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Mycopathologia

ISSN 0301-486X
Volume 174
Combined 5-6

Mycopathologia (2012) 174:451-456
DOI 10.1007/s11046-012-9567-2

Springer

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Mycopathologia (2012) 174:451–456
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Rapid Identification of *Histoplasma capsulatum* Directly from Cultures by Multiplex PCR

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Received: 1 April 2011 / Accepted: 6 July 2012 / Published online: 21 July 2012
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Abstract The multiplex PCR developed from a suspension of the yeast fungi correctly identified fifty-one clinical of *H. capsulatum* var. *capsulatum* strains isolated from clinical samples and soil specimens. The multiplex PCR was developed by combining two pairs of primers, one of them was specific to the *H. capsulatum* and the other one, universal for fungi, turned out to be specific to *H. capsulatum,* regardless of the fungus isolate studied. Primers designed to amplify a region of about 390-bp (Hc I–Hc II) and a region of approximately 600-bp (ITS1–ITS4) were used to identify a yeast isolated as *H. capsulatum* when both regions could be amplified. Absolute agreement (100 % sensitivity) could be shown between this assay and the cultures of *H. capsulatum* according to their morphological characteristics. Failure to amplify the target DNA sequence by PCR with primers Hc I–Hc II in the presence of the ITS1–ITS4 amplicon in isolates of *P. brasiliensis*, *Cryptococcus neoformans, Trichosporon spp, Candida glabrata, C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, or *Penicillium marneffei* was an unequivocal sign of the high specificity of this assay. The assay specificity was also found to be 100 %. Incipient yeast forms obtained from clinical samples were identified as *H. capsulatum* by the PCR assay described before the morphological characteristics were registered shortening the time of diagnosis.

Keywords Fungal identification · Histoplasmosis · Hc100 PCR · Intergenic spacer region · Molecular diagnosis · Multiplex PCR · Universal fungal primers

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Introduction

Histoplasmosis, a systemic fungal disease caused by *Histoplasma capsulatum* var. *capsulatum* [1, 2], is an important health problem worldwide. Although the majority of histoplasmosis cases present as a mild to moderate flu-like disease requiring only supportive therapy, approximately 5 % of patients develop a more serious pulmonary and extrapulmonary disease that can be life-threatening if diagnosis is delayed or if treatment is not initiated rapidly [3–7].

Diagnosis of histoplasmosis requires a high index of clinical suspicion and awareness of the uses and limitations of the tests commonly used to identify fungal diseases. It is based on histopathology and culture as well as antibody and antigen detection. Tissue biopsy should be done as soon as possible to look for *H. capsulatum.* Finding the distinctive 2–4 μm, oval, narrow-based budding yeasts allows a tentative diagnosis of histoplasmosis [3]. Other microorganisms can

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mimic the appearance of *H. capsulatum* in tissues, but generally, the clinical picture will separate histoplasmosis from the others [3]. Although histopathology may provide for rapid diagnosis, its sensitivity is <50 % in patients with disseminated disease or even lower in those with pulmonary histoplasmosis [8].

In microbiology laboratories, the standard stain used to demonstrate the tiny *Histoplasma* yeast form in clinical specimens is Giemsa [9]. The identification of *H. capsulatum* is based on the morphology of the colony, its mold-to-yeast conversion, and the organism's microscopic morphology. *Histoplasma* antigen detection in urine and/or serum has a variable range of sensitivity, depending on the clinical pattern, the chronicity of the affliction, and the underlying condition of the patient [10–13]. Serologic testing by immunodiffusion and complement fixation are also useful, although both false-positive and false-negative results may occur [7, 14].

Delay in diagnosis while awaiting results of fungal cultures may lead to a fatal outcome in the most severe cases. An improved recognition of positive cultures through molecular diagnostic techniques is needed.

