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PCR-RFLP as a useful tool for diagnosis of invasive mycoses in a healthcare facility in the North of Brazil



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ABSTRACT

Background: The incidence of invasive mycoses is increasing worldwide. PCR-RFLP was applied to the identification of 10 reference strains and 90 cultures of agents of invasive mycoses. In addition, the new approach was applied to detect fungal agents in 120 biological samples (blood, cerebrospinal fluid and bone marrow). PCR-RFLP results were compared with the ones obtained with conventional methods (culture, microscopy, and biochemical testing).

Results: The assays carried out with the reference strains (*Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida guilliermondii*, *Cryptococcus neoformans*, *Cryptococcus gattii* and *Histoplasma capsulatum*), demonstrated that the RFLP profiles were correctly predicted by the *in silico* investigation and allowed unequivocal identification of all chosen reference strains. The PCR-RFLP also identified 90 cultures of agents of invasive mycoses correctly, 2.5 times faster than the conventional assays. Evaluating PCR-RFLP with biological samples it was observed that the PCR was found to be 100% accurate and the RFLP profiles allowed the identification of the etiological agents: *C. neoformans* (n = 3) and *C. gattii* (n = 1) in CSF samples, *H. capsulatum* (n = 1) in bone marrow and *C. albicans* (n = 2) in blood cultures. The detection and identification by PCR-RFLP were found to be between two to ten times faster than the conventional assays.

Conclusion: The results showed that PCR-RFLP is a valuable tool for the identification of invasive mycoses that can be implemented in hospital laboratories, allowing for a high number of clinical analyses per day.

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1. Introduction

In recent years, the incidence of invasive mycoses has globally increased rendering in an important cause of morbidity and mortality, especially in immunocompromised patients, such as those having acquired AIDS, transplant patients using corticosteroids and/or patients undergoing chemotherapy in intensive care units [1,2,3]. Due to the high morbidity and mortality, and a tendency to rapidly spread to other organs, early diagnosis and a correct treatment of invasive fungal infections are essential with sensitive and rapid diagnostics in urgent need [4].

Currently, in developing countries including Brasil, the identification of invasive fungal infections comprises conventional culture,

biochemical methods, microscopic determination of micromorphology and immunological assays. However, these traditional methods (culture and direct) are most often used to detect agents that cause mycoses. The MALDI-TOF technique is being used as an alternative to precocious identification of fungal species; however, it is still not a reality in Brasil [5,6]. To overcome these limitations, molecular approaches can be used for the detection and identification of pathogenic fungi [7,8].

Methods employing polymerase chain reaction (PCR) are among the most promising alternatives and are increasingly being applied in routine detection of pathogenic microorganisms. The greatest advantages of PCR can be seen in its sensitivity, specificity and speed with test results being available within hours [9,10]. Real-Time PCR and microarray assays have been investigated for the detection and identification of pathogenic fungi [11,12,13]. However, these methods are the most of the time, unavailable in the routine of mycology laboratories of developing countries.

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Table 1
Sizes of PCR products of the ITS region 5 and DdeI restriction fragments based on nucleotide sequence deposited in GenBank.

Species	Access number in NCIS-Gene Bank	Product size and NL4 primers ITS5	Digestion products (DdeI)
<i>C. albicans</i>	BD309277	1153 bp	440, 361, 212, 140 bp
<i>C. tropicalis</i>	EU924133.1	1142 bp	435, 289, 212, 138, 68 bp
<i>C. parapsilosis</i>	FJ746057	1137 bp	567, 284, 212, 68, 6 bp
<i>C. krusei</i>	AB369918.1	1120 bp	556, 338, 200, 26pb
<i>C. guilliermondii</i>	JX993815	1224 bp	419, 359, 235, 211 bp.
<i>C. neoformans</i>	AF356652.1	1201 bp	602, 398, 104, 48, 49 bp
<i>C. gattii</i>	JN939487.1	1561 bp	603, 538, 320, 32 bp
<i>H. capsulatum</i>	AB436785.1	1239 bp	512, 432, 235, 60 bp

Santos et al. [14] presented a PCR-RFLP assay targeting the ITS region from the fungi rDNA that was able to differentiate the most important agents from invasive mycosis. The present work is the continuation of the assessment of this study by applying it in the routine of a hospital with patients at risk of being affected by invasive mycosis. The Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD) is a reference hospital for the treatment of tropical diseases in the North of Brasil. Its wards are mostly occupied by immunocompromised patients with AIDS, who are highly susceptible to infections with opportunistic pathogens.

