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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

checked for accurate insertion by restriction enzyme analysis, and the inserted fragment was sequenced on a MegaBACE DNA Analysis System (Amersham Biosciences, Buckinghamshire, England) (Sanger et al., 1977). The sequence homology was analyzed using the algorithms BLASTx and ClustalW available on the Internet: http://www.ncbi.nlm.nih.gov and http://www.ebi.ac.uk/clustalw/, respectively.

2.3. Subcloning and sequencing of rPb27 DNA

The sequence of the recombinant rPb27 was already cloned by our group in previous work into the expression vector pGEX 4 T-2 (GIBCO BRL), which produces a recombinant protein fused to glutathione S-transferase (GST) (Reis et al., 2008). In order to facilitate the purification procedure the rPb27 sequence was transferred to the expression vector pET-DEST 42 (Invitrogen, Carlsbad, USA) which express the recombinant protein with a C-terminal His-tag. The cloning procedure was based on Gateway® technology (Invitrogen, Carlsbad, USA), that uses a strategy based on the recombinational properties of bacteriophage lambda (Landy, 1989). The primer set for amplification of the rPb27 sequence included the forward: 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GAA GGA GAT AGA ATG GCA CGA GCG CTC AGT TC -3′ and reverse primer: 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GTT GTG GAA GAC AGC GCT GCA -3′. The PCR annealing temperature was 56 °C. The PCR products were separated in 1% agarose gel. The blunt-end PCR products were then cloned into pDONR 221 vector according to the manufacturer's protocol (Invitrogen, USA). The reaction mixture was incubated overnight at 25 °C. The reaction was then stopped with 10 min Proteinase K incubation. After this step, competent *E. coli* (TOP10) was transformed by electroporation with the pDONR 221/rPb27 construct according to the manufacturer's protocol (Invitrogen, USA). Positive clones were selected on LB medium containing 50 μg/mL kanamycin. Plasmid DNA was isolated using the high pure plasmid extraction kit, Mini-prep QIAprep Spin miniprep 150 (Qiagen, Hilden, Germany). The presence of the insert was confirmed by PCR and, finally, to confirm the fidelity of the sequence, DNA sequencing was performed. This construct is called the entry clone. The LR recombination reaction was then carried out between the entry clone and destination vector, pET-DEST42, according to the manufacturer's instructions (Invitrogen, USA). Competent *E. coli* (BL21) was transformed by electroporation with the products of LR recombination according to the manufacturer's protocol. Positive clones were analysed by culturing them on LB medium containing 100 μg/mL ampicillin. The recombinant plasmid pET-DEST42/rPb27 was checked for accurate insertion by restriction enzyme analysis, and the inserted fragment was sequenced on a MegaBACE DNA Analysis System (Amersham Biosciences, Buckinghamshire, England) (Sanger et al., 1977). The sequence homology with rPb27 was analyzed using the algorithm ClustalW available on the Internet: http://www.ebi.ac.uk/clustalw/.

2.4. Expression in E. coli and purification of rPb40 and rPb27

The recombinant proteins, rPb40 and rPb27, were expressed in *E. coli* using the expression vectors pET-21a (Novagen) and pET-DEST42 (Invitrogen, USA), respectively, which produce a recombinant protein with a C-terminal his-tag. Purification of these recombinant proteins was therefore undertaken by HiTrap™ Chelating HP according to the manufacturer's instructions (Amersham Biosciences, Uppsala Sweden).

The eluted material obtained were analyzed by SDS-PAGE 10% (Laemmli, 1970) and western blotting (Towbin et al., 1979), using anti-His tag antibodie (GE) to confirm the purity of them. The eluted proteins were quantified by the Bradford method (Bradford, 1976) and then, used in ELISA assays.

