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Research paper

Combined use of *Paracoccidioides brasiliensis* recombinant rPb27 and rPb40 antigens in an enzyme-linked immunosorbent assay for immunodiagnosis of paracoccidioidomycosis

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ABSTRACT

Paracoccidioidomycosis (PCM) is one of the most important endemic mycoses in Latin America; it's usually diagnosed by observation and/or isolation of the etiologic agent, *Paracoccidioides brasiliensis*, as well as by a variety of immunological methods, such as complement fixation and immunodiffusion. Although these approaches are useful, historically their sensitivity and specificity have often been compromised by the use of complex mixtures of undefined antigens. The use of combinations of purified, well-characterized antigens appears preferable and may yield good results. In the present study combinations of the previously described 27-kDa recombinant antigen (rPb27) and a recombinant 40-kDa-molecular-mass antigen (rPb40) from this fungus, that was identified by Goes et al. (2005) through the AST strategy as a homolog of *Neurospora crassa* calcineurin B, were used in an indirect enzyme linked immunosorbent assay (ELISA) for diagnosis and follow-up of patients with PCM. The complete coding cDNA of rPb40 and rPb27 were cloned into a pET-21a and a pET-DEST 42 plasmid, respectively, expressed in *E. coli* with a his-tag and purified by affinity chromatography. Among 109 PCM serum samples analyzed, a homogeneous IgG response to these proteins was observed. 62 serum samples from patients with other diseases, 18 from patients with other mycosis and 23 from healthy individuals were also studied. Detection of anti-rPb27 and anti-rPb40 antibodies in sera of patients with PCM by ELISA using a combination of the two purified proteins showed a sensitivity of 96% with a specificity of 100% in relation to control normal human sera and to sera from patients with other systemic mycosis and 93.5% to sera from patients with diverse infections. The use of this two proteins combination provided an excellent immunodiagnosis assay with great values of sensitivity and specificity, even in relation to sera from patients with other mycosis, making possible the standadization of a new methodology to diagnose this important mycosis, with a good confiability and reprodutibility.

1. Introduction

Paracoccidioidomycosis (PCM) is a rural and suburban endemic disease commonly manifesting as a pneumopathy of a chronic course, often associated with mucosal and skin lesions, and eventually with extra-pulmonary and disseminated lesions. Frequently, pulmonary fibrosis can appear as incapacitating sequel of the disease, and in absence of an effective therapy, PCM progresses and may be lethal (Brummer et al., 1993). The disease caused by *Paracoccidioides brasiliensis* is considered to be the most prevalent systemic fungal infection in Brazil and is present in many Latin American countries. It has been recently included in the list of neglected diseases whose impact on public health has not been quantified due to the lack of available data (Martinez, 2010).

The importance of a precise and rapid diagnosis of this mycosis resides on the prompter initiation of the specific therapy in order to avoid both increasing lung damage and

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dissemination of the fungus to other organs and the development of fibrosis. The definitive diagnosis of PCM is typically dependent on the visualization of the fungus in biopsy and clinical specimens, or its isolation by culture, but these techniques are insensitive and the latter is time-consuming (Brummer et al., 1993). Consequently, immunological testing is an important tool not only for disease diagnosis, but also for monitoring patients responses to treatment (Mendes-Giannini et al., 1989). Immunological tests are based on the detection of specific antibodies and include methods such as complement fixation, immunodiffusion and immunoenzymatic tests (Borges-Walmsley et al., 2002).

Serological tests have been widely used in PCM diagnosis, however, one of the main problems of such tests is the high cross reactivity with agents causing other mycoses because of the use of crude antigens prepared from the complete microorganism and its metabolic products. Another disadvantage is that the preparation of these antigens is a very complex process. Additionally, these antigens show a great variability, making it very difficult to standardize diagnostic techniques on different laboratories.

An alternative to obtaining suitable antigens is the cloning, expression and characterization of antigens derived from the fungus. The gp43, *P. brasiliensis* immunodominant antigen, was the first antigen cloned (Cisalpino et al., 1996); this recombinant antigen showed high reactivity when evaluated with sera from patients with PCM. McEwen et al. (1996) cloned the protein of 27 kDa (Pb27) and it has also been used to detect immune responses by ELISA (Ortiz et al., 1996, 1998). Another antigen, called as hsp60 was also cloned and used as a serodiagnostic marker (Cunha et al., 2002). One of the main advantages of using recombinant antigens is the reduction of the cross-reactions that occur with other mycoses when crude antigens are used, as previously indicated. Additionally, recombinant proteins facilitate production of antigenic preparations that display a little variability and can be used in different tests and different laboratories.