2. Materials and methods

The study was conducted in three stages: a) PCR-RFLP (NL4-ITS5, DdeI digestion) of reference strains; b) PCR-RFLP for identification of 90 cultures of clinical isolates comparing the results with conventional techniques; and c) determination of accuracy of PCR for detection and RFLP for the identification of causative agents of invasive mycoses in biological samples from 120 patients with clinical suspicion of invasive mycoses, prospectively. For the analysis of these latter, data was supplemented with epidemiological characteristics of patients with fungal infection.

2.1. Reference strains

The following fungal strains belonging to the collection of the Instituto Nacional de Pesquisas da Amazônia (INPA) were used as reference: *Candida albicans* FMT170, *Candida parapsilosis* FMT72, *Candida tropicalis* FMT44, *Candida krusei* ATCC6258, *Candida guilliermondii* ATCC6260, *Cryptococcus neoformans* FMT1420, *Cryptococcus gattii* FMT1170 and *Histoplasma capsulatum* FMT1400.

2.2. Cultures of clinical isolates

90 cultures of causative agents of invasive mycosis were obtained of the samples from patients with HIV/AIDS, referred to FMT-HVD in

the period between December 2005 and January 2008, and March and August 2012. These cultures were analyzed by conventional PCR and the PCR-RFLP, simultaneously.

2.3. Biological samples

120 biological samples were investigated including whole blood (7), blood culture (33), cerebrospinal fluid (CSF) (70) and bone marrow aspirate (10). Samples were taken from patients with suspected invasive mycosis who had been referred to the Mycology Laboratory of the FMT-HVD between September and December 2012, to the realization the conventional PCR and RFLP. Anonymized information of patients (sex, age, place of residence, clinical specimen investigated, HIV serology, viral load of HIV and CD4⁺ cells/mm³) was obtained from the computerized system “iDoctor hospital” used in FMT-HVD.

2.4. Detection and identification by conventional methods

Cultivation of biological samples was performed on Agar Sabouraud (BD Difco, Sparks, USA) and Agar Mycosel (BD Difco, Sparks, USA). Colonies growing on Sabouraud agar were identified by standard laboratory methods, including micro morphological and physiological tests (germ tube production, growth on Agar Niger and CHROMagar™ *Candida* (Becton-Dickinson, Sparks, USA)), incubation at 42°C, and testing for carbohydrate and nitrogen assimilation and carbohydrate fermentation.

2.5. Detection and identification by PCR-RFLP

Generation of PCR products and subsequent digestion was performed as described by Santos et al. [14]. DNA was extracted from samples (200 µL of fungal biomass or biological sample) using the QIAamp Blood and Tissue kit (Qiagen, Hilden, Germany) following the recommendations of the manufacturer. The DNA was quantified by absorbance at 260 nm using Genequant (Eppendorf, Hamburg, Germany) and 20 ng of the extracted DNA served as a template for

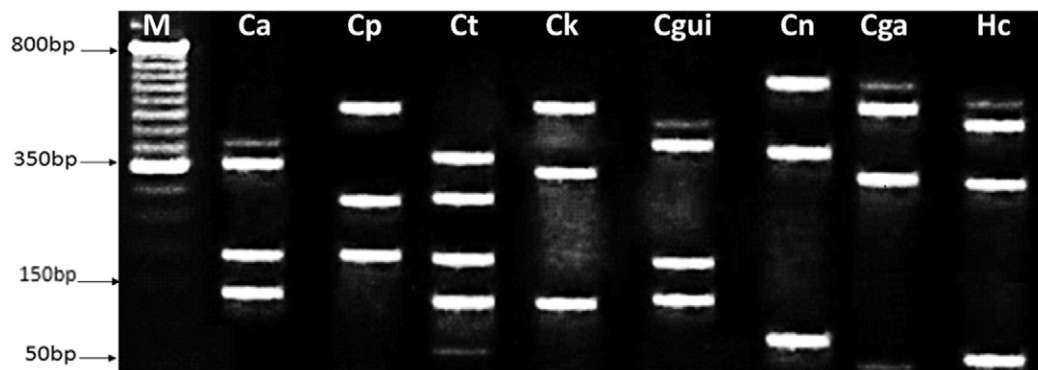


Fig. 1. PCR digestion products with DdeI. PCR products were obtained from DNA isolated from standard strains: *C. albicans* (Ca) FMT170, *C. parapsilosis* (Cp) FMT72, *C. tropicalis* (Ct) FMT44, *C. krusei* ATCC6258 (Ck), *C. guilliermondii* ATCC6260 (Cgui), *C. neoformans* FMT1420 (Cn), *C. gattii* FMT1170 (Cga) and ss FMT1400 (Hc).

PCR amplification. PCR reactions had a final volume of 25 µL containing PCR buffer (final concentration: 1 × 10 mM Tris-HCl, pH 8.3, 50 mM KCl); 1.5 mM MgCl₂; 200 nM primers ITS5 (5'-GGAAGTAAAAGTCGTAACAA GG-3) and NL4 (5'-GGTCCGTGTTCAAGACGG-3'; both primers were described by Irobi et al. [15]), 50 µM dNTPs, and 1 U Ampli-Taq DNA polymerase.

