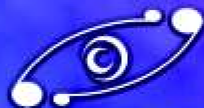
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Identification of fungemia agents using the polymerase chain reaction and restriction fragment length polymorphism analysis

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Abstract

Prompt and specific identification of fungemia agents is important in order to define clinical treatment. However, in most cases conventional culture identification can be considered to be time-consuming and not without errors. The aim of the present study was to identify the following fungemia agents: *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Cryptococcus neoformans*, *Cryptococcus gattii*, and *Histoplasma capsulatum* using the polymerase chain reaction and restriction fragment length polymorphism analysis (PCR/RFLP). More specifically: a) to evaluate 3 different amplification regions, b) to investigate 3 different restriction enzymes, and c) to use the best PCR/RFLP procedure to indentify 60 fungemia agents from a culture collection. All 3 pairs of primers (ITS1/ITS4, NL4/ITS5 and Primer1/Primer2) were able to amplify DNA from the reference strains. However, the size of these PCR products did not permit the identification of all the species studied. Three restriction enzymes were used to digest the PCR products: *Hae*III, *Dde*I and *Bfa*I. Among the combinations of pairs of primers and restriction enzymes, only one (primer pair NL4/ITS5 and restriction enzyme *Dde*I) produced a specific RFLP pattern for each microorganism studied. Sixty cultures of fungemia agents (selected from the culture collection of Fundação de Medicina Tropical do Amazonas - FMTAM) were correctly identified by PCR/RFLP using the prime pair NL4/ITS5 and *Dde*I. We conclude that the method proved to be both simple and reproducible, and may offer potential advantages over phenotyping methods.

Key words: PCR/RFLP; Fungemia agents; Identification; *Dde*I

Introduction

The incidence of invasive fungal infections has increased in recent years and has been important cause of morbidity and mortality in immunocompromised patients such as recipients of bone marrow transplants, patients with hematological malignancies (with or without chemotherapy) and AIDS patients (1-3).

The conventional methods for the identification of fungemia agents are often based on the examination of phenotypic characteristics. This approach can be time-consuming and its dependence on the variable expression of phenotypic characteristics can lead to inconsistent results (4-6).

The polymerase chain reaction (PCR) is being increasingly applied within hospital laboratories for the routine detection of human genes and pathogenic microorganisms (7). Modern PCR techniques such as multiplex PCR and multiplex real-time PCR have been used for the detection and identification of pathogenic fungi (8-11). However, these methods have some weaknesses such as: a) the multiplex techniques are still limited to the identification of only a few fungal species per reaction (8-9), b) the multiplex PCR is still presenting problems of low sensitivity and specificity for fungal detection in biological samples (8-10), and c) the multiplex real-time PCR is not a well-disseminated method because of the necessity of specific and expensive equipment (11).

The PCR/RFLP (restriction fragment length polymorphism) is relatively simple to perform. It only requires standard molecular biology equipment, provides instantly recognizable results, and is easily transferable to most

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diagnostic laboratories (12-14).

The ribosomal genes are popular targets for PCR-based systems for the detection and identification of fungal pathogens (15-17). The internal transcribed space regions (ITS1 and ITS2) are highly variable sequences that have been used for the identification of fungi at the species level in different formats (17-20).

A number of procedures using PCR/RFLP amplification techniques have been suggested for the identification of *Candida* species. However, few identification studies included fungemia agents.

The aim of the present study was to evaluate three PCR primers and three restriction enzymes for the identification of fungemia agents on the basis of size and variation of the rDNA internal space regions.

Material and Methods

Seven reference strains of fungemia agent species (obtained from the FMTAM fungal collection) were included: *Candida albicans* FMT170, *Candida parapsilosis* FMT72, *Candida tropicalis* FMT44, *Candida glabrata* FMT66, *Cryptococcus neoformans* FMT1420, *Cryptococcus gattii* FMT1170, and *Histoplasma capsulatum* FMT1400. These microorganisms were identified by conventional methods (20) and their identities were confirmed by the sequencing of their ITS1 gene from ribosomal DNA.

Clinical strains. Between December 2005 and January 2008, 60 isolates causing invasive infection in HIV patients from the FMTAM were collected. For the studies of variability we used clinical strains of *C. albicans* (8 isolates), *C. tropicalis* (3), *C. parapsilosis* (3), *C. glabrata* (2), *Cryptococcus neoformans* (31), *C. gattii* (9), and *Histoplasma capsulatum* (4). These isolates were characterized by morphology, germ tube production and assimilation tests (API-20C System-BioMeireux, France) (20).

