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Differential *PbP27* expression in the yeast and mycelial forms of the *Paracoccidioides brasiliensis* species complex

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ABSTRACT

p27 is an antigenic protein produced by *Paracoccidioides brasiliensis*, the etiologic agent of paracoccidioidomycosis (PCM). Despite its unknown function, it has been suggested as a putative virulence factor, proposed as a suitable target for the design of diagnostic tools and vaccines, and considered as an enhancer in antifungal treatment of PCM. We evaluated sequence polymorphisms of *PbP27* gene sequence among isolates, finding some polymorphisms associated with the isolates' phylogenetic origin. In order to determine if there was a differential expression pattern between morphological states and among isolates, we also evaluated *PbP27* expression, at transcriptional and translational levels, in mycelia and yeast cultures in 14 isolates belonging to the *P. brasiliensis* species complex (S1, PS2, PS3, and "*Pb01-like*", proposed to be named *Paracoccidioides lutzii*) by two techniques, real time RT-PCR (RT-qPCR) and protein dot blot. For the latter, four protein extracts from different cell localizations (SDS or β-mercaptoethanol, cytoplasmic and extracellular proteins) were analyzed for each isolate. p27 was present in the four extracts evaluated, mainly in the SDS extract, corresponding to an extract containing proteins loosely attached to the cell wall. This information correlates with immunohistochemical analysis, where positive staining of the yeasts' cell wall was observed. We found that p27 was present in all isolates, mainly in the yeast form. This pattern was corroborated by RT-qPCR results, with higher expression levels found in the yeast form for most of the isolates. The results provide new insights into the expression patterns of this protein, and further characterize it in view of potential uses as a diagnostic and/or therapeutic tool.

1. Introduction

Paracoccidioidomycosis (PCM) is one of the most important systemic mycoses in Latin America, with a restricted geographic distribution ranging from Mexico to Argentina, excluding some countries as Nicaragua, Chile, Guyana and the Caribbean islands (Brummer et al., 1993; Restrepo et al., 2001). Usually this infection is asymptomatic, affecting males and females at the same rate (Brummer et al., 1993). However, the clinical form is in 90% of the cases a chronic disease with higher incidence in males, characterized by primary lung compromise that can disseminate to mucosae, skin and other organs. The remaining 10% of the cases, usually described in children and young persons, are characterized by an acute/subacute form with compromise of the reticuloendothelial system (Borges-Walmsley et al., 2002). In absence of treatment, this disease is mostly fatal (Borges-Walmsley et al., 2002; Brummer et al., 1993).

The etiologic agent of this mycosis is *Paracoccidioides brasiliensis* sensu lato (s.l.), a dimorphic fungal pathogen that grows as a mold at temperatures below 26 °C, whereas at temperatures between 35 °C and 37 °C it grows as multibudding yeasts, the pathogenic form of this fungus (Borges-Walmsley et al., 2002). High phenotypic diversity among *P. brasiliensis* s.l. isolates has been reported in morphology, rate of growth, and virulence (Borba Cde et al., 2008; Burger et al., 1996; Carvalho et al., 2005; Molinari-Madlum et al., 1999), and recent phylogenetic studies have revealed that this species actually contains at least four cryptic phylogenetic species: two paraphyletic species (S1 [Species 1] and PS2 [Phylogenetic Species 2]), and two monophyletic species (PS3 [Phylogenetic Species 3] and the "*Pb01-like*" species for which the name *Paracoccidioides lutzii* sp. nov. has been proposed) (Matute et al., 2006; Teixeira et al., 2009).

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A number of studies in *P. brasiliensis* s.l. have focused on the identification of specific antigenic proteins that can be used for the serological diagnosis of PCM, for follow up of patients in response to therapy, and for possible development of immunization alternatives. In this way, the immunodominant antigen gp43, the first one described (Puccia et al., 1986), has been found useful for diagnosis (Puccia and Travassos, 1991) and to protect against experimental infection in mice (Marques et al., 2006; Pinto et al., 2000; Taborda et al., 1998). Other antigens also assayed for diagnosis, follow up of patients and/or immunization include gp70 antigen (da Silva et al., 2004; de Mattos Grosso et al., 2003) and the heat shock protein 87-kDa antigen (Díez, 2004; Gomez et al., 1998, 1997).

Another such antigen is a 27-kDa protein (p27), obtained from a *P. brasiliensis* cDNA library (McEwen et al., 1996), with potential use for diagnosis due to a high sensitivity and specificity shown (Diez et al., 2003; Fernandes et al., 2011a; Ortiz et al., 1998, 1996). In addition, it has been shown to promote protective immunity in BALB/c mice against infection with *P. brasiliensis* yeast forms (Fernandes et al., 2011b; Reis et al., 2008), enhancing the PCM antifungal therapy (Fernandes et al., 2011b), and has been suggested as a putative virulence factor (Matute et al., 2008; Ortiz et al., 1996; Reis et al., 2008). Nonetheless, the function of this protein remains unknown.

A common approach for recognizing putative virulence factors in dimorphic fungi is to establish a correlation between increased protein expression and the pathogenic form of the fungus (Rappleye and Goldman, 2006), since it has been shown that the dimorphic ability of these fungi is crucial for their pathogenicity (Nunes et al., 2005; Rappleye and Goldman, 2006), and that those isolates unable to transform into yeast are not virulent (Borges-Walmsley et al., 2002). In this study, *PbP27* expression was examined at the transcriptional and translational levels in the mycelia and yeast forms of different isolates of the species complex of *P. brasiliensis* in order to detect potential differential expression patterns. We also characterized the nucleotide polymorphisms among different isolates.