Nucleic acid amplification diagnostic techniques including polymerase chain reaction (PCR) are increasingly used in clinical practice. Most reports on histoplasmosis diagnosis by PCR have been focused on clinical samples [15, 16]. Target sequences within the 18S rRNA genes are often used for diagnostic PCR to achieve high sensitivity, since multiple gene copies are usually present within a single genome. Accordingly, there were several reports targeting the 18S rRNA *Histoplasma* gene to detect and monitor experimental or clinical infections [16–18]. However, rRNA genes are conserved regions bearing the risk of nonspecific amplifications among the related species. This suggested the need for an additional PCR target [16]. A distinctive target gene of *H. capsulatum* was sought in order to develop a diagnostic PCR assay with high specificity. The product of this gene, a protein of 100-kDa-like of *H. capsulatum* (Hcp100), is probably a regulatory protein involved in the processes required for fungal adaptation and its survival in the intracellular hostile conditions of the macrophages. The protein was described as being essential for the existence of *H. capsulatum* in human cells [19].

Ribosomes are critical for survival in all forms of life, from bacteria to humans; their physical parameters have been conserved. However, some components within the ribosomal factories have changed during the evolutionary process. These similarities, as well as the changes within the genetic material, can be used as a tool for the identification of microorganisms such as fungi as well as the performance of phylogenetic studies [17, 20]. Ribosomal RNA amplification has also been used as an internal control in PCR amplification [16, 21, 22]. The most popular approach as an internal amplification control in prokaryotes is the use of specific primers targeting conserved sequences of 16S and 23S ribosomal DNA [21], and 18S rDNA partial sequence [16], or universal fungal primers ITS1–ITS4 in *H. capsulatum* [22]. An internal amplification control is necessary for PCR analyses because of false-negative results [21, 23].

A sensitive and specific technique is described in this study: a multiplex PCR developed for the early identification of *H. capsulatum* isolates in culture from yeast fungi. This technique combines two pairs of primers: the Hc I–Hc II (species-specific primers) and the ITS1–ITS4 (universal fungal primers) in a single PCR tube. The ITS1–ITS4 primer pair was included as a positive control to monitor the amplification of all fungal samples.

Materials and Methods

Strains Used in this Study

Fifty-one *H. capsulatum* var. *capsulatum* isolates were included in this study. Forty-five strains were obtained from the culture collection of the Mycology Center, School of Medicine, University of Buenos Aires. Isolates of *H. capsulatum,* 1 from the cerebrospinal fluid, 5 from bronchoalveolar lavage, 1 from bone marrow, 19 from blood culture, 5 from oral mucosa, 1 from mucosa nasal, 8 from skin, 4 from sputum, and 1 from soil specimen were converted to yeast fungi with successive subcultures using Brain Heart Infusion (BHI, BioKar Diagnosis, Beauvais, France) agar at 37 °C. In addition, clinical specimens (6 blood cultures) recently recovered from patients suspected to have histoplasmosis were simultaneously cultured on Sabouraud Dextrose Agar (SDA, BioKar Diagnosis, Beauvais, France) and BHI agar at both temperatures 28 and 37 °C, respectively, in order to accelerate the time of yeast fungi recovery and then the identification.

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The other microorganisms tested included closely related fungi (i.e., strains of *Paracoccidioides brasiliensis*), those which are similar to the tissue phase of *H. capsulatum* (*C. glabrata* and *Penicillium marneffei*), and a variety of yeasts (*Candida krusei*, *Candida parapsilosis*, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Cryptococcus neoformans*, and *Trichosporon* spp.), commonly encountered in clinical mycology laboratories. The molds were identified by their growth characteristics, as well as their microscopic and colonial morphology. Yeast isolates were identified by conventional mycological methods: colony morphology on the chromogenic medium (CHROMagar Company, Paris, France) [24], morphology on agar 1 % milk and Tween 80 [25] and carbohydrate assimilation assay using the commercially available kit API ID 32C (BioMérieux, France).

A pure culture from each isolate was maintained on BHI agar. A total of 3 or 4 mm loopful from each fungus after 72 or 96 h incubation on BHI was put on 200 µl of sterile distilled water in microcentrifuge tubes. The suspension was vortexed vigorously for 1 min and tested as template for PCR.