Preparation of template DNA

Strains were cultivated on solid Sabouraud agar plates for 3-15 days at 30°C. Sabouraud broth medium (10 mL) was inoculated and the cells were grown in 50-mL tubes (Falcon, Becton Dickinson, USA) at room temperature with shaking overnight. After centrifugation at 2268 *g* for 10 min, the supernatant was removed and the pellet was washed by adding 20 mL washing buffer (150 mM NaCl, 10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) at room temperature. Fungal DNA was extracted with the QIAamp tissue kit (Qiagen, Germany) using the silica column technology according to manufacturer instructions for DNA extraction from yeast, including digestion with Lyticase and RNAase.

Polymerase chain reaction

The reaction mixture for the primary PCR consisted of 10 µL DNA extract in a total volume of 50 µL with final concentrations of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 µM of each primer, 1.5 U of recombinant DNA polymerase (Invitrogen, USA) and a 200-µM concentration of each deoxynucleoside triphosphate (Promega, USA). The following pairs of primers were studied: a) ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') described by Williams et al. (12), b) ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3) and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') described by Irobi et al. (13), and c) primer 1 (5'-GTCAAACTTGGTC ATTTA-3') and primer 3 (5'-TTCTTTTCCTCCGCTTATT GA-3') described by Trost et al. (14). Thirty-four amplification cycles were performed in a thermocycler (Mastercycler, Eppendorf, USA) after initial DNA denaturation at 94°C for 3 min. Each cycle consisted of a denaturation step at 94°C for 60 s, an annealing temperature depending on the melting temperature of the pair of primers (suggested by the fabricant) for 60 s, and an extension step at 72°C for 60 s with a final extension at 72°C for 10 min following the last cycle.

Restriction digest

The PCR product was digested individually with 10 U of the restriction enzymes *Hae*III, *Bfa*I, and *Dde*I (New England Biolabs, UK) during an overnight incubation at 37°C.

Electrophoresis analysis

The PCR products were analyzed by 2% agarose gel electrophoresis and the gels were stained with ethidium bromide. Gene Ruler DNA ladder Mix (SM0331, MBI Fermentas, Germany) was used as the DNA marker. The PCR products were purified using the QIAquick PCR Purification kit (Qiagen) according to manufacturer instructions. The PCR products were diluted in 40 µL of the buffer included in the kit.

Results

Selection of the rDNA region

The regions of ribosomal DNA described for the three different pairs of primers were successfully amplified from all the reference strains tested (Figure 1).

As expected, a PCR product was consistently obtained for each microorganism. Using the pair of primers ITS1/ITS4, the reference strains identified as *C. glabrata*, *C. gattii* and *C. neoformans* yielded unique product sizes of approximately 400, 600, and 650 bp, respectively, and a product of approximately 550 bp was obtained for the remaining species. Using the pair of primers NL4/ITS5, only *C. glabrata* DNA strain yielded unique product sizes of approximately 800 bp and a product of approximately 1100 bp was obtained for the remaining species. Using the pair of primers Primer1/Primer3, *C. glabrata*, *C. gattii*, and *C. neoformans* strains yielded product sizes of approximately 200, 300 and 300 bp, respectively, and a product of approximately 350 bp was obtained for the remaining species.

Selection of the restriction enzyme

The PCR products were treated with the restriction enzymes *Bfa*I, *Dde*I and *Hae*III for further differentiation. Figure 2 shows a typical gel electrophoresis of the patterns obtained after digestion of the PCR products.

The PCR products obtained using the pair of primers ITS1/ITS4 after treatment with *Hae*III, *Dde*I and *Bfa*I produced 5, 6, and 5 RFLP patterns, respectively. The products obtained using the pair of primers NL4/ITS5 after treatment with *Hae*III, *Dde*I, and *Bfa*I produced 6, 7, and 0 RFLP patterns, respectively. The products obtained using the pair of primers Primer1/Primer3 after treatment with *Hae*III, *Dde*I, and *Bfa*I produced 6, 0, and 0 RFLP patterns, respectively. Among the combinations of pairs of primers and restriction