2. Materials and methods

2.1. Strains and culture conditions

Fourteen isolates belonging to the four cryptic phylogenetic species previously described (S1, PS2, PS3, and "*Pb01-like*" cluster) (Matute et al., 2006; Teixeira et al., 2009) were included in this study (Table 1). For maintenance, the mycelial form was grown in the modified synthetic McVeigh and Morton medium (Restrepo and Jimenez, 1980) at room temperature (18–23 °C). To obtain the yeast form, mycelia was grown in anaerobic conditions in Sabouraud agar supplemented with 0.2% asparagine and 1% thiamine at 37 °C and subcultured every 4–5 days until a complete reversion was observed. For experiments, all isolates were cultured in Brain Heart Infusion broth supplemented with 0.2% asparagine and 1% glucose, and incubated in constant agitation at 20 °C for 8–10 days and at 37 °C for 4–6 days, for mycelial and yeast forms respectively. Two independent cultures were grown for each form of each isolate in study. Aliquots of each culture were used for RNA, DNA and protein isolation.

Table 1
Isolates evaluated in this study.

Isolate[a]	Country	Source	Species
A1 (A1)	Argentina	Acute PCM	S1
Pb339 (B18)	Brazil	Chronic PCM	S1
T4B14 (B3)	Brazil	Armadillo	S1
Pb18 (B17)	Brazil	Chronic PCM	S1
T15LN1 (B10)	Brazil	Armadillo	S1
BT84 (B15)	Brazil	PCM	PS2
T10B1 (B7)	Brazil	Armadillo	PS2
Uberlandia (B13)	Brazil	Dog Food	PS2
Pb2 (V2)	Venezuela	Chronic PCM	PS2
ATCC 76533 (C13)	Colombia	Chronic PCM	PS3
ATCC 60855 (C4)	Colombia	Chronic PCM	PS3
P204 (C7)	Colombia	Chronic PCM	PS3
P206 (C17)	Colombia	Chronic PCM	PS3
Pb01 (–)	Brazil	Acute PCM	*"Pb01-like"*

[a] Isolates were provided by the Corporación para Investigaciones Biológicas – CIB collection. Name in brackets is according to the nomenclature given by Matute et al. (2006).

2.2. Nucleic acids isolation

RNA isolation was performed as previously described (Garcia et al., 2010). Briefly, cells were harvested, disrupted by grinding in liquid nitrogen and mixed with Qiazol reagent (Qiagen, Duesseldorf, Germany) for RNA extraction according to the supplier's instructions. In order to verify the RNA integrity, samples were electrophoresed in 1.2% denaturing formaldehyde agarose gels and visualized with UV light. The presence of intact 28S and 18S rRNA bands was used as a criterion to verify that there was no significant degradation.

DNA was removed by Amplification Grade DNase I treatment (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's procedure. The absence of DNA contamination after the RNase-free DNase treatment was verified by PCR amplification of the β-tubulin gene using oligonucleotides BTubE2F: 5′-AGATGTTC GATCCTAAGAACATGA-3′ and BTubE2R: 5′-ATAGCTGAGCAGGTAA GGTAACG-3′.

For DNA preparation, cells were mixed with lysis buffer (1 mM EDTA, 10 mM TrisHCl [pH 8.0], 1% Sodium Dodecyl Sulfate (SDS), 2% Triton X100, 100 mM NaCl) and disrupted using glass beads or maceration of frozen cells for yeast and mycelia respectively (Morais et al., 2000; van Burik et al., 1998). Extraction was carried out with phenol chloroform-isoamyl alcohol (25:24:1) as previously described (Sambrook and Russell, 2001). RNA was removed by RNase I treatment at 37 °C for 30 min. DNA integrity was examined by electrophoresis on 1% agarose gels.

RNA and DNA quantification and additional quality evaluation were carried out using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA).

2.3. PCR conditions and sequencing

The sequence of the *PbP27* gene from the Pb18 isolate, obtained from the Broad Institute *P. brasiliensis* Database (Locus PABG_07332.1), was used to design the following primers for DNA sequencing: P27F, 5′-GACGAGCTGAAAACTGTTGT-3′ and P27R, 5′-CTAGTTGTGGAAGACAGCG-3′. Amplification reactions were composed of 1x Taq Buffer (100 mM Tris–HCl pH 8.5; 500 mM KCl and 1% (v/v) Triton X-100) (Tucantaq, Corpogen, Bogotá, Colombia), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer, 1 U of Taq Polymerase (TucanTaq DNA Polimerasa, Corpogen, Bogotá, Colombia) and 0.4 μg of DNA. A reaction without DNA was included as a negative control to rule out possible contamination. PCR amplifications were performed on a MyCycler IQ (Bio-Rad, Hercules, CA, USA) with an initial denaturing cycle at 94 °C for 5 min, followed by 35 cycles of 94 °C (30 s), 58 °C (30 s) and 72 °C (30 s), with a final cycle at 72 °C for 5 min. PCR products were electrophoresed on 1% agarose gels visualized by staining with ethidium bromide and sequenced using the BigDye™ Terminator sequencing chemistry on an ABI 3730Xl DNA sequencer (Applied Biosystems). Sequence assembly and editing was performed manually on CLC Genomics Workbench (http://www.clcbio.com).