In all these cases described above the antigens were used individually. Considering the large number of antigenic epitopes expressed by *P. brasiliensis*, Diez et al. (2003) used the combination of two recombinant proteins (Pb27 and p87) in the diagnosis of PCM. These authors showed an increase on sensitivity and specificity when using the two proteins together. Accordingly, in this paper we described the application of a mixture of defined *P. brasiliensis* antigens to the diagnosis of PCM using indirect ELISA strategy. The antigens chosen for study were rPb27 (McEwen et al., 1996) and rPb40 (Goes et al., 2005) – a 40-kDa-molecular-mass antigen from this fungus, which was previously identified through the AST strategy, as a homolog of *Neurospora crassa* calcineurin B – recombinant proteins.

2. Methodology

2.1. Study population

Patients (age range = 18–75 years) previously diagnosed with active PCM from Centro de Treinamento e Referência em Doenças Infecto-Parasitárias Orestes Diniz (CTR-DIP), Hospital das Clínicas of the Universidade Federal de Minas Gerais (UFMG), Brazil, were enrolled in this study. The patients were divided into three groups: untreated (No treated), treated for 3 months to 5 years (Treated), and patients with relapse of the disease (Relapsed). The therapeutic schedule consisted of the administration of amphotericin B during hospital stay, and sulfonamides or ketoconazole as long-term medication. Sera from patients infected by *Schistosoma mansoni*, *Toxoplasma gondii*, *Mycobacterium tuberculosis*, *Plasmodium falciparum*, *Trichuris trichuira*, *Ascaris lumbricoides*, *Amoeba* sp., *Ancylostoma duodenalis*, *Leishmania* sp., *Trypanosoma cruzi*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Fonsecaea pedrosoi, Aspergillus fumigatus* and *Sporothrix schenckii* all parasitologically and microbiologically confirmed, were assayed and they did not present a clinical history of infection by *P. brasiliensis*. The negative control group (NHS) comprised healthy volunteers from Instituto de Ciências Biológicas, UFMG, with no history of PCM. All groups used in this study are described in Table 1. This study was approved by the Research Ethics Committee (COEP) of UFMG, the protocol number is ETIC 523/07, and informed consent was obtained from each patient before blood collection.

2.2. Cloning and sequencing of rPb40 cDNA

The sequence of the recombinant 40-kDa-molecular-mass antigen (Goes et al., 2005) from the fungus *P. brasiliensis* was cloned into a pET-21a (Novagen) expression vector. A *P. brasiliensis* cDNA library was generated from yeast forms that were RNA extracted with Trizol Reagent according to the manufacturer's protocol (Invitrogen Life Technologies). rPb40 cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) methods using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). The oligonucleotides used in PCR were designed taking into account the sequence described by Goes et al. (2005). The forward primer was 5′-TTGTCGACC-TATGGAAAATGCCTTTCTCG-3′ and the reverse was 5′-TTGCGGCCGCACCCTCCAAGAAATCATCTT-3′, which served as the sense and anti-sense primers, respectively. The amplified rPb40 DNA fragment was digested by enzymatic digestion of Sal I/Not I restriction sites. The digested product was purified and inserted into the Sal I/Not I restriction site of pET-21a (Novagen). The recombinant plasmid pET-21a/rPb40 was

Table 1
Sources of serum specimens tested by ELISA.

Source	Number of samples
Healthy subjects	23
Untreated PCM patients	25
Treated PCM patients	74
Relapsed PCM patients	10
Sera from patients infected with *Ascaris lumbricoide*	5
Sera from patients infected with *Ancylostoma duodenalis*	5
Sera from patients infected with *Trypanosoma cruzi*	6
Sera from patients infected with *Trichuris trichuira*	5
Sera from patients infected with *Mycobacterium tuberculosis*	14
Sera from patients infected with *Amoeba* sp.	5
Sera from patients infected with *Toxoplasma gondii*	10
Sera from patients infected with *Taenia* sp.	3
Sera from patients infected with *Leishmania* sp.	9
Sera from other systemic mycosis patients	18