Synthetic Peptides Mimic gp75 from *Paracoccidioides brasiliensis* in the Diagnosis of Paracoccidioidomycosis

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Abstract Paracoccidioidomycosis (PCM) is a systemic granulomatous disease, endemic in Latin America, caused by the thermal dimorphic fungus *Paracoccidioides brasiliensis*. Although some fungal antigens have already been characterized and used for serological diagnosis, cross-reactions have been frequently observed. Thus, the examination of fungal forms in clinical specimens or isolation of *P. brasiliensis* by culture is still the most frequent method for the diagnosis of this mycosis. In this study, a random peptide phage display library was used to select mimotopes of *P. brasiliensis*, which were employed as antigens in an indirect enzyme-linked immunosorbent assay. The protective monoclonal antibody against experimental PCM (anti-gp75) was used as molecular target to screen a phage display library. That approach led to a synthetic peptide named P2, which was synthesized and tested against PCM patients' sera to check whether it was recognized. There was significant recognition of P2 by sera of untreated PCM patients when compared with normal human sera. Sera from treated PCM group, patients with other mycosis or co-infected with HIV had much lower recognition of P2 than untreated patient group. The test showed a sensitivity of 100 and 94.59% of specificity in relation to human sera control. These data indicate a potential use of P2 as diagnostic tool in PCM. Its application for serological diagnosis of PCM may contribute to the development and standardization of simpler, faster and highly reproducible immunodiagnostic tests at low cost.

Keywords gp75 · *Paracoccidioides brasiliensis* · Paracoccidioidomycosis · Peptides · Phage display

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Introduction

Paracoccidioides brasiliensis is the agent of paracoccidioidomycosis (PCM), a prevalent disease in Latin America. Despite the large number of cases frequently diagnosed, the exact epidemiology of PCM is not

clearly known since there is no compulsory notification of identified cases. Due to the lack of available data, PCM was recently added to the list of neglected diseases [1].

The infection probably begins by inhalation of fungal propagules that, once in the lungs, transform into the pathogenic yeast forms [2] with ability to disseminate through the lymphatic system and the bloodstream to any location in the host's organism [3]. Two clinical forms of the PCM are recognized: acute form (AF), which is more rare and severe, with involvement of the reticuloendothelial system; and chronic form (CF), which may develop in multiple ways, ranging from benign and localized (unifocal) to severe and disseminated (multifocal) disease, depending on the degree of depression of cellular immunity [4, 5].

Regarding its diagnosis, serology has an important role in identifying the suspected cases of PCM. However, difficulties in the immunodiagnosis have been associated with cross-reactions with antibodies present in sera from patients with other mycosis or diseases, mainly due to antigen preparation used [6]. To improve serodiagnosis of PCM, great efforts have been made, such as using recombinant proteins [7, 8] that in most cases, reduced cross-reaction [9]. Nevertheless, production of these molecules still represents a high cost, being used only in research centers.

Considering that for PCM diagnosis, only the epitopes able to induce specific humoral response may be sufficient in immunologic reactions, herein, we employed the phage display methodology to define peptides that mimic natural epitopes. It consists of a collection of millions of randomized peptides displayed on bacteriophage surfaces that can be used to identify ligands of molecules of interest [10–12]. Peptides that mimic epitopes (mimotopes) have been identified by screening phage libraries with monoclonal antibodies (mAb) in several models and those synthetic peptides have been used for diagnostic applications with promising results [13–16].

In this study, a phage display approach was used for the first time to identify ligands mimicking antigenic epitopes from *P. brasiliensis*. The protective monoclonal antibody 5E7C (anti-gp75) was used here to select binding peptides [17]. After selection and analysis procedures, one synthetic peptide selected (called P2) was significantly recognized by untreated PCM patients' sera when compared with controls.

These results open the way to new approaches using peptides for the diagnosis of PCM.

Materials and Methods

Mice

Male BALB/c mice, 6–8 weeks old, were obtained from the animal facility, Federal University of São Paulo (UNIFESP), SP, Brazil. Animal handling and housing were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Bacterial Strain and Growth Conditions

The *E. coli* ER2738 strain was obtained from New England Biolabs, Inc. (Beverly, Massachussets, USA) exhibiting rapid growth and suited for propagation of phages (M13). These bacteria have a mini-transposon that confers tetracycline resistance. It was grown in Luria–Bertani agar containing 20 μg tetracycline/mL at 37°C overnight with shaking.