Sequences are available on request. Base substitutions were confirmed by sequencing both strands. Sequence alignments were performed using MUSCLE software (Edgar, 2004).

2.4. cDNA synthesis and real time PCR (RT-PCR)

Equal amounts of DNA-free total RNA (0.3 μg) of each sample and its duplicate were reverse transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo(dT) primers. Real time PCR amplification assays were carried out with an iQ™5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in 25 μl reactions containing 0.25 μM of each primer, 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 2 μl of cDNA. Each sample was assayed in triplicate. The thermocycling conditions comprised an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 56.5 °C for 30 s and 72 °C for 45 s. Finally, to verify the specificity of the reaction, a melting curve analysis was performed by monitoring the decrease in fluorescence as the amplification products were heated from 55 °C to 95 °C at 0.5 °C/10 s.

Relative quantification was performed using the method described by Muller and coworkers (Muller et al., 2002) to evaluate *PbP27* gene expression, with β-tubulin being used as the normalizer gene (Goldman et al., 2003). This method takes the different efficiencies of PCR amplification for the target (*PbP27*) and the reference (*β-tubulin*) into consideration and transforms the logarithmic scaled raw data unit Cycle Threshold (Ct, which is the number of cycles required to detect a fluorescence signal over a settled threshold) into the linear unit of normalized expressions (Simon, 2003). Primers used were BTubE2F (5′-AGATGTTCGATC CTAAGAACATGA-3′) and BTubE2R (5′-ATAGCTGAGCAGGTAAGG TAACG-3′) for normalizer amplification, and P27-RT F (5′-CCTCGTGATCCATGTTGACCA-3′) and P27-RT R (5′-TGTGCCCAAA TTGGCTGACT-3′) for target gene. For the latter, primers were corroborated to anneal within conserved regions of the gene, first with the gene sequences already available from isolates Pb18, Pb01, Pb3 (*P. brasiliensis* Sequencing Project, Broad Institute of Harvard and MIT http://www.broadinstitute.org/) and Pb339 (GenBank: U41503.1) and subsequently with the gene sequencing results for the 14 isolates in this study. For data analysis, Q-Gene software was used to calculate mean normalized expression (Simon, 2003), and a *t*-test was performed to determine if there were differences between the fungal forms, with a significance level of 95%.

2.5. Preparation of proteins

Protein extracts were obtained following the method previously described (Gil et al., 1996; Gonzalez et al., 2005; Penalver et al., 1996) with some modifications. Briefly, cell cultures were centrifuged, washed with phosphate buffered saline and mixed with lysis buffer (100 mM Tris–HCl pH 7.4, containing 10% (V/V) protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA)). Initial supernatant fluid, which corresponds to cell free extract, was recovered and dialyzed against distilled water at 4 °C and then concentrated by assisted evaporation to obtain the extracellular extract. Yeast cells were mechanically disrupted by shaking in a vortex mixer using glass beads, and mycelia were disrupted by maceration in liquid nitrogen (Morais et al., 2000; van Burik et al., 1998). Cell breakage was assessed by microscopic examination.

Cytoplasmic extract was obtained after sedimentation of debris containing proteins from membranes and cell walls (9200 g for 30 min at 4 °C) and recovery of the supernatant. The pellet was washed three times with chilled distilled water, resuspended in 10 mM phosphate buffer (pH 7.4) containing 1% (v/v) β-mercaptoethanol (βME) plus protease inhibitor cocktail as described above and incubated at 37 °C for 30 min in a rotatory shaker. Thereafter, extract containing cell walls components were sedimented and the supernatant fluid was recovered, dialyzed against chilled distilled water at 4 °C and concentrated by assisted evaporation (βME extract). βME-extract was washed three times with chilled distilled water, resuspended in a 2% SDS solution and boiled for 5 min. Subsequently, preparations were centrifuged at 9200 g for 15 min. Proteins present in the supernatant were precipitated with 75% (v/v) ethanol at 4 °C for 16 h, sedimented (9200 g for 35 min) and resuspended in distilled water (SDS extract) (Gil et al., 1996; Penalver et al., 1996). Total protein concentration in the different samples was determined by the method of Bradford (Bradford, 1976).

2.6. SDS PAGE and Western blot analysis

Assays in 12% SDS polyacrylamide gel electrophoresis (SDS–PAGE) were performed as previously described (Laemmli, 1970) in a MiniProtean Tetra Cell system (Bio-Rad, Hercules, CA, USA) using protein extract samples in concentrations from 50 to 500 ng in each line and a PageRuler Prestained Protein Ladder as molecular weight standard (Fermentas Inc., Glen Burnie, MD USA). Cytoplasm protein extract from isolate Pb339 was selected as a positive control due to its demonstrated capacity to produce antigenic proteins, especially gp43 (Camargo et al., 1998, 2003). Cytoplasmic and debris control extracts from human macrophages cell line (MH-S) and Brain Heart Infusion (BHI) broth extracts were added as negative controls. Gels were stained with Coomasie brilliant blue and duplicates were transferred to nitrocellulose membranes, using a Trans-Blot SD Semi Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA), following the manufacturer's recommendations.

