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Characterization of *Paracoccidioides brasiliensis* by FT-IR spectroscopy and nanotechnology

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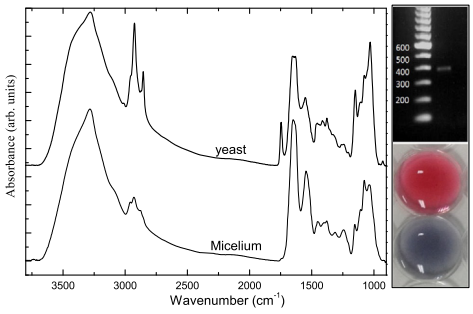
HIGHLIGHTS

- The development of new approach's for identification of *P. brasiliensis* is needed.
- PCR associated with a colorimetric methods is safer and cheaper than other methods.
- Characterize and compare chemical composition of yeast and mycelia forms by FT-IR.

GRAPHICAL ABSTRACT



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ABSTRACT

Paracoccidioides brasiliensis, the etiological agent of paracoccidioidomycosis, is a dimorphic fungus existing as mycelia in the environment (or at 25 °C *in vitro*) and as yeast cells in the human host (or at 37 °C *in vitro*). Because mycological examination of lesions in patients frequently is unable to show the presence of the fungus and serological tests can misdiagnose the disease with other mycosis, the development of new approach's for molecular identification of *P. brasiliensis* spurges is needed. This study describes the use of a gold nanoprobe of a known gene sequence of *P. brasiliensis* as a molecular tool to identify *P. brasiliensis* by regular polymerase chain reaction (PCR) associated with a colorimetric methods. This approach is suitable for testing in remote areas because it does not require any further step than gene amplification, being safer and cheaper than electrophoresis methods. The proposed test showed a color change of the PCR reaction mixture from red to blue in negative samples, whereas the solution remains red in positive samples. We also performed a Fourier Transform Infrared (FT-IR) Spectroscopy analysis to characterize and compare the chemical composition between yeast and mycelia forms, which revealed biochemical differences between these two forms. The analysis of the spectra showed that differences were distributed in chemical bonds of proteins, lipids and carbohydrates. The most prominent difference between both forms was vibration modes related to 1,3-β-glucan usually found in mycelia and 1,3-α-glucan found in yeasts and also chitin forms. In this work, we introduce FT-IR as a new method suitable to reveal overall differences that biochemically distinguish each form of *P. brasiliensis* that could be additionally used to discriminate biochemical differences among a single form under distinct environmental conditions.

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1. Introduction

Paracoccidioides brasiliensis is a thermo-dimorphic fungus that switches between a filamentous hyphae form (under environmental mild temperature or at 25 °C *in vitro*) and a cellular yeast form (inside the mammalian host or at 37 °C *in vitro*). This fungus is a pathogenic microorganism that causes a systemic disease named paracoccidioidomycosis (PCM), an important mycosis in Latin America [1–3]. This mycosis is a public health problem due to its morbidity and mortality, especially for agricultural workers. The disease affects mostly male between 30 and 50 years old as a chronic mycosis, but about 5–10% of the patients develop the acute and more severe form of the disease, which affects both genders and all ages indistinctly. The major risk factor for acquisition of infection is the activities related to the management of soil contaminated with fungus [4]. Infection occurs by inhalation of propagules or fragments of mycelia from the environment. Inhaled propagules differentiate to pathogenic yeast in the human lungs, the primary site of the disease, but the fungus can spread to other organs.

Many aspects of the biology of this fungus remain unknown. The most prominent difference between both forms is probably the cell wall polysaccharide, being 1,3-ß-glucan usually found in mycelia and 1,3-α-glucan found in yeasts [5], but a plethora of other biochemical differences have already been described between them [6–8]. However, an overall chemical composition for each form is not known and the definition of a chemical signature that distinguish one form from the other, or the same form exposed to different condition (i.e. drug treatment, environmental stresses, etc) could be useful to various applications. Fourier Transform Infrared (FT-IR) Spectroscopy is widely used to characterize the presence of chemical bonds, chemical groups of complex molecules and their interactions (hydrogen bonding by example). For more than a decade, FT-IR has been increasingly applied to characterize or distinguish biological samples [9].