Production of *P. brasiliensis* Exoantigen

All experiments were carried out with *P. brasiliensis* yeast cells from isolate Pb18 kindly provided by Prof. Vera L. G. Calich from the Department of Immunology, São Paulo University. To obtain secreted protein produced by *P. brasiliensis*, yeast cells cultured for 5 days in modified solid YPD [0.5% yeast extract (Oxoid), 0.5% casein peptone (Difco), 1.5% glucose (Synth) and 1.5% agar (Difco), pH 6.5] were transferred to Erlenmeyer containing 50 mL of modified liquid YPD and maintained under agitation for 3 days at 37°C. Then, the fungal cells were transferred to Fernbach flask containing 450 mL of the same medium and cultured for 7 days. The cells were killed with 0.4 g/L of timerosal (Sigma), and the supernatant (crude exoantigen) was collected by filtration through filter paper. The exoantigen protein content was determined by the Bradford method [18], analyzed by SDS–PAGE [19] and stored at −20°C.

Preparation of Monoclonal Antibody (mAb)

Both 5E7C (anti-gp75) and 17C (anti-gp43) mAb [17, 20] were obtained from ascitic liquid of BALB/c mice.

Purification of mAbs was performed by affinity chromatography of ascitic fluidic in a column prepared with Sepharose coupled with protein A (Pharmacia, Uppsala, Sweden). The purified mAb was always tested by immunoblotting to verify the recognition of its specific proteins in *P. brasiliensis* exoantigen.

Phage Display Library

Experiments were performed with Ph.D.-7 library (New England Biolabs, Inc., Beverly, Massachussets, USA) that consisted of 1.2×10^9 independent clones. This library contains peptide sequences (7-mer), inserted into the NH3 terminus of the pIII minor coat protein of the M13 bacteriophage. The randomized segment of seven amino acids (CX7C–C, cysteine; X, any amino acid residue) is flanked by a pair of cysteine residues, which are oxidized during phage assembly to form a disulfide linkage. The first residue of the peptide–pIII fusion is the first randomized position that is preceded by Ala–Cys. The short linker sequence (Gly–Gly–Gly–Ser) is localized between the displayed peptide and pIII. This specific library carries the *lacZα* gene, and phage plaques appear blue when plated on media containing Xgal and IPTG.

Selection of Binding Peptides by Phage Display

Selection was performed according to the manufacturer's instructions (New England Biolabs, Inc., Beverly, Massachusetts, USA). First, library was cleaned with mAb 17C (anti-gp43) that belongs to the same isotype of the mAb 5E7C (IgG2a) to eliminate non-specific phage clones to the target molecule (mAb 5E7C). Briefly, 100 μg/mL of monoclonal antibody 17C was added into all screening wells of 96-well ELISA plate, and the plate was kept at 4°C overnight. The wells were blocked with blocking buffer (0.1 M NaHCO$_3$ [pH, 8.6], 5 mg/mL BSA, 0.02% NaN$_3$) at 4°C for 60 min followed by six washes with TBS-T (TBS 50 mM, Tris–HCl [pH, 7.5], 150 mM NaCl—plus 0.1%, v/v; Tween-20). Next, 10^{11} plaque-forming units (PFU) were added to each well, and the plate incubated at room temperature for 1 h. The unbound phages remained in the supernatant and were used for subsequent stages of selection of 5E7C ligand phage clones. Those phage clones were amplified, titled in plates containing LB/IPTG/Xgal medium and used for selection of 5E7C ligand phage

clones. The preselection-obtained phage clones were incubated with 5E7C mAb adsorbed in ELISA plates (100 μg/mL). All procedures of blocking and washing were performed as described above. Phages that bound to mAb 5E7C were eluted with glycine–HCl (0.2 M [pH, 2.2], 1.0 mg/mL BSA) by gently rocking for 10 min and were then neutralized with 1 M Tris–HCl (pH, 9.1) to pH 7. The phage solution was taken for dilution with LB medium, and the title of the phages was determined. Subsequently, the eluted phages were amplified by infection of *E. coli* ER2738 and concentrated by precipitation with PEG/NaCl (20%, w/v polyethylene glycol-8000, 2.5 M NaCl). The same panning procedures were used to the second and third generations, except for the washing step in which Tween-20 0.5% (v/v) was used. After the third round of panning, positive phage clones were selected.