Thereafter, membranes were blocked with 5% skin milk plus 1% BSA in a PBS solution containing 0.05% (v/v) Tween 20 (PBST) for 2 h at room temperature, washed three times with PBST and then incubated for 1 h at room temperature with BJ4 (an anti p27 monoclonal antibody obtained by collecting supernatant from subcloned hybridomas (Díez, 2004)) diluted 1:1000 in PBST 5% skin milk plus 1% BSA. Following this, membranes were washed three times with PBST and incubated with Peroxidase-conjugated AffiniPure Goat Anti-mouse IgG antibodies (1:1000 dilution in PBST 5% skin milk plus 1% BSA) (Jackson ImmunoResearch Laboratories, Inc) for 1 h at room temperature. Blots were washed three times with PBST and then immersed in a fresh mixture of 4-chloro-1-naphthol (4C1N) (Sigma–Aldrich, St. Louis, MO, USA) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma–Aldrich, St. Louis, MO, USA) dissolved in ethanol; both suspensions were mixed and H_2O_2 was added. The reaction was stopped with distilled water.

2.7. Dot blot analysis

Five microliters of each protein extract at 50 ng/μl were applied to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) in duplicate. Subsequent procedure was performed as described above for Western blot.

Image analysis was performed with the Gel Doc XR + 2.0.1 System (Bio-Rad, Hercules, CA, USA) using the colorimetric application with white epi illumination and a standard filter as an emission filter. To determine spots' intensities, the relative volume quantity was determined using the positive control (Pb339 cytoplasm protein extract) as the reference volume. A replicate of this experiment was done.

2.8. Immunohistochemical analysis

Six skin and oral mucosa biopsy samples from 3 Colombian patients with PCM (diagnosed and confirmed by silver staining and

culture) were embedded in Optimal Cutting Temperature compound (OCT), stored in liquid nitrogen, and subsequently sectioned in a cryostat (Figocut 2700, Reichert-Jung Nussoloch, Germany). Thick slices were cut and collected onto Super Frost microscope slides (Labcraft, Houston, Tx, USA) previously coated with 20 µl of 0.1% (v/v) poly-L-lysine (Sigma–Aldrich, St. Louis, MO, USA). Negative controls consisting of two lung biopsies from patients with histoplasmosis and aspergillosis and two skin biopsies from healthy volunteers were also used.

For indirect immunofluorescence (modified from Van Moorden, 1986) (Van Noorden, 1986), slides were washed with PBS buffer, placed into a moist chamber and blocked with superblock® (Pierce, Rockford, IL) for 1 h at 37 °C. Slides were washed with PBS and incubated with BJ4 monoclonal antibody for 1 h at 37 °C. For this, different dilutions of the monoclonal antibody (1:10–1:100) were used. This solution was replaced after washing with fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG Fc (Jackson Immuno-research Laboratories, USA) and incubated 1 h at 37 °C. After further washes with PBS, slides were mounted in a 50% (v/v) glycerol/PBS buffer and fluorescence was evaluated by fluorescence microscopy. A negative control in which primary antibody was replaced with fresh PBS was also included. Additionally, indirect immunofluorescence with P1B, a monoclonal antibody raised to detect a 87-kDa antigen (Gomez et al., 1998) was done as a control.

3. Results

3.1. PbP27 gene sequence analysis

As an initial step of this analysis, we compared *PbP27* gene sequences belonging to different isolates in order to evaluate polymorphisms. A 620 bp region of the *PbP27* gene was sequenced for the 14 isolates of *P. brasiliensis* and analyzed. This analysis revealed the presence of several single nucleotide polymorphisms in the region analyzed (Table 2). We found 26 nucleotide polymorphisms, resulting in 13 silent substitutions, and 13 non-synonymous substitutions. The latter were categorized according to the amino acid classification proposed by Hanada et al. (2007), leading to five conservative and eight non-conservative substitutions. We did not find any insertions or deletions.

As shown in Fig. 1 (box) and Table 2 (position 45, underlined), there is a clustering of the PS3 isolates. These isolates share a polymorphic site that is exclusive for the group, except for the Pb339 isolate, belonging to the S1 group that also showed this polymorphism. PS2 and S1 isolates grouped together in another cluster.

The sequence of this gene was highly conserved among isolates belonging to cryptic species S1, PS2 and PS3, which exhibited low variation between isolates (Fig. 1). Pb01 showed the most divergent sequence compared to the sequences from the other isolates (23 out of the 28 polymorphisms found, including all the non-synonymous substitutions) (Table 2 and Fig. 1). The *PbP27* sequence of the Pb01 isolate was compared with the available genome sequences (*P. brasiliensis* Sequencing Project, Broad Institute of Harvard and MIT http://www.broadinstitute.org/), corroborating the polymorphisms found.

3.2. Differential p27 gene expression observed by RT-PCR

PbP27 transcript was detected in all evaluated isolates in yeast and mycelia forms. To determine if there were differences in the *PbP27* levels between yeast and mycelia, normalized *PbP27* expression was compared between the two phases (Fig. 2). For most isolates a significant difference between the two phases ($p < 0.05$) was

Table 2
Distribution of polymorphisms on p27 sequence. Non-synonymous polymorphisms are shown in bold and italic. Non-conservative changes are additionally underlined. Isolates T4B14, T15LN1 and Uberlandia exhibit the same sequence, shown here as the consensus sequence.