Multiplex PCR

Multiplex PCR was carried out by using two pairs of primers. Primer pairs Hc I (5′-GCG TTC CGA GCC TTC CAC CTC AAC-3′) and Hc II (5′-ATG TCC CAT CGG GCG CCG TGT AGT-3′) were used to amplify a 391-bp nucleotide sequence of a gene coding for a 100-kDa-like protein (Hc100PCR), specific to *H. capsulatum* (accession number AJ005963) [16]. Primer pairs ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT G-3′) were universal fungal primers, used to produce approximately 600-bp-sized amplicons that contained conserved regions among fungi [26].

The *H. capsulatum* PCR was performed as follows: the reaction mixture consisted of 10 µl of suspensions from of yeast culture in a total volume of 50 µl with final concentrations of 1× PCR Buffer (Invitrogen, Argentina), 2.5 mM $MgCl_2$, 1 µM concentration of each primer Hc I, Hc II, ITS1, and ITS4 (Invitrogen, Argentina), 1.25 U of Taq DNA polymerase (Invitrogen, Argentina), and 100 µM concentration of each deoxynucleoside triphosphate (Invitrogen, Argentina). Reaction mixtures with primer sets were thermally cycled (MultiGene Gradient Thermal Cycler Edison; NJ, USA) once at 95 °C for 5 min, 30 times at 95 °C for 20 s, 56 °C for 15 s, and 72 °C for 65 s, and then once at 72 °C for 5 min.

Controls

A negative control (without DNA) was used to detect contamination. An internal PCR control was used in all amplifications to verify the efficiency of the test and to ensure that PCR inhibition was absent. The universal fungal primers ITS1 and ITS4, derived from highly conserved regions of the fungal rRNA gene, were used for this purpose.

Electrophoresis

PCR products were electrophoresed through 1.5 % agarose (Invitrogen, Argentina) dissolved in Tris–borate-EDTA buffer (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA [pH 8.4]). Electrophoresis was conducted at 80 V for 90 min, with 10 µl of each PCR amplicon plus 1 µl of tracking dye added to each well; the bands were visualized with a UV transilluminator after ethidium bromide staining.

Results

Fifty-one clinical isolates of *H. capsulatum* var. *capsulatum* were converted to yeast fungi using BHI agar at 37 °C. The multiplex PCR was developed by combining two pairs of primers: one of them was specific to *H. capsulatum* and the other one, universal for fungi, turned out to be specific to *H. capsulatum* var. *capsulatum,* regardless of the fungus isolate studied. Primers designed to amplify a region of about 390-bp (Hc I–Hc II) and a region of approximately 600-bp (ITS1–ITS4) were used to identify a yeast isolated as *H. capsulatum* var. *capsulatum* when both regions could be amplified by the use of both pairs of primers (Fig. 1).

This assay was 100 % sensitive for the identification of *H. capsulatum* var. *capsulatum* in at least all of the cultures recognized as this dimorphic fungus taking into account their morphological characteristics. For specificity testing, *P. brasiliensis*, *Cryptococcus neoformans, Trichosporon spp, C. glabrata, C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, or *P. marneffei* were examined (Fig. 1). Failure to amplify the target

