctagagccacattgtgaatac-3') and CO2 (5'-gcattagttagtatcgtctttttattaaata-3') for *C*. orthopsilosis (M.P. Vercher, unpublished results).

The amplification was performed in an automated thermocycler in a final volume of 25 µl containing 2.5 µl of 10x buffer, 1 µl of 50 mmol of $MgCl_2$, 2.5 U of Eco Taq polymerase (MBI fermentas), 2.5 µl of dNTP (2.5 mmol) (Sigma, St. Louis, MO, USA), and optimum concentrations of each primer (4 µmol). One microlitre of DNA suspension (30 to 50 ng) was amplified in a PCR thermal cycler (PTC-150 MinicyclerTM) by using 1 cycle at 95°C for 5 min and then 35 cycles as follows: 30 s of denaturation at 95°C, 30 s of annealing at 56°C, and 90 s of primer extension at 72°C. At the final cycle, an additional 10 min of incubation at 72°C was added for complete polymerization.

The resultant fragments of amplified DNA were analyzed by electrophoresis through 1% agarose gels, run in Tris-acetate-EDTA buffer (TAE) for 1 h at 90 volts. A 100-bp ladder (Fermentas) was used as a size marker. Gels were stained in a solution of ethidium bromide (10 µg/ml) and photographed by Gel printer plus and image analysis software ScionImage (TDI. S.A. Madrid, Spain).

RESULTS

Identification of Candida isolates based on carbohydrates assimilation using ID 32 C system identified our isolates as *C. glabrata* (19 strains) and *C*. *parapsilosis* (3 strains). Molecular identification of this two species has been carried out by PCR amplification of RPS0 gene intron fragment. For *C*. *glabrata*, primers CG1 and CG2 (Garcia et al., 2010) produce a specific amplicon of 406 pb (Figure 1). This amplicon was sequenced and presented a 100% identity with RPS0 intron (Figure 1) . We obtained 100% of positive PCR products from 19 strains of *C. glabrata* (an amplicon size of 406 bp). Since the intron sequences are poorly conserved among microorganism strains, we decided to use this 406 bp amplicon, which we termed CgRPS0- INT, to identify *C. glabrata* from different sources. Our results showed that these primers can specifically identify only *C. glabrata* strains isolated from the oral cavity and vaginal site.

The second strain object of this study was *C. parapsilosis*. The size of the amplicon was 150 pb (Figure 1). Amplification with specific primers CP1 and CP2 (Garcia et al., 2010) only identify as *C*. *parapsilosis* the control strain ATCC 20019, shown by an amplicon of 150 bp on the gel. No amplicon was obtained for the two oral isolates (H11 and H12). These strains were further identified as *C*. *orthopsilosis* using specific primers recently designed at the Department of Microbiology of the Valencia University, Spain (Vercher, 2009).

For the two Candida species, only one amplicon was obtained (406 pb for *C*. *glabrata* and 150 pb for *C*. *parapsilosis*). As shown in Figure 2, the 406 bp fragments were amplified in all 19 *C* . *glabrata* isolates tested, confirming the high sensitivity of the method.

DISCUSSION

PCR approaches for the identification of *Candida* species are important in both epidemiological and taxonomic studies. Genes containing intron sequences could prove useful to design specific primers for the identification of yeast strains at the species level. The present study extends the work of Baquero et al. (2002) and Garcia et al. (2010) that used RPS0 intron based primers to identify different *Candida* species by designing a set of primers for the identification of yeast species of relevant clinical interest. These primers are based on the RPS0 gene and are mainly derived from intron sequences.

According to the previous results, PCR primers based on RPS0 introns are an important tool for the identification of yeasts and fungi of clinical and environmental interest. For the few yeast species with no RPS0 intron, primers from the less conserved regions of the gene can be designed. Alternatively, another gene intron could be eventually used for these few species. These data indicate that sequences of intron genes can be useful to specifically identify Candida strains by PCR.

The discriminatory power of the described test (Garcia et al., 2009) is strongly supported by the fact that, two strains of *C*. *parapsilosis* were confirmed as non *C*. *parapsilosis* by this molecular identification, whereas they were identified as *C*. *parapsilosis* with the Api ID 32 C system. The confirmation of the identity of these two strains as *C*. *parapsilosis* group III or *C*. *orthopsilosis* by PCR amplification with primers specific for *C*. *orthopsilosis* RPS0 intron.

Conclusion

Molecular approaches may have interest in epidemiological and taxonomic studies and will be considered as an early identification of *Candida* species responsible for all candidiasis, in this work, we showed the value of RPS0 gene intron for identification of pathogen yeast species and we concluded that, this molecular identification will be considered as an early identification of *Candida* species responsible of all candidiasis.

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