Position	16	17	24	32	44	45	50	73	96	100	106	118	120	121	124	135	136	160	180	181	183	187	206	207	208	211
Consensus	L	A	N	G	N	R	V	K	R	T	L	P	Y	D	T	Q	K	R	K	P	L	Q	S	S	A	A
76533	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
T10B1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–
BT84	–	–	–	–	–	–	–	–	–	–	L	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
60855	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P206	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–
PB2	–	–	–	–	–	–	–	–	–	–	L	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PB18	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PB339	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P204	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PB01	L	–	***D***	G	***D***	–	***I***	***T***	R	***A***	–	P	Y	***N***	***A***	Q	K	R	***R***	P	L	***G***	***R***	***Q***	***S***	***V***

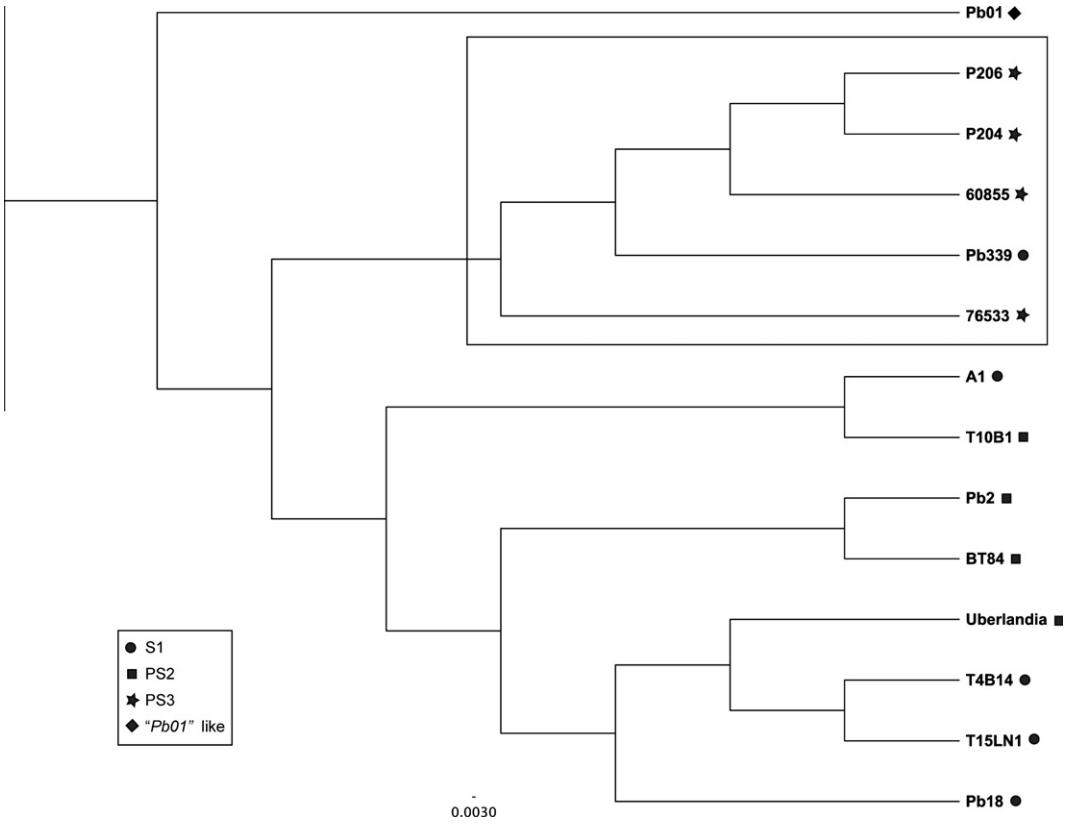

Fig. 1. Dendrogram of the *PbP27* sequences' multiple alignment obtained with the MUSCLE software. Symbols placed next to each isolate name represent the corresponding cryptic species of *P. brasiliensis* (●: S1, ■: PS2, ★: PS3 and ♦: "*Pb01*-like"). Box: PS3 clustering for a particular polymorphic site. Dendrogram was generated using the Figtree tool version 1.3.1.

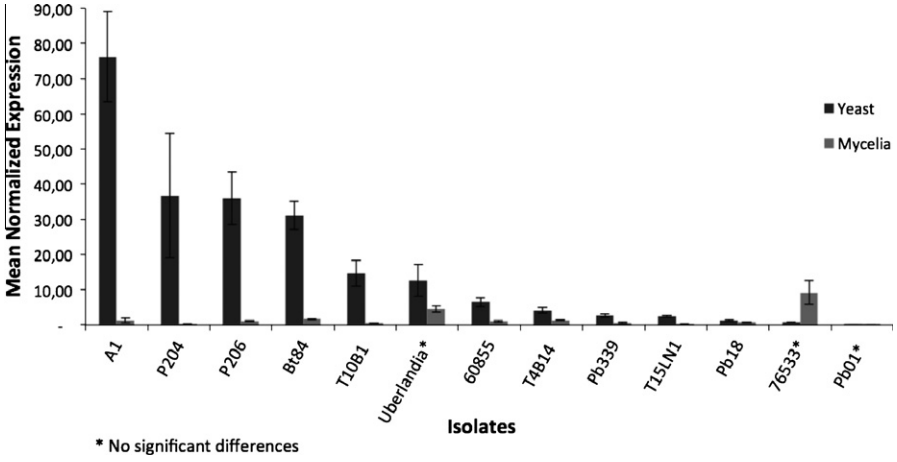

Fig. 2. Mean normalized *PbP27* expression in yeast and mycelial forms. Black bars correspond to yeast form's expression, while gray bars correspond to mycelia. * No significant differences.

detected (Supplementary Table 1), with an up regulation in the yeast form. There were no statistically significant differences between the forms' expression for isolates Uberlandia, Pb01 and 76533.