DNA Sequencing

Phage clones were prepared for DNA sequencing as described in the Ph.D 7-mer phage display peptide library kit. The method used for the PCR product sequencing was automatic, accomplished in the ABI Prism 3100 Genetic Analyzer equipment, with the commercial kit Big Dye Terminator 3.1 (Applied Biosystems), according to the manufacturer's instructions. Briefly, 4.0 pmol of specific initiator oligonucleotide (5′-TAATAC GACTCAC TATAGG GCAAGC TGATAA ACCGAT ACAATT-3′), 1.5 μL of Big Dye Mix, 2 μL of sequencing buffer (five times) and 4.5 μL of the purified samples for sequencing were used. The thermocycling program used 34 cycles, with denaturation at 96°C for 10 s, annelation at 50°C for 5 s and extension at 60°C for 4 min. The DNA was precipitated (for 15 min at 25°C) with addition of 40 μL of ethanol 80% (Merck) following centrifugation with 4,000 rpm for 1 h at 25°C. After discarding the supernatant, all samples were washed with 150 μL of ethanol 70% (Merck) and re-suspended in 10 μL of HiDi formamide (Applied Biosystem). Samples were denatured at 90°C for 2–5 min and immediately placed in ice.

Graphic Analyses of Antigenicity and Structural Parameters of the Peptides Sequences

The sequences of peptides selected were analyzed by Jameson-Wolff antigenic index methods, Kyte-Doolittle

hydropathicity plot, Eisenberg's alpha helix amphi-pathic regions, Emini's and motifs surface probability plot and Sette major histocompatibility complex II [21–23] using the Protean program (protein sequence analysis) of Lasergene biocomputing for Windows, 1994 (DNASTAR Inc., Madison, Wisconsin, USA).

Binding

The interaction between the selected phage clones and the 5E7C mAb was evaluated by in vitro binding assays. The phage clones selected were incubated in 96-well plates previously sensitized with 100 μg/mL of each mAb (17C, anti-gp43 or 5E7C, anti-gp75). After blocking with blockage buffer, wells were washed six times with TBST 0.1%. Selected phage clones (2×10^{11} of each) were diluted in TBST 0.1% (final volume of 100 μL) and added to sensitized wells with mAbs. After 2 h of incubation at room temperature, the wells were washed 10 times with TBST 0.1%, and phage clones bound to the antibody were eluted, as described above. Phages eluted were recovered through infection in E. coli ER2738. Serial dilutions were prepared, and the bacteria were plated in LB IPTG/Xgal medium for quantification of the number of bound phage clones.

Peptide Synthesis

Peptides were kindly synthesized by Maria Aparecida Juliano at the facility of Department of Biophysics, Federal University of São Paulo (UNIFESP), SP, Brazil. The 9-fluorenylmethoxycarbonyl method [24] was performed using an automated benchtop simultaneous multiple solid-phase peptide synthesizers (PSSM 8 system; Shimadzu, Tokyo, Japan).

Animal Immunization Using Synthetic Peptides

Group of 5 BALB/c mice was immunized four times at 10 days intervals with 20 μg of synthetic peptide. The first immunization was performed with emulsified peptide in complete Freund's adjuvant (CFA) and the subsequent immunizations with incomplete Freund's adjuvant (IFA). The emulsion (50 μL) was subcutaneously inoculated in the footpad. Control mice received only adjuvant. Before each immunization, blood samples were collected by the ocular plexus and the serum was separated and kept at −20°C.

Immunoblotting

Proteins of exoantigen from P. brasiliensis were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions [19]. The proteins were transferred to nitrocellulose (NC) membranes [25], and non-specific sites were blocked with PBS containing 5% skim milk. Sera from P2 peptide-immunized animals were added to each NC strips for 1 h at room temperature. Strips were washed five times for 5 min each with PBS containing 0.1% Tween 20. After treatment with affinity-purified peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) (Zymed) for 1 h at room temperature, the reactive Ig was detected by ECL detection reagent (GE Healthcare, UK).

Human Serum Specimens

Individual serum specimens from 33 individuals with acute or chronic localized PCM and 31 treated PCM patients were obtained from São Paulo Hospital (São Paulo, Brazil). All patients' sera were positive for whole exocellular P. brasiliensis antigens by immunodiffusion test. Untreated refers to patients under treatment with antifungal drugs. All patients in this group had low titers by immunodiffusion.