Traditional methods for the diagnosis of PCM require either microscopic identification of *P. brasiliensis* yeasts in skin lesions, bronchial washings, sputum and biopsies, or serology identification of major antigens, such as GP43, an antigenic glycoprotein known for its diverse function during infection and modulation of immune response, which is based on detection of specific antibodies [6]. However, the mycological examination is frequently unable to show the presence of the fungus, and because symptoms are similar to other fungal or bacterial diseases, such as histoplasmosis or tuberculosis [10–12], paracoccidioidomycosis is often misdiagnosed.

The classic ELISA method is usually chosen because it has high sensitivity. However, antibodies-based diagnosis is subject to cross reactions, and paracoccidioidomycosis is especially mistaken with histoplasmosis, leishmaniasis or Chagas disease, which are also endemic in Brazil [13]. The implementation of ELISA is difficult in remote areas.

Several molecular techniques to identify and diagnose the paracoccidioidomycosis have being incorporated into clinical laboratories routine in order to increase the effectiveness of current microbiological and immunobiological methods, especially regular PCR or PCR combined with other methodologies. PCR is an important tool for the detection of fungi in patients with negative serological reactions when the concentration of antigen and/or antibody appears low.

More recently, new diagnose methods based on the unique properties of metal nanoparticles has been used to improve sensitivity and specificity values of current methods. Particularly, anisotropic nanoparticles of noble metals such as gold and silver have been receiving much attention due to its optical properties in the visible range of electromagnetic spectrum. Their applications are based on three fundamental characteristics of the optical response of metallic nanostructures: a high sensitivity to changes in the local vicinity chemistry; the location of the electromagnetic fields of the incident radiation below the diffraction limit and the subsequent generation of high near-field intensity [14]. These properties allow its application in biology, particularly molecular biology.

Mirkin et al. (1996) described the use of gold nanoparticles with thiolated oligonucleotides for the colorimetric detection of DNA targets [15]. The non-cross-linking method consists in aggregation of the oligonucleotide-functionalized gold nanoparticles induced by an increasing salt concentration with complementary oligonucleotides [16]. Solution of non-functionalized gold nanoparticles aggregates instantaneously after NaCl addition, which is observed by a color change of the solution from red wine to blue. However, nucleic acid sequences protect gold nanoparticles against aggregation, possibly through electrostatic interactions between the negatively charged phosphate groups of the nucleic acid and the polarized gold nanoparticles [17]. These properties were further exploited and applied in the detection and characterization of gene expression [18,19]. This method has also been successfully applied to detect eukaryotic gene expression without retrotranscription or polymerase chain reaction (PCR) amplification [18]; and in a fast and straight forward assay for *Mycobacterium tuberculosis* DNA detection in clinical samples [20].

The GP43 gene was first characterized in *P. brasiliensis* by Cisalpino et al. [21]. This gene encodes a glycosilated protein and a major antigen that react with 100% of sera from patients with PCM [22]. GP43 glycoprotein binds to murine laminin to entail an increased invasiveness and destruction of tissues infected [23], and is abundantly detected in yeast phase [24].

FT-IR spectroscopy has established as a powerful method for the rapid differentiation and identification of microorganisms, and presents a new addition to genetic methods [25,26]. The FTIR analysis allowing the rapid characterization of bacterial isolates and typical marker bands were used to detected and identified bacterial cell components [26,27].

In this work we propose a new molecular method that associates nanotechnology with molecular biology that can contribute to diagnose PCM in remote areas, as well as in epidemiological studies. This method is based on the detection of *P. brasiliensis* using gold nanoparticles functionalized with DNA in PCR assays in substitution to the further steps of eletrophoresis, which, in turn, avoid the use of ethidium bromide staining and visualization of DNA by UV light. We also propose a new approach to biochemically distinguish both forms of *P. brasiliensis* by Fourier Transform Infrared Spectroscopy that can be used in various applications in cell and molecular biology to compare general differences between cells that are, for example, exposed to distinct experimental or environmental conditions.

2. Experimental

2.1. Culture of P. brasiliensis

The filamentous hypha and yeast forms of the Pb18 isolate were cultivated in liquid Ham's F12 medium supplemented with 2% glucose either at 25 °C to obtain mycelia or 37 °C to obtain yeast cells, until the middle of the log phase before harvesting.

2.2. FT-IR: sample preparations and spectral conditions

Filamentous hypha and yeast forms were washed three times in cold distilled water and fixed in 60% isopropanol for 24 h. After two additional washings, the fungi were resuspended in ultrapure water. Analyses were performed with three independently