3.3. p27 is present in the SDS protein extract

Evaluation of some protein extracts by Western blot with the monoclonal monospecific antibodies (BJ4) showed reactivity with a specific single band corresponding to p27, and did not present cross-reactivity with other proteins in the extracts (Fig. 3). Given these results, we proceeded to evaluate quantitatively all isolates' protein samples in a dot blot assay, as this is a simple, cost-effective and straightforward technique.

As revealed by dot blot assay, the p27 protein was primarily found in the cytoplasm and SDS extracts containing cell wall proteins. To a lesser extent, p27 was detected in the extracellular and βME extracts (Fig. 4).

Furthermore, p27 detection was not the same for all isolates, showing different patterns in the protein extracts tested

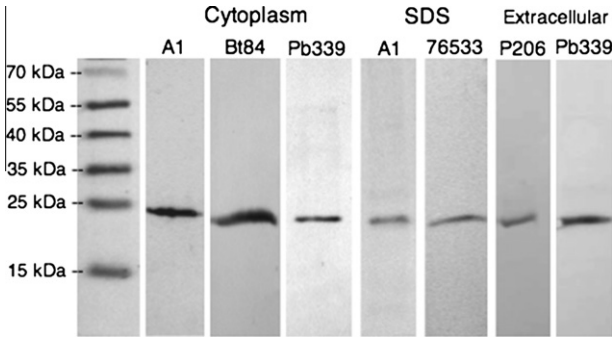

Fig. 3. Examples of p27 protein detection by Western blot in extracellular, cytoplasm and SDS cell wall containing extracts for some isolates. Molecular marker: PageRuler Prestained Protein Ladder weight standards (in kDa). For A1, Bt84 and Pb339 cytoplasmic extracts the concentrations were 400 μg, 500 μg and 300 μg respectively. For A1 and 76533 SDS extracts the concentrations were 50 μg and 100 μg respectively. For P206 and Pb339 extracellular extracts the concentration was 400 μg in both cases.

(Table 3). For the SDS extract, protein p27 was more easily detected in extracts from the yeast forms than in those from the mycelial ones (Fig. 4 and Table 3).

Accordingly, positive and strong staining of the cell wall for the yeast cells with scarce extracellular distribution was observed by immunofluorescence staining in material from biopsy samples (Fig. 5). The optimal dilution of the BJ4 monoclonal antibody was found to be 1:50. Negative control slides were unreactive and indirect immunofluorescence with P1B monoclonal antibodies showed fluorescence in the cell wall together with some cytoplasmic regions of the cells with a generalized granular appearance. Also extracellular labeling was observed with this antibody (data not shown).

4. Discussion

In this study, we gathered information on expression of the *PbP27* gene and its corresponding protein, an antigenic protein of the human pathogen *P. brasiliensis*, and evaluated sequence polymorphisms. We combined real-time RT-PCR and dot blot analysis to examine differential expression in the yeast and mycelial forms of *P. brasiliensis*. These procedures informed us at different levels about protein expression (Beyer et al., 2004). As expected, no relation was found between the gene or protein sequences' polymorphism and expression levels. Future evaluation of the upstream DNA sequences of *PbP27* gene for polymorphisms in the promoter sequences that could be related to differential regulation could provide valuable information to understand the variable expression level profiles observed.

We found by RT-PCR that the *PbP27* gene is expressed in all evaluated isolates (*P. brasiliensis* s.l.), in which a general trend was the higher *PbP27* expression in yeast cells. These findings were in concordance with the results obtained by dot blot in which p27 protein was present in all isolates, mainly detected in the SDS extract with the strongest reactivity detected in the yeast forms. No correlation between a specific cryptic species, isolate source or country of origin and a *PbP27* expression level pattern was