2.5. Antibody detection by indirect ELISA (Enzyme Linked Immunosorbent Assays)

Flat-bottomed microtiter plates (Maxisorp, Nunc) were coated with 1 μg/100 μl per well of purified rPb40 or rPb27. When using the combination of rPb40 and rPb27, the plates were coated with 0.5 μg/100 μl per well of each one. Both antigen samples were diluted in 0.5 M carbonate buffer, pH 9.6, and left overnight at 4 °C. The plates were washed five times with washing buffer (0.05 M PBS containing 0.05% Tween 20 – PBS-T), and blocked with 150 μl of blocking solution (1.6% casein in 0.15 M PBS, pH 7.4), at room temperature. After 1 h of incubation, plates were washed five times with PBS-T, and then filled with 100 μl of a 1/400 dilution of infected or uninfected human sera in 0.15 M PBS containing 0.25% casein, pH 7.4, and re-incubated for 1 h. The plates were washed ten times with PBS-T and incubated for an additional hour with 100 μl of a 1/5000 dilution of goat anti-human IgG peroxidase-conjugated antibody (Sigma) in 0.15 M PBS containing 0.25% casein, pH 7.4. The plates were then washed ten times with PBS-T and the peroxidase activity was assayed with 100 μl of o-phenylenediamine dihydrochloride (OPD) solution (3.4 mg of OPD and 20 μl of hydrogen peroxide to 10 ml of citrate/phosphate buffer, pH 5.0) from Sigma. Color development was stopped with 20 μl of 2 N H_2SO_4. An optical density of 490 nm was registered in an automated ELISA reader (Bio-Rad 2550 Reader EIA).

2.6. Statistical analysis

Statistical differences among groups were determined using the non-parametric Kruskal–Wallis test. The cut-off level for serum antibody reactivity was calculated as the control group mean ± 2 S.D. of control group.

3. Results

Sera of 109 patients with *P. brasiliensis* infection from endemic areas in Brazil were tested by ELISA assays using rPb40 (Fig. 1A), rPb27 (Fig. 1B) or the two proteins together (Fig. 1C).

In the Fig. 1A, its shown that sera from untreated PCM patients (No treated) presented a higher mean reaction in relation to sera from uninfected individuals (NHS) against rPb40 protein ($P<0.05$). However, the mean reaction of the sera from treated PCM patients and from patients with a relapse of this disease was similar ($P>0.05$) to the mean reaction of uninfected individuals against rPb40 antigen.

The rPb27 protein was recognized by a large number of PCM patients' sera (Fig. 1B). Untreated PCM patients and patients with a relapse of this disease presented a higher