Control sera from healthy blood donors ($n = 37$) and sera from patients with aspergillosis (Asp, $n = 8$), histoplasmosis (HC, $n = 17$) and candemia (CA, $n = 20$) were kindly provided by Medical Mycology laboratory from Federal University of São Paulo. Prof. Roberto Martinez, Faculdade de Medicina de Ribeirão Preto Universidade de São Paulo, kindly provided sera from patients co-infected with HIV and PCM ($n = 10$), and HIV and histoplasmosis ($n = 10$). Sera from individuals infected with other mycosis were negative against P. brasiliensis antigens by immunodiffusion. This study was approved by the Research Ethics Committee (CEP) of UNIFESP with protocol number 0484/07.

Detection of Human Serum Antibodies Against P2 Peptide

ELISA was carried out to detect circulating antibodies that recognized P2 peptide in PCM patients' sera. First, 96-well polyvinyl plates (Costar) were sensitized with 50 μg/mL of P2 diluted in PBS (50 μL/

well). After overnight incubation at 4°C, the plates were blocked with 200 μL of PBS-molico 5% for 1 h at 37°C. Then, wells were washed three times with PBS-molico 5% containing Tween 0.1%. Patients' sera were added at dilution 1:20 in PBS-Molico 5% (50 μL/well) for 1 h at 37°C. Following, wells were washed with PBS-molico 5% containing Tween 0.1% (200 μL/well), and anti-IgG human secondary antibody coupled to the peroxidase (Sigma) diluted at 1:1,000 in PBS-molico 5% was added in each well. The reactive sera were detected by addition of substrate solution (1 mg of o-phenylenediamine [OPD] in 5 mL of 0.1 M citrate–phosphate buffer [pH, 5.0] plus 10 μL of 30% H_2O_2), and the reaction interrupted by the addition of 50 μL of 4 N H_2SO_4 per well. Optical density was measured in an automatic MCC/40 reader (Labsystem Multiscan Dynatech, Chantilly, Virginia, USA) at 492 nm.

Statistical Analysis

The statistical analyses were accomplished by the Student's *t* test using the INSTAT program (GraphPad 4.0, San Diego CA) or by analysis of variance (ANOVA) followed by the Tukey–Kramer test. $P < 0.05$ indicated statistical significance. The cutoff was determined by receiver operating characteristic (ROC) curve.

Results

Selection of Phages Displaying Peptides Recognized by Anti-gp75 Antibodies

The specificity of anti-gp75 (5E7C) and anti-gp43 mAbs (17C) was checked as shown in Fig. 1a. Figure 1b shows the enrichment of the phages after selection cycles. The amount of phages recovered was 4.2×10^{11} and 8.9×10^{11} pfu/mL in the first and second rounds (Fig. 1b), respectively. After the third round, in which more stringent conditions were used, 1.8×10^{11} phage clones were obtained. These results indicate the presence of clones bearing the peptide sequence recognized by 5E7C.

Phages displaying peptide sequences that mimicked the epitope of the 75-kDa protein of *P. brasiliensis* were analyzed, and the peptides P9, P13, P18, P19, P25 and P36 were selected for further

studies. Table 1 shows the sequences of selected peptides.

Binding assays were performed to evaluate the interaction between the selected phages (P9, P13, P18, P19, P25 and P36) and mAb 5E7C. As shown in the Fig. 1c, phages displaying P9, P18, P19, P25 and P36 bound with similar patterns to both 5E7C and 17C mAbs. On the other hand, phages presenting P13 peptide significantly bound to the 5E7C monoclonal antibody when compared with anti-gp43 mAb. These data suggest that the peptide sequence expressed by P13 seemed to be the most promising candidate to be a mimotope for anti-gp75 mAb and was chosen for more detailed analysis.