PCR was performed using a thermocycler (Kyratrec's SuperCycle, Republic of Korea) under the following PCR conditions: initial denaturation of 5 min at 94°C, 40 cycles of 30 s at 94°C (denaturation), 30 s at 50°C (annealing), for 30 s and 1 min at 72°C (extension), followed by a final extension for 10 min at 72°C. PCR products were visualized by electrophoresis on a 2% agarose gel and stained with SYBR® Green (SYBR Safe DNA Gel Stain, Invitrogen, Carlsbad, USA). A DNA Ladder Mix of 100 bp (SM0331, MBI Fermentas, St. Leon-Rot, Germany) served as size marker. For RFLP, PCR products from individual isolates were digested with 10 U of the restriction enzyme DdeI (Thermo Fisher Scientific, Vilnius, Lithuania) for 3 h at 37°C, and subjected to electrophoresis as described above. The sizes of the PCR products and restriction fragments generated from the isolates were compared with the corresponding nucleotide sequences deposited in the GenBank database (Table 1).

2.6. *In silico* evaluations

National Center for Biotechnology Information (NCBI) sequences were subjected to *in silico* restriction digestion utilizing the BLAST package (<http://www.ncbi.nlm.nih.gov/BLAST>). The BioEdit software (<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>) was used for editing and aligning the sequences.

3. Results

3.1. PCR-RFLP's profiles obtained with the reference strains

Eight reference strains were submitted to PCR-RFLP in order to investigate if the profiles described *in silico* (Table 1) were similar to that obtained experimentally. As shown in Fig. 1, the PCR-RFLP profiles were correctly predicted by the *in silico* investigation. In addition, RFLP patterns distinct all chosen reference strains allowing their unequivocal identification.

3.2. PCR-RFLP's profiles obtained with cultures from clinical isolates

PCR-RFLP was evaluated for identifying cultures obtained in the routine of a healthcare facility in the North of Brasil, 90 clinical cultures of fungi previously isolated from clinical samples were investigated. These same cultures were also submitted for identification by conventional methods (phenotypic assays). It was observed 100% accordance with the conventional and PCR-RFLP identification. Both methods take, on average, 5 and 2 d, respectively.

3.3. PCR (NL4-ITS5) sensibility/specificity and PCR-RFLP's identification of fungal agents in biological samples

PCR-RFLP was also evaluated for the detection/identification of fungal agents directly in biological samples. It was carried out an accuracy study comparing both the PCR-RFLP and conventional methods. 120 biological samples were assessed, including whole blood (7), blood culture (33), CFS (70) and bone marrow aspirate (10).

Seven biological samples were considered positive by conventional methods. None of these culture-positive results was PCR-negative corresponding to a 100% sensibility (Table 2). On the other hand, 12 samples were considered positive only by PCR but tested negative by conventional methods. In addition, to the high sensitivity, PCR-RFLP took only two days while the conventional identification took

Table 2

Sensibility and specificity between PCR (ITS5-NL4) and conventional methods for the detection of fungi in 120 biological samples.

		Culture	
		Positive	Negative
PCR	Positive	7	12
	Negative	0	101
Sensibility 100%			
Specificity 89.4%			

between 3 and 25 d (with an average of 5 d), for the fungal agents identification.

All the 12 patients considered positive only by PCR had been previously diagnosed with cryptococcosis and submitted to an antifungal treatment against cryptococcal meningitis (Table 3). The digestion of the PCR products allowed the identification of *C. neoformans* in all these 12 samples.

The seven patients diagnosed by both (conventional and PCR methods) were mostly men (n = 5); age range between 18 and 58 and the majority of them lived in the East Zone of Manaus. Five of them were HIV positive presenting viral loads between <50 to 170,660 copies/mL and lymphocytes CD4⁺ between 5 and 491 cells/mL. The RFLP patterns allowed identifying their fungal agents as *C. neoformans* (n = 3) in CSF samples, *C. gattii* (n = 1) in CSF sample, *H. capsulatum* (n = 1) in a sample of bone marrow and *C. albicans* (n = 2) in blood cultures. The conventional assays and PCR-RFLP showed identical species identifications (Table 4).

4. Discussion

The present study demonstrated that PCR-RFLP is a useful tool for routine detection and identification of agents of invasive mycoses in a tertiary healthcare facility in the state of Amazonas-Brazil. It was observed that: a) the products of PCR and RFLP profiles obtained with the standard strains were similar to those achieved with the *in silico* assays; b) all cultures of 90 clinical isolates were identically identified by conventional methods and PCR-RFLP; and c) the application of PCR (ITS4-NL5) to investigate the fungi detection directly in biological samples resulted in 100% sensitivity and the RFLP profiles allowed correct identification of the causative agents. Whereas the majority of previously published works were limited to identification of standard strains and cultures of clinical isolates, this study demonstrates the effective detection and identification of fungi by PCR-RFLP directly from clinical samples of biological origin.