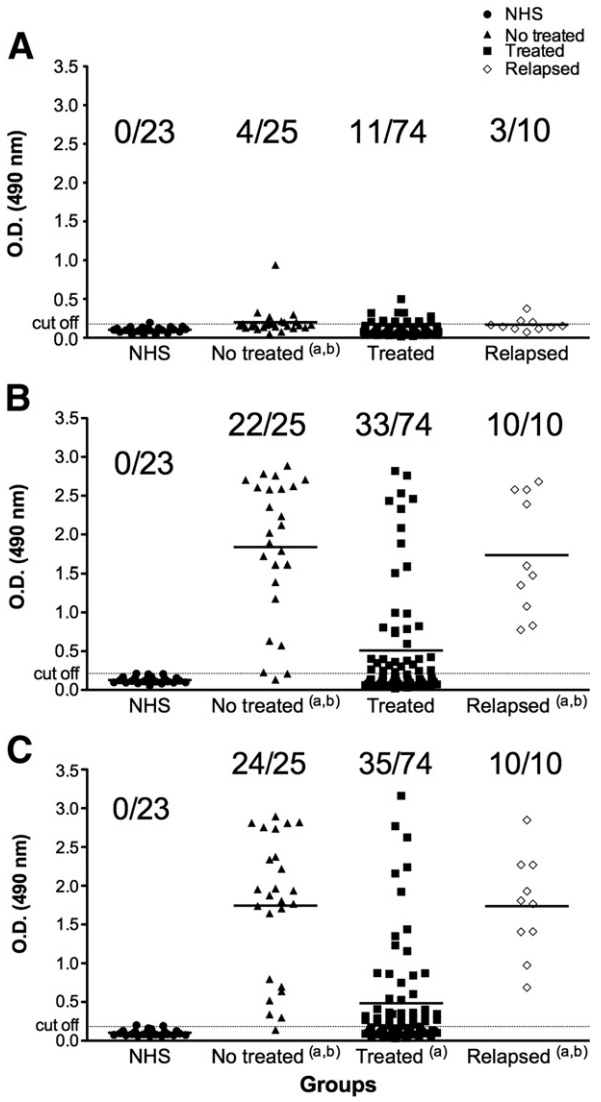

Fig. 1. Human IgG reactivity to rPb40 (A), rPb27 (B) and both proteins (C) from control group (normal human sera, NHS, $n=23$), untreated PCM patients (no treated, $n=25$), PCM patients treated (treated, $n=74$) and relapsed group (relapsed, $n=10$) of sera measured by ELISA. To evaluate the reactivity of sera from PCM patients and healthy individuals, these sera were tested by ELISA against rPb40, rPb27 and both. The dotted line represents the test cut off (the mean of control group plus 2 times the standard deviation of the test). [a] Significant ($P<0.05$) differences in relation to the NHS group. [b] Significant ($P<0.05$) differences in relation to the Treated group. The ratio positive/total of sera samples is indicated at the top of each graph.

mean reaction in relation to sera from uninfected individuals (NHS) against rPb27 protein ($P<0.05$). But, sera from treated PCM patients showed a similar mean reaction to the sera from uninfected individuals ($P>0.05$).

When used individually, the reactivity to the rPb27 (Fig. 1B) antigen was superior to the reaction to the rPb40 one (Fig. 1A) – thus, only 3 of the 25 untreated PCM serum samples recognized rPb40, whereas 22 (88%) recognized the purified rPb27.

A number of different combinations of the rPb27 and rPb40 antigens concentrations were tested (data not shown), and the most effective, 0.5 μg/well of each antigen, is shown in the Fig. 1C. All PCM patients groups (Treated, No treated and Relapsed) presented higher mean reaction in relation to uninfected individuals (NHS) ($P<0.05$). However the mean reaction of groups "No treated" and "Relapsed" were higher than that of "Treated" group ($P<0.05$). A total of 24 untreated PCM sera were reactive when this antigen combination was used. This gave an overall sensitivity for the combined antigen ELISA of 96% (Table 2), which was superior to the sensitivity of either antigen used alone. Positive and negative predictive values are also included in Table 2; these values also demonstrated the superiority of the combined antigen approach.

In the Fig. 1 it is observed that the treatment of patients induced a reduction in reactivity against rPb27 (Fig. 1B) and rPb40 plus rPb27 (Fig. 1C). While all patients that presented a disease relapse showed high optical density, higher than cut off point, when using rPb27 (Fig. 1B) and rPb40 plus rPb27 (Fig. 1C) antigens.

To evaluate the specificity of the ELISA using the recombinant proteins together or individually, the sera from uninfected individuals and from patients infected by *Ascaris lumbricoides*, *Ancylostoma duodenalis*, *Trypanosoma cruzi*, *Trichuris trichuira*, *Mycobacterium tuberculosis*, *Amoeba* sp., *Toxoplasma gondii*, *Taenia* sp., and *Leishmania* sp. were assayed in ELISA against rPb40 (Fig. 2A), rPb27 (Fig. 2B) and rPb40 plus rPb27 (Fig. 2C). The specificity of the ELISA in reference to sera from healthy subjects was 100% when the proteins (rPb40 and rPb27) were used together or individually (Figs. 1, 2, Table 2). In relation to sera from patients with other diseases, rPb40 plus rPb27 combination proved to be more specific than rPb27 alone (Fig. 2B,C and Table 2). This combined approach presented 93.5% of specificity in relation to patients with diverse diseases.

The Fungi share a lot of component proteins, so cross reaction among a great variety of fungal infections is commonly seen in immunodiagnosis. To test the specificity of the assay against sera from patients with other fungal

Table 2
Sensitivities, specificities, and predective values of ELISAs using individual and combined antigens.