P2 Synthetic Peptide Construction and Analyses of Antigenic and Structural Parameters

Considering that gp75 immunopurified with 5E7C showed phosphatase activity in vitro [17], the P13 cyclic peptide (CHSSLLNPC) selected by phage display against 5E7C was aligned and analyzed with phosphatases sequences deposited in the database of *P. brasiliensis* genome project (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html) (data not shown). Besides, aiming to improve the antigenic potential of the peptide selected sequence, five amino acids similar to the phosphatase sequence showing greater homology were added to the N-terminal and C-terminal, flanking the initial sequence of nine amino acids. Using this approach, two sequences of peptides were obtained: NANEAHSSLLNNALSI (P2) and DEHSRSLLNPRQETW (P5). Figure 2a shows graphic analyses of the antigenic and structural parameters of the P2 and P5 sequences. As observed, P2 peptide sequence presented highest antigenic indexes and hydrophilicity profiles and therefore was selected to be synthesized and used in subsequent experiments.

By ELISA, P2 peptide was recognized by 5E7C mAb mainly at the lower dilutions of the fluid ascitic containing anti-gp75 mAb. These data are strong evidence that P2 synthetic peptide may represent a mimotope of gp75.

Evaluation of Antigenic Potential of P2

Humoral immune response induced by P2 synthetic peptide was evaluated by subcutaneous immunization of BALB/c mice. After three immunizations,
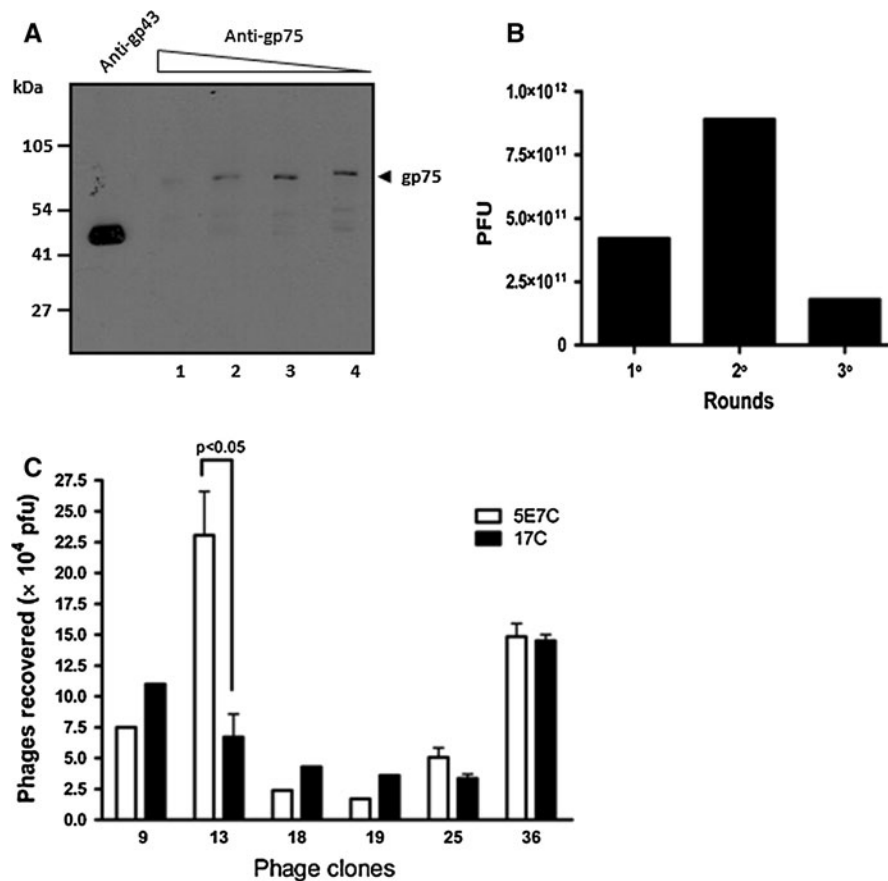
Fig. 1 Selection of the gp75 mimotope. **a** Immunoblotting showing the specific recognition of anti-gp43 and anti-gp75 mAbs against exoantigen proteins. The purified anti-gp43 mAb was used at 1:50 dilution. The anti-gp75 mAb was diluted at 1:400 (*lane 1*), 1:200 (*lane 2*), 1:100 (*lane 3*) or 1:50 (*lane 4*). **b** Monitoring of specific phages to anti-gp75 mAb during three rounds of biopanning. **c** Binding assay showing that phage displaying P13 is a putative mimotope to anti-gp75 mAb.