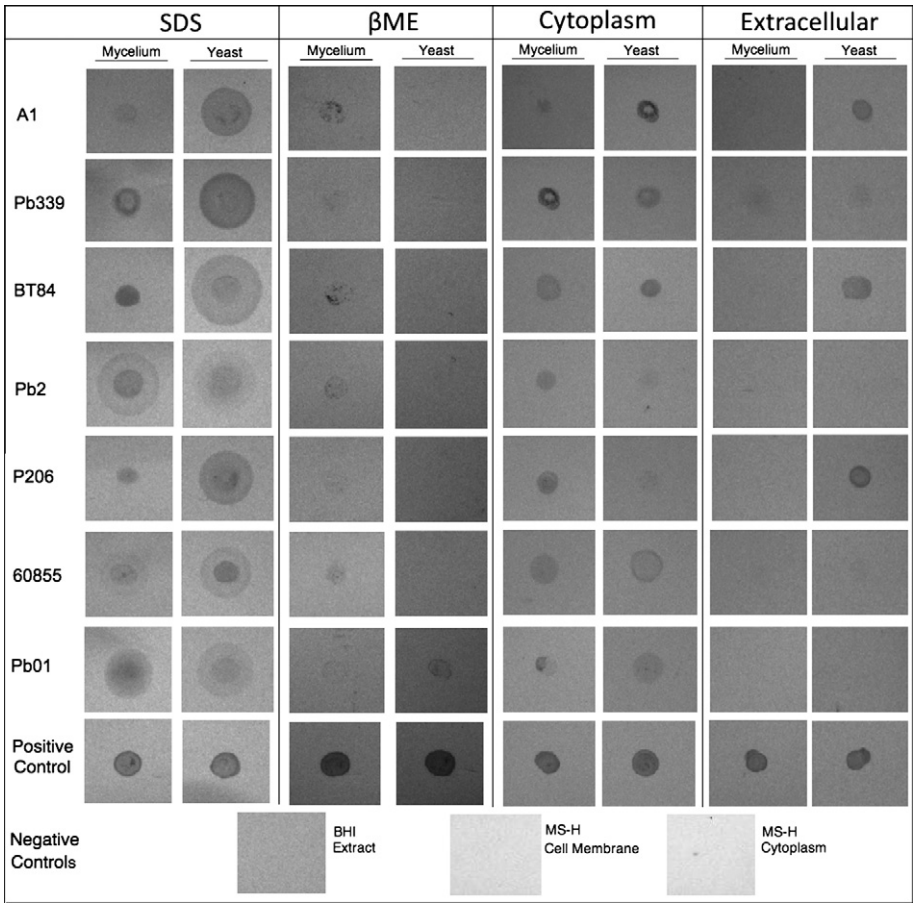

Fig. 4. p27 detection by dot blot assay in the four protein extracts from different *P. brasiliensis* isolates (isolate name is shown on the left). Positive control: Cytoplasmic protein extract from strain Pb339. Negative controls: Brain Heart Infusion broth, cytoplasmic and cell wall extracts from Human macrophages cell line MS-H.

Table 3
p27 detection in the four protein extracts of *P. brasiliensis* isolates by dot blot assay. Values for relative volume quantity were determined using the positive control (Pb339 cytoplasm protein extract) as the reference with a volume quantity value of 3.

	Cell wall SDS		Cell wall βME		Cytoplasmic		Extracellular	
	Yeast	Mycelia	Yeast	Mycelia	Yeast	Mycelia	Yeast	Mycelia
Pb339	2.844	0.55	0	0.278	0.483	0.18	0.11	0.258
Bt84	2.728	0.526	0	0.838	0.34	0.357	0.783	0
A1	2.097	0.724	0	0	0.915	0.064	0.674	0
P206	2.002	0.261	0	0.034	0.199	0.52	0.894	0
Pb2	1.561	1.179	0	0.485	0.199	0.291	0	0
60855	1.266	0.232	0	0.59	0.199	0.378	0	0
76533	0.919	0.617	0.103	0.33	0.273	0.287	0	0
Pb01	0.749	1.485	0.962	0.019	0.199	0.199	0	0
Pb18	0.619	0.136	0	0.468	0.236	0.072	0.428	0
T10B1	0.483	0.589	0	0	0.199	0.116	0.683	0
T15LN1	0.451	0.795	0	0	0.07	0.261	0	0
Uberlandia	0.46	0.561	0	0.69	0.475	0.087	0.355	0
P204	0.33	0.852	0.535	0.144	0	0.052	0.59641	0
T4B14	0.266	0.822	0	0.109	0	0.1644	0.348	0

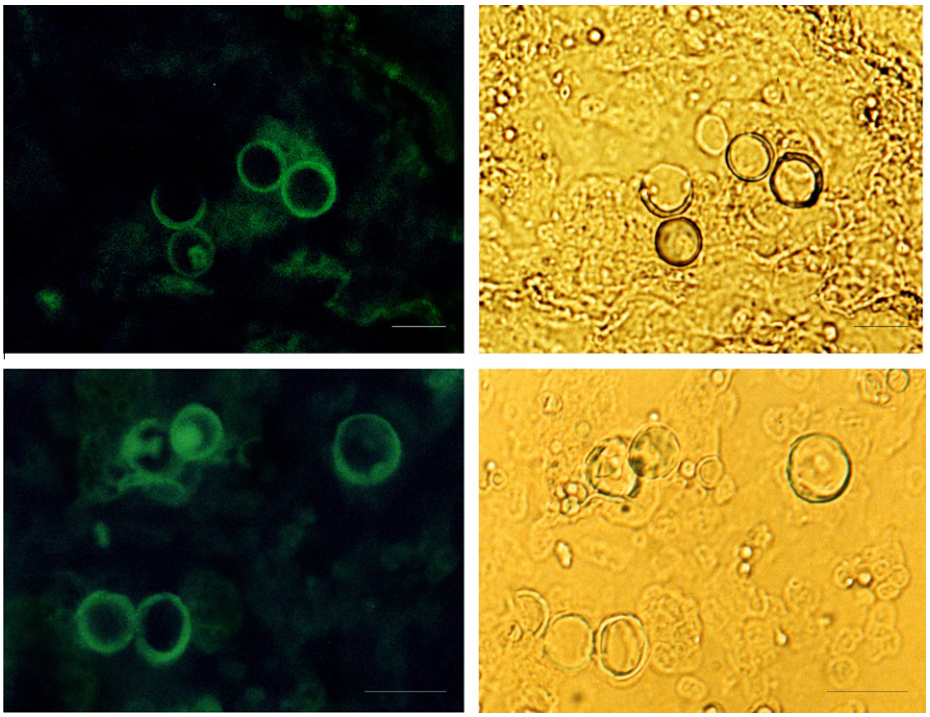

Fig. 5. Immunofluorescence reactivity of *P. brasiliensis* using BJ4 monoclonal antibody. Yeast cell forms in biopsy material from PCM patients. Bars represent 40 μm.

observed, although high variability of expression was seen among isolates.