Parameter [a]	Value obtained with antigen(s)		
	rPb40	rPb27	rPb40 + rPb27
Sensitivity	54	89	96
Specificity vs NHS	100	100	100
Specificity vs other diseases	95	92.5	93.9
Specificity vs other mycosis	75	78	100
Predictive value of positive result (vs NHS)	100	100	100
Predictive value of positive result (vs other disease)	87	80	83
Predictive value of positive result (vs other mycosis)	54	58	100
Predictive value of negative result (vs NHS)	52	88	95.8
Predictive value of negative result (vs other disease)	95	100	100
Predictive value of negative result (vs other mycosis)	90	100	100

[a] NHS, normal human serum. All values are percentages.

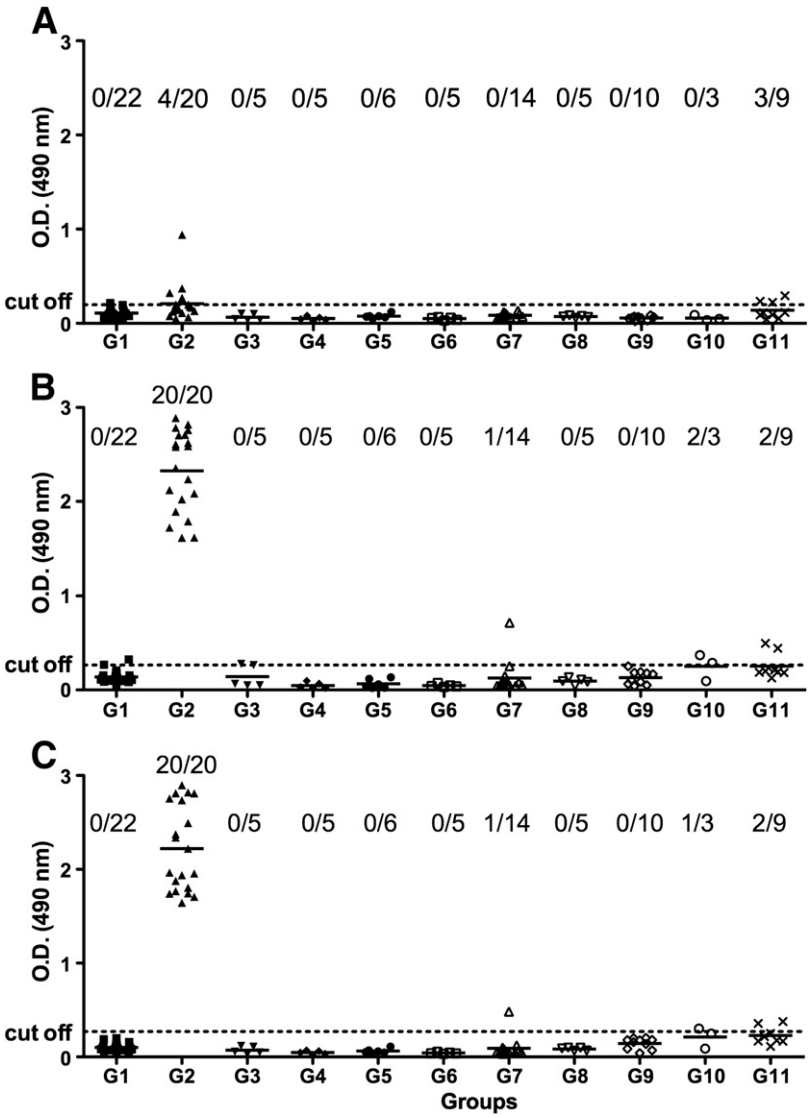

Fig. 2. Human IgG reactivity to rPb40 (A), rPb27 (B) and both proteins (C) from control group (normal human sera, G1), untreated PCM patients (G2), and patients with other diseases (G3–G11) of sera measured by ELISA. To evaluate the reactivity of sera from PCM patients, healthy individuals, and patients with other diseases, these sera were tested by ELISA against rPb40, rPb27 and both proteins. The dotted line represents the test cut off (the mean of control group plus 2 times the standard deviation of the test). G1 – negative control (uninfected people) ($n=22$); G2 – positive control (untreated PCM patients) ($n=26$); G3 – sera from patients infected with *Ascaris lumbricoides* ($n=5$); G4 – sera from patients infected with *Ancylostoma duodenalis* ($n=5$); G5 – sera from patients infected with *Trypanosoma cruzi* ($n=6$); G6 – sera from patients infected with *Trichuris trichiura* ($n=5$); G7 – sera from patients infected with *Mycobacterium