Phages presenting P9, P13, P18, P19, P25 and P36 peptides were incubated with 5E7C or 17C mAbs (negative control). Phages that bound to those antibodies were recovered through infection in *E. coli* ER2738. After incubation in LB IPTG/Xgal medium, they were quantified. It is observed that only the P13 phage clone specifically bound to mAb 5E7C when compared with anti-gp43 mAb ($P < 0.05$). Statistical analysis were performed with Student's *t* test ($P < 0.05$)

Table 1 Amino acid sequences of peptide inserts identified with the anti-gp75 antibody

Phage clones	Peptide sequences
P9	CHMRNTIHC
P13	CHSSLLNPC
P18	CNSKLHAFC
P19	CYNPRLHIC
P25	CMEPKSRYC
P36	CSNRMATTC

reactivity of the mice sera was detected by ELISA. Serial dilutions of a pool of sera from mice after the last immunization were employed, and the recognition

of P2 by polyclonal sera was observed (Fig. 3a). Sera from control mice injected only with adjuvant did not react with P2 synthetic peptide.

Anti-P2 polyclonal antibodies were recognized by immunoblotting a band of 75 kDa in *P. brasiliensis* exoantigen (Fig. 3b). This result is further evidence that P2 synthetic peptide presented characteristics of a mimotope of gp75.

Recognition of P2 Synthetic Peptide by PCM Patients' Sera

Given that previous works showed that infected mice sera and PCM patients' sera react with a band of 75 kDa
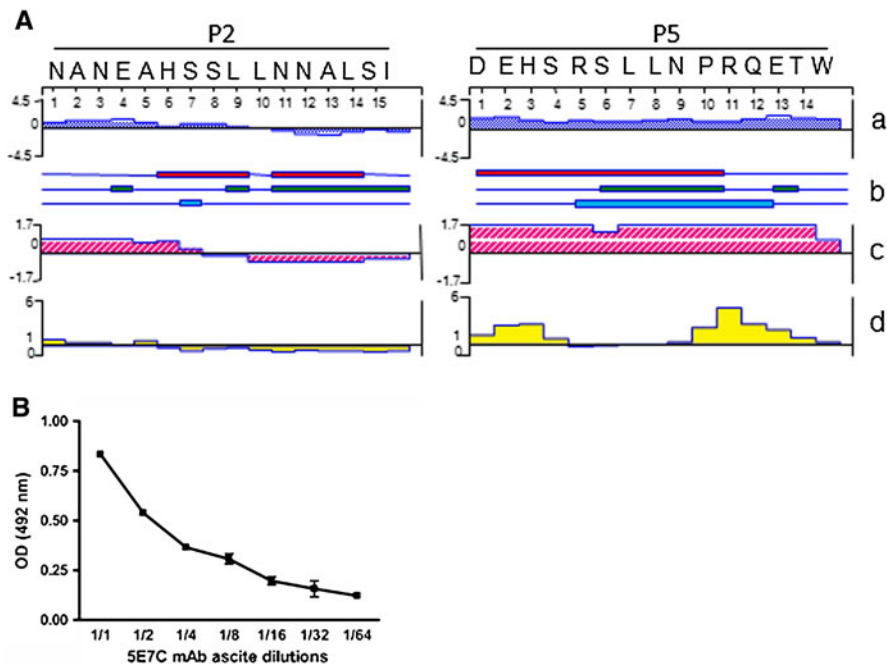
Fig. 2 Analysis of antigenicity, structure and specificity of the synthetic peptides obtained by phage display. **a** Analysis of P2 or P5 sequences. *a* Kyte-Doolittle hydrophilicity plot; *b* the *red box* detaches the Eisenberg alpha amphipathic regions, the *green* correspond to Eisenberg beta amphipathic and the *blue* shows Karplus-Schulz rexible regions; *c* Jameson-Wolff antigenic index; and *d* Emini surface probability plot. Positive values correspond to the hydrophilic structure. **b** The reactivity of 5E7C to P2 peptide was evaluated by ELISA. The ascitic liquid containing mAb was submitted to serial dilution starting at 1:50. (Color figure online)

by Western blot assay [26, 27], and considering the hypothesis that P2 peptide may represent a mimotope of gp75, we asked whether P2 peptide could be recognized by PCM patients' sera. In this proposal, ELISA was developed using P2 as antigen. Figure 4 shows that there was recognition of P2 peptide by sera from PCM untreated individuals. On the other hand, there was no reaction with sera from normal individuals (NHS) when compared with treated patients ($P < 0.005$). The cutoff was estimated by the ROC curve, and the value determined by this method was 0.4233. The sensitivity was 100% and specificity 94.59%.