The PCR-RFLP assay can be useful for laboratories that do not have real-time thermocycling capability. PCR-RFLP is a simple molecular

Table 3

Epidemiological characteristics of patients with false positive PCR diagnosis.

No Pac	Age	Gender	Manaus area	HIV	C. Viral cop/mL	HIV	Linf. CD4 cells/mL	Sample
28	38	F	West	Positive	7.135		171	CSF
33	40	M	East	Positive	40.759		131	CSF
50	34	M	East	Positive	<50		384	CSF
53	49	F	East	Positive	<50		289	CSF
59	63	M	East	Positive	23.587		65	CSF
71	38	F	East	Positive	36.146		226	CSF
78	54	F	East	Positive	11.367		10	CSF
80	59	M	East	Positive	43.601		160	CSF
97	45	M	East	Positive	<50		307	CSF
99	39	F	East	Positive	1.551		282	CSF
105	41	M	East	Positive	3.670		376	CSF
117	48	M	North	Positive	32.609		250	CSF

Table 4
Epidemiological characteristics of patients with true positive PCR diagnosis.

No Pac	Age	Gender	Manaus area	HIV	C. Viral HIV cop/mL	Linf. CD4 cells/mL	Sample	Identification by conventional culture and PCR-RFLP
01	34	F	North	Positive	<50	238	CSF	<i>C. neoformans</i>
17	18	M	East	Negative	102	203	CSF	<i>C. gatti</i>
40	58	F	East	Positive	<50	174	Blood culture	<i>C. albicans</i>
47	25	M	North	Negative	170.660	151	Bone marrow	<i>H. capsulatum</i>
48	28	M	East	Negative	<50	167	Blood culture	<i>C. albicans</i>
56	18	M	East	Positive	45.525	5	CSF	<i>C. neoformans</i>
77	31	M	East	Positive	1.449	34	CSF	<i>C. neoformans</i>

assay, requires only standard equipment used for molecular biology and provides results suitable for the simultaneous analysis of several species [14,15].

RFLP profiles obtained with the standard strains investigated in this work (Fig. 1) were consistent with the results predicted theoretically and in practice found by Santos et al. [14], Irobi et al. [15], Fallahi et al. [16], and Kordbacheh et al. [17]. There are a vast number of other fungal species that can cause invasive mycosis and that are not considered by the method described. Therefore, data must be analyzed with caution due to the risk of misdiagnosis.

In regard to conventional identification of cultures of invasive mycoses agents, it should be mentioned that the identification of *Candida* spp. uses enzymes, assimilation and fermentation trials. It is expensive when performed with commercial kits and slow when performed without them. The identification of *Cryptococcus* spp. is possible using the culture medium canavanine glycine bromothymol blue agar (CGB), however, test results are only obtained after an average of 3 d, and are subject to error. The identification of *H. capsulatum* is undoubtedly the most time consuming with cultivation requiring, on average 25 d to develop visible growth. Subsequent identification is micro morphological, confirmatory assay identification (pleomorphism test) may take over 25 d [7,8,18]. An attractive possibility for the use of PCR-RFLP, therefore, consists in the identification of colonies obtained from clinical samples. The method allows the establishment of a database containing RFLP profiles from relevant microorganisms. The identification of 90 fungal cultures isolated from clinical specimens presenting results similar to the results of conventional methods demonstrates this statement.

Regarding the use of PCR (NL4-ITS5) for detecting agents that cause invasive mycoses, the data presented in this study confirms that this test has adequate sensitivity and can be used with biological materials from different sources (blood, CSF and bone marrow).

This study also revealed that PCR detected *C. neoformans* in CFS samples that were considered negative by the conventional methods. Interestingly, all these samples were obtained from AIDS patients having undergone antifungal therapy against cryptococcal meningitis. This fact tempts to speculate that the CFS of these patients contained dead fungal cells or residual DNA from previous infection causing positive PCR results and explaining lack of success to culture the pathogens. It remains to be assessed whether nucleic acids from therapy-killed fungal cells remain intact longer in this highly sheltered environment [19]. If so, the interpretation of PCR results obtained from this type of clinical samples has to be interpreted with care and always to observe that the control sample in this case was efficient. Elimination of this residual nucleic acid can be obtained by sample treatment with viability dye, such as propidium monoazide might overcome this limitation [20,21].

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Conflict of interest

The authors have no conflict of interest to declare.

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