tuberculosis* ($n=14$); G8 – sera from patients infected with *Amoeba* sp. ($n=5$); G9 – sera from patients infected with *Toxoplasma gondii* ($n=10$); G10 – sera from patients infected with *Taenia* sp. ($n=3$); G11 – sera from patients infected with *Leishmania* sp. ($n=9$). The ratio positive/total of sera samples is indicated at the top of each graph.

diseases, we perfomed a ELISA using the proteins together and individually against sera of patients with other systemic mycosis (Histoplasmosis, Chromoblastomycosis, Aspergillosis, Cryptococcosis and Sporotrichosis). It wasn't observed any cross reaction against other systemic mycosis serum samples when it was used the combination approach (rPb27 plus rPb40), giving to this assay a 100% of specificity in relation to other systemic mycosis sera (Fig. 3C and Table 2). On the other hand, when the proteins were used alone it was observed cross reaction against other systemic mycosis, presenting 66.7% and 77.8% of specificity when using rPb40 and rPb27, respectivelly (Fig. 3 and Table 2).

4. Discussion

The detection of antibody by serological methods has an important role in the diagnosis of PCM (de Camargo et al., 1984; Cano and Restrepo, 1987; Hamilton, 1998). However, the complexity of the antigens traditionally used resulted, regardless of the methodology employed, in problems associated with a lack of antigen standardization and specificity (Hamilton, 1998). To circumvent this complexity of antigens many works using protein purification methods and recombinant protein technology were performed and have identified a series of candidate antigens (Giannini et al.,