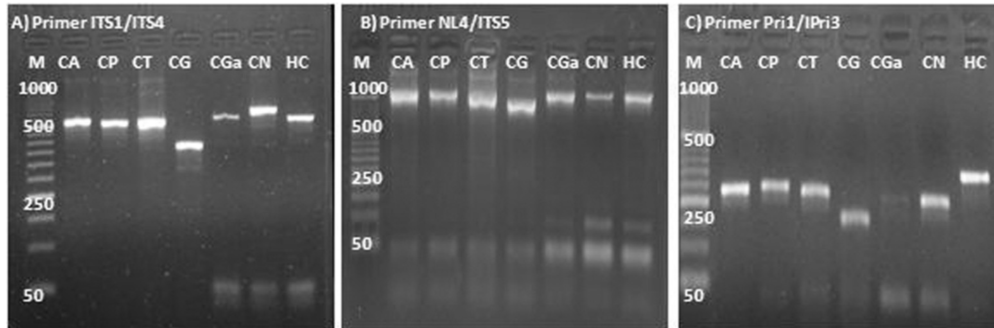

Figure 1. PCR products obtained with primers ITS1/ITS4 (A), NL4/ITS5 (B) and Pri1/Pri3 (C) of reference strains: *Candida albicans* (CA), *Candida parapsilosis* (CP), *Candida tropicalis* (CT), *Candida glabrata* (CG), *Cryptococcus gattii* (CGa), *Cryptococcus neoformans* (CN), and *Histoplasma capsulatum* (HC).

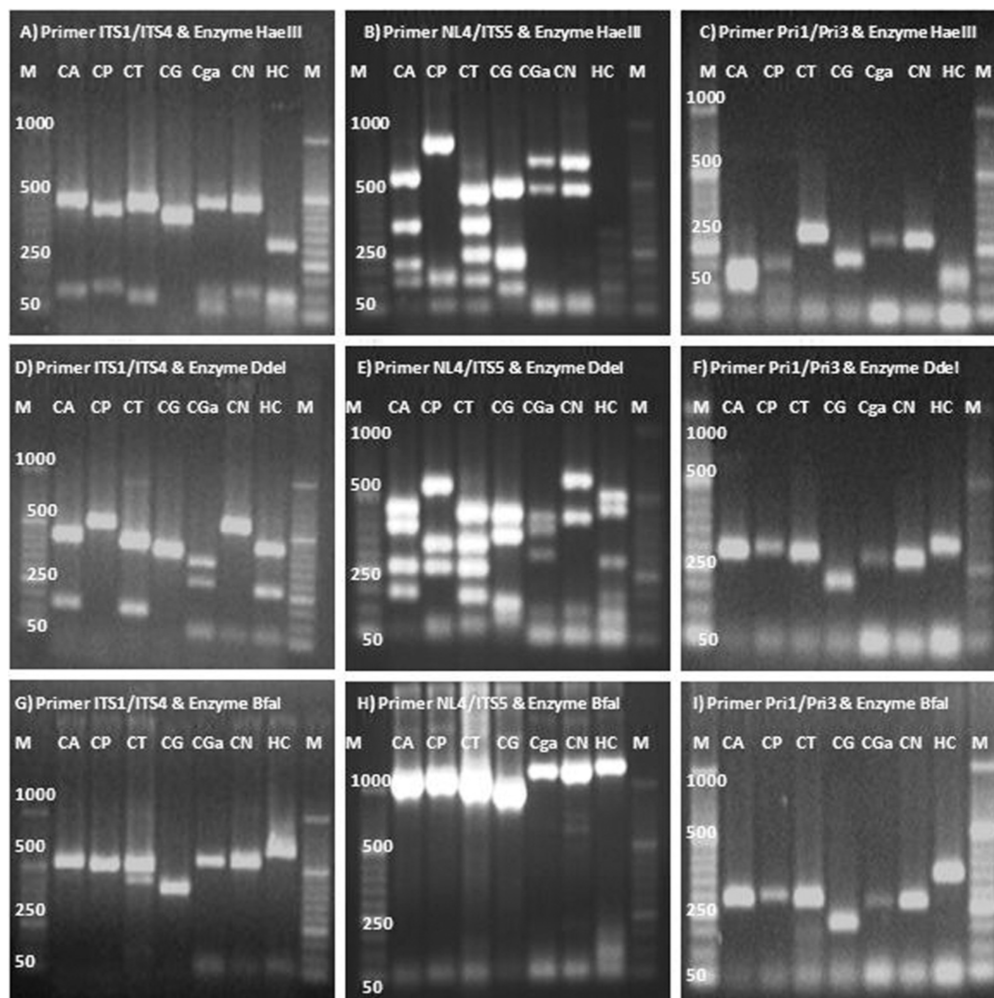

Figure 2. RFLP patterns of the PCR products obtained with primers ITS1/ITS4 (A, D, G), NL4/ITS5 (B, E, H) and Pri1/Pri3 (C, F, I) shown in Figure 1 after digestion with the endonucleases *Hae*III (A, B, C), *Dde*I (D, E, F) and *Bfa*I (G, H, I). Reference strains: *Candida albicans* (CA), *Candida parapsilosis* (CP), *Candida tropicalis* (CT), *Candida glabrata* (CG), *Cryptococcus gattii* (CGa), *Cryptococcus neoformans* (CN), and *Histoplasma capsulatum* (HC).