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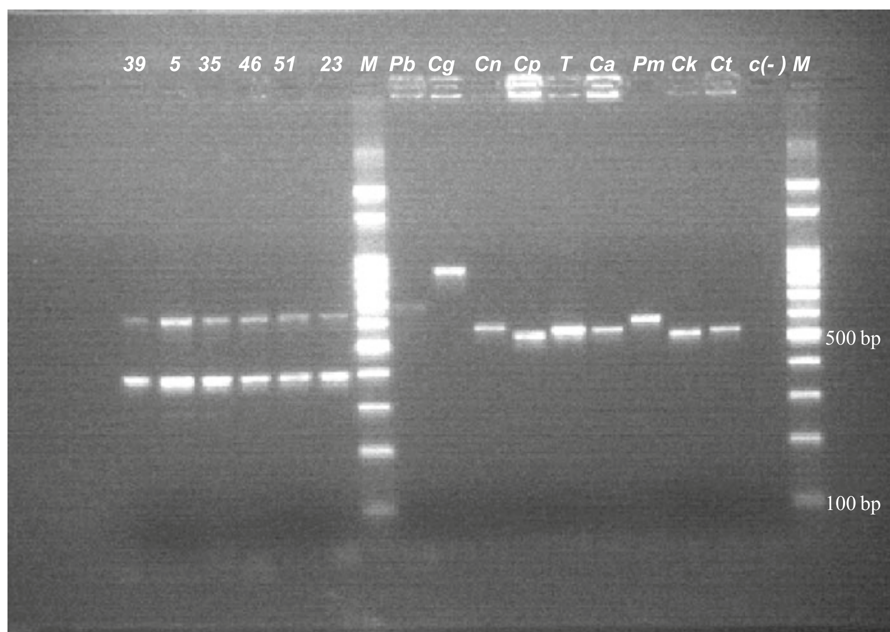

Fig. 1 Agarose gel of amplified products from yeast-like fungi as template in the PCR assay. The gene encoding 100-kDa-like protein of *H. capsulatum* and rDNA were co-amplified simultaneously in a single PCR tube. Lane abbreviations: M: molecular weight markers (the numbers on the right side of the gel are in base pairs). 39, 5, 35, 46, 51 and 23: isolates of *H. capsulatum; Pb:* isolate of *P. brasiliensis; Cg:* isolate of *C. glabrata; Cn:* isolate of *C. neoformans; Cp:* isolate of *C. parasilopsis; T:* isolate of *Trichosporon spp; Ca:* isolate of *C. albicans; Pm:* isolate of *P. marneffei; Ck:* isolate of *C. krusei; Ct:* isolate of *C. tropicalis* and *c*(−): negative control

DNA sequence by PCR with primers Hc I–Hc II in the presence of the ITS1–ITS4 amplicon in the isolates was an unequivocal sign of the high specificity of this assay (Fig. 1). Incipient yeast forms were obtained from clinical samples on BHI agar with simultaneous cultures at two different temperatures. A total of 3 or 4 mm loopful of yeast fungi was identified as *H. capsulatum* by the PCR assay described herein before the morphological characteristics were registered.

Discussion

The detection of *H. capsulatum* infections is still largely dependent on the culture of the organism collected from the clinical sample; precise identification relies on visualization of the typical morphology and the demonstration of dimorphism. The identification of fungal species grown in cultures of clinical specimens is essential for appropriate clinical decision-making and monitoring of antifungal therapies. Unfortunately, saprophobic fungi such as members of the genera *Chrysosporium*, *Corynascus*, *Renispora*, and *Sepedonium* produce structures that resemble the tuberculated macroconidia of *H. capsulatum* var. *capsulatum*, hindering its visual identification [3, 7]. In addition, many laboratories do not have direct access to trained mycologists capable of confirming a visual identification of these species.

The multiplex PCR assay described herein enabled us to detect *H. capsulatun* var. *capsulatum* DNA from culture. PCR methods are particularly promising because of their simplicity, specificity, and sensitivity. The application of PCR on the detection and identification of *H. capsulatum* var. *capsulatum* form culture was previously established with a gene encoding the M antigen [22] and in a real-time format targeting 18S rRNA [27] using different procedures of DNA extraction.

This study identified DNA of *H. capsulatum* var. *capsulatum* obtained from suspensions of yeast fungi in one-step PCR assay. We used whole yeast cells as template for multiplex PCR. Omission of the DNA extraction procedure from the yeast form significantly decreased the time required to make an accurate identification by PCR [28, 29], avoided the use of potentially toxic organic solvents (such as phenol,

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isoamyl alcohol or chloroform), and no mechanical procedures or enzymatic breakdown of the wall was performed. Although fungal cell breakage and the release of genomic DNA are undoubtedly less efficient without the preliminary extraction of DNA, adequate template was nevertheless available to yield positive results by PCR tests.