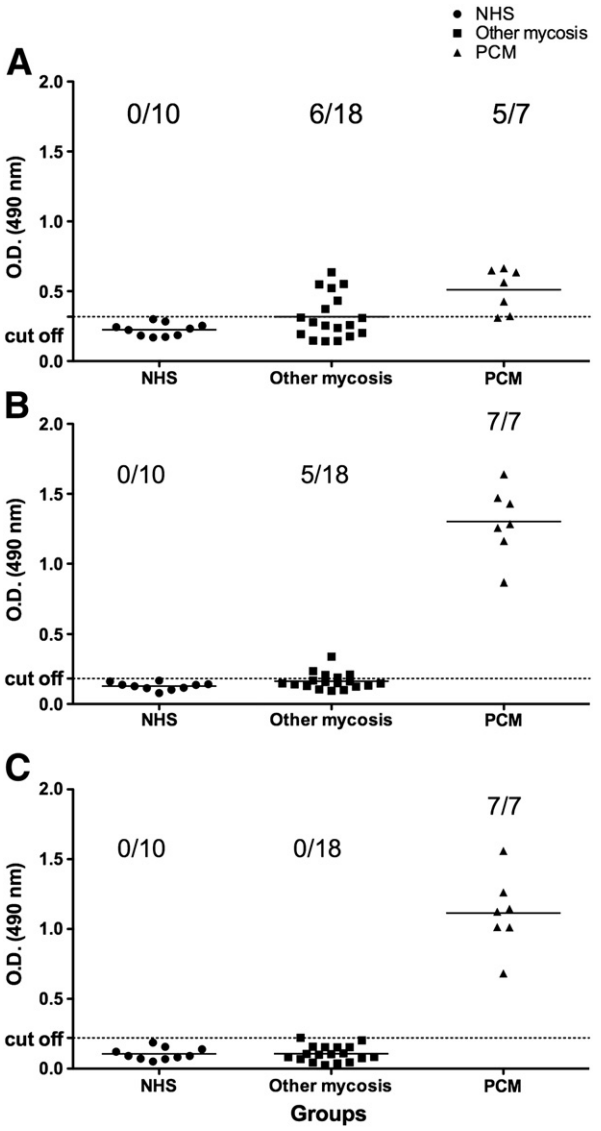

Fig. 3. Human IgG reactivity to rPb40 (A), rPb27 (B) and both proteins (C) from control group (normal human sera, NHS, $n=10$), untreated PCM patients (no treated, $n=7$) and patients with other systemic mycosis (other mycosis, $n=18$) of sera measured by ELISA. To evaluate the reactivity of sera from PCM patients, patients with other systemic mycosis and healthy individuals, these sera were tested by ELISA against rPb40, rPb27 and both proteins. The dotted line represents the test cut off (the mean of control group plus 2 times the standard deviation of the test). The ratio positive/total of sera samples is indicated at the top of each graph.

1990; Cisalpino et al., 1996; McEwen et al., 1996; Ortiz et al., 1998; Cunha et al., 2002; Diez et al., 2002) that may be used in place of complex antigenic extracts.

On the other hand, the use of individual purified or recombinant antigens in diagnostic tests can result in low sensitivity. In this regard, the use of combinations of characterized antigens may be advantageous. Diez et al. (2003), described the use of a multiantigenic approach, with proteins p27 (Pb27) and p87 for the diagnosis of PCM, using a standard ELISA format and demonstrated that this combined use is an efficient strategy to improve specificity and sensitivity of the assay.

Although many researches about immunodiagnostic of PCM were performed, until now they aren't employed in the definitive diagnosis of this mycosis that is still obtained by direct visualization of the fungus in biological materials. This direct visualization is a very difficult and laborious procedure, mainly depending on the site of infection. Accordingly, researches about new alternatives for serodiagnosis of PCM continue. In the search for a new strategy to diagnose PCM we described in this report the development of a multiantigenic approach for the serodiagnosis of this mycosis, using a standard ELISA format.

To perform the ELISA we chose to use two well-characterized proteins. The 27-kDa protein was originally cloned and used as an immunological marker in 1996 (McEwen et al., 1996); subsequently, Ortiz et al. (1998) used it in antibody detection; this protein was also used in combination with other protein to detect antibodies in PCM patients (Diez et al., 2003), and finally this protein showed a significant degree of protection in the lungs, livers and spleens of mice immunized with it and posteriorly challenged with a virulent strain of *P. brasiliensis* (Reis et al., 2008). The rPb40 protein was identified as an immunogenic component of the F0 fraction of *P. brasiliensis* (Goes et al., 2005). This fraction was able to promote significant decrease of organ colony-forming units (CFUs) in the lungs after challenge infection with virulent *P. brasiliensis* strain, without fungi dissemination to the spleen or liver (Diniz et al., 2004).

By using a cocktail comprised of the rPb40 and rPb27 antigens in the ELISA, we reached an excellent sensitivity and specificity, even when it was used sera from patients with other mycosis. These results were more effective than others performed in previous studies (Mendes-Giannini et al., 1989; Ortiz et al., 1998; Diez et al., 1999, 2002, 2003; Reis et al., 2005).

The combined use of these proteins was able to improve the sensitivity and specificity in relation to the single use of them. The rPb40 protein when used alone in ELISA showed a low sensitivity, while rPb27 showed a higher one. But when the proteins were associated the sensitivity of the assay was higher than when only rPb27 was used, it happened because of the complemanteriety of these two proteins, since the sera that don't recognize rPb27, is able to react to rPb40.

These results demonstrated an increase in sensitivity and specificity compared to results when the antigens were used separately. 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. Besides this, patients undergoing treatment for more than 1 year showed a reduced antibody response against rPb27, alone or combined with rPb40, while patients with relapsed disease showed an increased antibody response against it. These results suggest that the presence of anti-rPb27 antibodies might be an indicator of active disease.

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