When sera from treated PCM patients were tested, no significant recognition to P2 peptide was determined, considering the established cutoff. The mean reaction of treated group was lower than untreated group ($P < 0.005$). Sera from patients co-infected with HIV and PCM also did not recognize the peptide, probably due to the impaired immune response presented by these individuals. Nevertheless, one of the sera tested showed high reactivity to P2 peptide with an average OD of 1.729.

To verify the recognition of P2 by sera from patients infected with other mycosis, we tested sera from patients with candidemia, histoplasmosis, aspergillosis or co-infected with HIV and histoplasmosis. Most sera were below the cutoff line; however, eight sera from patients with histoplasmosis and four sera from patients with candidemia showed cross-reaction against P2, suggesting that epitope P2 may be shared by proteins from other fungi.

Discussion

The diagnosis of PCM is considered technically simple, and techniques are basically conventional methodology such as the identification of yeast cells by histopathology, isolating of the fungus by culture or by detection of antibody in clinical samples [28]. However, none of the methods presently used is sufficiently fast, cheap, sensitive or specific.

The gold standard of PCM diagnosis is the demonstration of the parasite with morphological
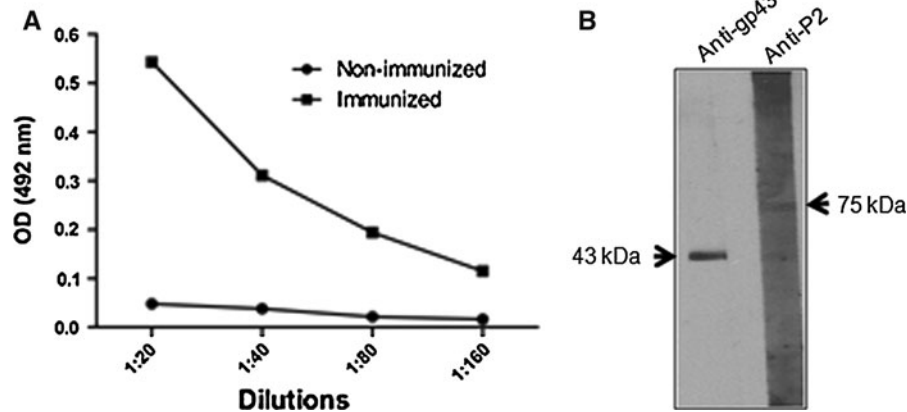
Fig. 3 Antigenic potential of P2. **a** Five BALB/c mice were immunized with P2 peptide emulsified in adjuvant. Fifteen days after last immunization, ELISA was performed to verify the production of polyclonal antibodies by animals. The sera of immunized mice reacted with P2, showing antigenic potential of synthetic peptide. **b** Recognition of gp75 by anti-P2 polyclonal antibodies. Nitrocellulose membrane with exoantigen proteins immobilized on the surface was tested with anti-P2 polyclonal antibodies. The band with molecular weight of 75 kDa was recognized by sera containing anti-P2 antibodies. Anti-gp43 were used as reaction control
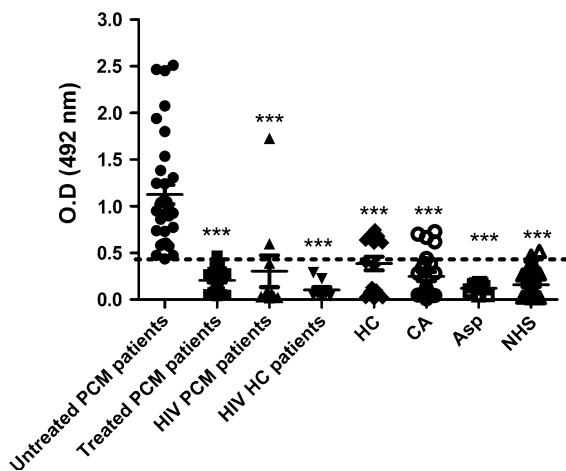
Fig. 4 Recognition of P2 synthetic peptide by serum of PCM patients. Sera of untreated PCM patients ($n = 33$), treated PCM patients ($n = 31$), patients co-infected with HIV and PCM ($n = 10$), patients co-infected with HIV and HC ($n = 10$), aspergillosis (Asp, $n = 8$), histoplasmosis (HC, $n = 17$), candidemia (CA, $n = 24$) and human normal serum (NHS, $n = 37$) were tested to verify the reactivity against P2 peptide. Untreated PCM group significantly recognized P2 as compared with other groups ($P < 0.005$). ANOVA was followed by the Tukey–Kramer test