Because suspensions of yeast were used as template, the gene encoding 100-kDa-like protein of *H. capsulatum* and rDNA were co-amplified simultaneously with their respective target sequences in a single PCR tube. PCR amplification with universal fungal primers was used in the present study to rule out any failures in obtaining free fungal DNA or to exclude the presence of PCR inhibitors. It is well known that in a PCR without this control, a negative response (no band) can mean that there was no target sequence present in the reaction and this could be due to a failure in obtaining free fungal DNA or inhibitors in the cultures. Therefore, a false-negative result might be obtained. On the other hand, a negative result could also mean that the reaction was inhibited due to malfunction of the thermal cycler, incorrect PCR mixture, poor polymerase activity, and not least, the presence of inhibitory substances in the sample matrix [21, 23]. Conversely, in a PCR with universal fungal primers, a control signal should always be produced even though there is no target sequence present. Then, we suggest that the rDNA amplification could be used as an internal amplification control in a noncompetitive form [21] in the multiplex PCR described. However, as pointed out, the internal amplification controls have not been widely used in fungal PCR, and further research should include this control [23].

DNA amplification by PCR of the *H. capsulatum*, *P. brasiliensis*, *C. neoformans*, *Trichosporon spp, P. marneffei,* and five different *Candida* species with ITS1 and ITS4 evidenced some differences in amplicon size, which depended on the fungal species studied. The intact yeast cells were consistently amplified probably because numerous cells were sampled and the rDNA being amplified was present in multiple copies (>100) per genome [17]. However, sufficient template was available to yield positive PCR results with the specific primers Hc I and Hc II in all the isolates of *H. capsulatum* var. *capsulatum* studied.

The specificity of this assay was demonstrated by the fact that no specific band of the gene encoding for the 100-kDa-like protein was amplified when suspensions from the yeast form of other pathogenic fungal species were used as the DNA template. No cases of African histoplasmosis were observed in our study. As previously reported [16], we do not know whether the gene encoding for this protein is present in the genome of *H. capsulatum* var. *duboisii*. However, we must note that the gene was present in the genome of *H. capsulatum* var. *capsulatum,* and it was not found in the genome *P. brasiliensis* or *P. marneffei* studied.

Several reports indicated that some strains of *H. capsulatum* lacked microconidia, while others did not produce tuberculate macroconidia, and macroconidia often remained smooth and devoid of tubercles [30–32]. Variants included pleomorphic colonies (lacking conidiation) and glabrous, leathery to waxy colonies. Most of these variants remained sterile and failed to produce conidia on routine mycologic media. The identification by the PCR procedure described herein could be an alternative tool in atypical isolates or variants of *H. capsulatum,* which convert from the mycelial to yeast.

An early diagnosis was achieved by DNA amplification of the incipient yeast form isolate from clinical samples by the PCR assay described in the present study. Furthermore, molecular detection of *H. capsultaum* might improve diagnosis and the outlook for patients with histoplasmosis.

This assay was sensitive and specific for the differentiation of *H. capsulatum* from other cultured fungi that may be encountered in clinical mycology laboratories. No cross-reactivity was observed in this assay regarding genetically related fungi such as *P. brasiliensis* or fungi with forms that may be morphologically similar to the tissue form of *H. capsulatum*or culture at 37 °C, such as *Cryptococcus neoformans, Trichosporon spp, C. glabrata, C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, or *P. marneffei.*

Finally, the multiplex PCR developed by our research team might be an important tool available to microbiological laboratories using this kind of technology, to be used for the confirmation of incipient culture isolates suspected to be *H. capsulatum*.

Acknowledgments This research was supported by Grant UBACyT 20020100100554, School of Medicine. University of Buenos Aires.

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