enzymes, only one (primer pair NL4/ITS5 and restriction enzyme *Dde*I) produced a specific RFLP pattern for each microorganism studied. The observed patterns were approximately 450, 400, 275, and 200 bp (for *C. albicans*); 550, 350, and 275 bp (for *C. parapsilosis*); 425, 350, 275, and 200 bp (for *C. tropicalis*); 425, 375, and 100 bp (for *C. glabrata*); 400, 400, and 325 bp (for *C. gattii*); 550, 400, and 100 bp (for *C. neoformans*), and 475, 425, 300, and 100 bp (for *H. capsulatum*).

Evaluation of the detection limit and the time required for microorganism identification by PCR/RFLP

In order to study the detection limit of the PCR/RFLP, different concentrations (10^6, 10^5, 10^4, 10^3, and 10^2/mL) of cells of the fungemia agents were submitted to the DNA extraction and PCR amplification using the pair of primers ITS1/ITS4. The minimum concentration of cells necessary for the amplification of *C. neoformans* and *H. capsulatum* was 10^4 cells/mL; however, for *C. albicans* amplification a concentration of 10^5 cells/mL was necessary. The genotyping identification proved to be more adequate than phenotyping methods (gold standard) for *Candida* species identification. *Cryptococcus* sp (40 strains) and *H. capsulatum* (4 strains) were identified identically using both conventional methods and PCR/RFLP. The time necessary for the identification of a fungemia agent using PCR/RFLP was approximately 2 days in a routine laboratory: 1 day for extraction and amplification and 1 additional day for enzyme restriction and electrophoresis assay.

Discussion

In the present study, we investigated three of the most important methodologies described for the identification of *Candida* species by PCR/RFLP (12-14) in order to evaluate them for the identification of fungemia agents. The results obtained demonstrated that it is possible to use a single RLFP/PCR procedure, on the basis of size and structural differences in the rDNA space regions, for correct identification. The present study was also the first to describe the differentiation of *Cryptococcus* species using the primer pair NL4/ITS5 and the restriction enzyme *Dde*I.

Although PCR/RFLP can be considered to be an “old tool” for PCR product analysis, it is still being used for studies of microorganism characterization because of its simplicity, reliability, easy adaptation for identifying several genera or species, and because it does not require expensive materials or equipment (12-14). These characteristics are important in cost-effective studies and indicate that the use of this technique will probably become routinely accepted in mycology laboratories before the DNA sequencing or real-time PCR methods are used.

In the present study, some species could be identified by evaluating the size of the PCR products with different primers. However, the enzyme digestion of the PCR products was necessary for the identification of most species. The combination of the PCR products using the pair of primer NL4/ITS5 and digestion with the restriction enzyme *Dde*I was the only one able to produce a single PCR/RFLP pattern for each one of the reference species.

The PCR/RFLP presented differs from those previously reported by Irobi et al. (13) that identified 7 medically important *Candida* species. In this new study, we included three different species: *C. neoformans*, *C. gattii* and *H. capsulatum*. It is important to note that the new method proposed here was able to effectively differentiate between *C. neoformans* and *C. gattii*. Both the reference and patient strains gave exactly the same RFLP patterns and the results obtained in the present study were reproducible and consistent for the isolates of the fungemia cultures tested.

The traditional methods for identifying fungemia agents are often based on the examination of phenotypic characteristics. This approach can be time-consuming and the reliance on the variable expression of phenotypic characteristics can lead to inconsistent results (3-6) and consequently genotype-based approaches may be preferable. The phenotypic identification of *Histoplasma capsulatum*, *Cryptococcus* sp and *Candida* sp cultures takes approximately 30, 10, and 5 days, respectively. The method described, PCR/RFLP, was able to identify cultures containing more than 1 x 10^5 cells within about 48 h. This detection limit is sufficient for the investigation of blood cultures or small/young cultures from agar plates.

The present methodology was developed and evaluated in order to be applied in the public health system of developing countries. The information presented in this manuscript could be useful for hospitals of medium and high complexity that need to identify fungemia agents and already have the standard materials for PCR.

In summary, three PCR amplification sites and three different cutter restriction enzymes were evaluated for the identification of fungemia species. Seven reference strains and 60 clinical isolates were successfully analyzed. The reliability of the technique was demonstrated by the examination of a large number of isolates.

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