characteristics of yeast cells of *P. brasiliensis* in clinical specimens [29]. Nevertheless, the direct mycological diagnosis demands an experienced examiner and depends both on the sample quality and on the reasonable amount of fungi in the preparation. Another disadvantage is the time of fungus growth, which is often slow. Therefore, to shorten the time needed and diminish the material required to perform these techniques, serological tests have been developed to detect both antibodies [30, 31] and antigens [32–34]. Moreover, serological tests are important tools either when other procedures are not available or for post-therapy follow-up [9, 35].

Immunodiffusion (ID) is the most common method employed for being simple, inexpensive and useful to monitor the disease treatment. However, its very low sensitivity leads to false-negative results and may not exclude the possibility of PCM. Sensitive tests, like ELISA and Western blot, increase the sensitivity of immunological methods for diagnosis [36], but their specificity depends on the nature of antigen used. Specifically in PCM, most serologic diagnostic assays use crude antigenic preparations that are not completely standardized. This fact is possibly responsible for cross-reactions with other infections, mainly histoplasmosis and Jorge Lobo's disease [37].

Production of good antigenic preparations has improved specificity and sensitivity in serological tests. Thus, some purified antigens from *P. brasiliensis* were characterized and used for antibody detection. Among those antigens, gp43 is the most important component studied [38]. The antigen is recognized by virtually 100% of PCM patients' sera [26], but its carbohydrate composition contains epitopes recognized by heterologous sera, mainly by histoplasmosis

[6]. Gp43 is widely secreted by the fungus in liquid medium, but its purification depends on specific mAb [20] or HPLC systems [39], limiting its production to referral centers and/or research.

Importantly, the application of new methods for obtaining antigens able to improve serological diagnosis of PCM, such as production of synthetic peptides and recombinant proteins, has been rare. Herein, a new approach was employed to prepare synthetic peptides useful for standardization of serological tests for PCM. A phage display library was used to select phage clones that specifically bound to anti-gp75 mAb. Resulting phages were analyzed, and promising candidates were tested by binding assays. Phage displaying a peptide, here named P13, significantly bound to the 5E7C monoclonal antibody (Fig. 1c). Its sequence was aligned with sequences of phosphatases contained in the *P. brasiliensis* genome databases in order to improve the antigenic potential of the P13 peptide sequence. Five amino acids corresponding to the phosphatase sequence most homologous in portions N and C-terminals were added flanking the initial sequence of nine amino acids, and the peptide obtained was called P2. Our results showed that there was a significant recognition of P2 by untreated PCM patients' sera (acute or chronic forms) when compared with control NHS (Fig. 4). It must be stressed that so far, no synthetic peptide has been used for serological diagnosis of PCM. P2 may be the first with those characteristics and thus be considered a promising alternative to standardize serology for this endemic mycosis in Latin America.

This work points the relevant advances and applications of a synthetic peptide (P2) on immunodiagnosis of PCM. The chemical imitation of antigens offers possibilities in the diagnosis of parasitic infections since this strategy is cheaper, simpler, reproducible, useful for large scale testing and, in most cases, specific and sensitive. Another advantage would be a better standardization of tests among laboratories. Thus, the same antigen could be used in all tests to avoid possible variations in protein production of exoantigen, inherent to the fungus and the different culture media and growth conditions. Still, synthetic peptides can be linked to beads to make a rapid test with latex. This specific new approach has been studied in our laboratory with promising results.

Currently, studies using large sample of patients' serum have been conducted to determine sensitivity and specificity of serological methods using P2 as antigen. These tests are important and fundamental for evaluation of its applicability on serodiagnosis of PCM. Probably, a little engineering in P2 peptide may be employed to improve its recognition by PCM patients' sera and/or virtually eliminate some cross-reactions with sera from patients with other deep mycoses. Moreover, the approach applied in this work can be used to obtain different peptides applicable not only to diagnosis, but also for immunization and/or treatment of PCM.

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