Differences in antigen expression among isolates and in the same isolate cultured under the same conditions have been reported in *P. brasiliensis* (Berzaghi et al., 2005; Franco et al., 1996). Gp43, the immunodominant antigen of this fungus, has a variable secretion pattern even in clonal cultures (Berzaghi et al., 2005); the amount of gp43 accumulated in the extracellular fluids of the same isolate could also be affected by the incubation time, culture medium, fungal phases as well as multiple sub culturing and after animal passage (Rocha et al., 2009). This variability is also reported for *Candida albicans* and seems to be related to multiple environmental and organism-related factors (Berzaghi et al., 2005). Nonetheless, the mechanisms underlying this antigenic instability remain unknown and require further studies (Berzaghi et al., 2005). For p27, we would like to perform, in the future, additional experiments on p27 expression kinetics, not only in the yeast and mycelia forms of different isolates of the described phylogenetic species but also in the conidia form and during the transition processes, in order to understand its variable expression profile. In the future, it would also be useful to evaluate all protein extracts by Western blot in order to corroborate results obtained here on protein expression differences among isolates.

The gene sequence analysis of this protein in the different isolates of *P. brasiliensis* revealed that it is a highly conserved protein, showing low levels of variation among species S1, PS2 and PS3 and a divergent sequence belonging to Pb01 isolate. To determine the levels of polymorphism among "*Pb01-like*" isolates more sequences are needed. However, it is known that the "*Pb01-like*" phylogenetic species shows high levels of genomic variation (Carrero et al., 2008; Teixeira et al., 2009). It was proposed that antigenic differences among Brazilian central-western region isolates (where *Pb01-like* isolates are considered to be endemic) could be associated with the phylogenetic divergence of them from most of the

P. brasiliensis isolates (Batista et al., 2010). Moreover, this clade tends to show different responses to the host, and exclusive phenotypic characteristics such as virulence levels, resistance to fungicides and rates of proliferation (Teixeira et al., 2009). It should be important to figure out if *PbP27* sequence differences found in Pb01 have a biological effect in this protein since this isolate, interestingly, displays the lowest *p27* expression level.

Isolate A1 was identified as a good candidate for antigen preparation. This isolate exhibited the highest *PbP27* gene expression level among the isolates tested, and p27 protein was easily detected in protein extracts of this isolate. Isolate Pb339 has been widely used to produce antigenic preparations for PCM serological diagnosis (Berzaghi et al., 2005) and although it showed a moderate *PbP27* expression level, in the protein assay it showed an acceptable detection level.

The procedure for obtaining protein extracts described by Peñalver and coworkers and Gil and coworkers (Gil et al., 1996; Penalver et al., 1996) has been successfully used also by other authors such as Gonzalez et al. in 2005. Using this methodology and determining proteins' presence in the SDS extracts, Gonzalez et al. (2005) demonstrated the presence of two new polypeptides capable of interacting with extracellular matrix proteins on the surface of *P. brasiliensis* yeast cells. In the present study, we found a primary cytoplasmic and cell wall localization of p27 protein consistent with previous reports (Reis et al., 2008). The protein was predominantly found in the SDS cell extract, which corresponds to proteins loosely attached to other cell wall components by non-covalent bonds (Pitarch et al., 2008). This location is expected due to the antigenic nature of p27. Also, immunohistochemical analysis demonstrated that p27 is detectable in skin and oral mucosa biopsy samples containing *P. brasiliensis* yeast cells, with a limited extracellular distribution. Both dot blot and immunohistochemical analysis results confirmed p27 as a cell wall associated protein. Indeed, cell wall proteins are a target of preference for the design of antifungal drugs and vaccines as the cell wall plays an active role in the host-pathogen interaction and has fungal specificity (Pitarch et al., 2008).

In addition, we detected the protein in the extracellular extract of some isolates, suggesting that it can be secreted. On one hand, secreted proteins pass through the cell wall, where they are transiently associated with cell wall-bound moieties and contribute to the total cell wall proteinaceous component (Chaffin et al., 1998). On the other hand, from studies developed in *Candida albicans* it is known that several cell wall proteins, not thought to be secreted, can be found in the cell cultures' supernatants, probably coming from the outer wall layers, and may be released by lysed cells or as a consequence of the processes of synthesis and degradation of the cell wall structure, required for wall expansion during cell growth (Chaffin et al., 1998), as evidenced during the early stages of cell wall regeneration in protoplasts (Pitarch et al., 2006). Moreover, for some proteins, localization (cell wall or secreted proteins) depends upon growth conditions (Chaffin et al., 1998). Considering the results obtained from *in situ* immunolocalization assay, p27 is a cell wall protein, found in the culture supernatants for the reasons mentioned above.

5. Concluding remarks

Antigenic proteins have been widely studied in other fungal pathogens such as *Histoplasma capsulatum* (Deepe and Gibbons, 2001), *Cryptococcus neoformans* (Biondo et al., 2002; Huang et al., 2002), *Coccidioides spp.* (Kirkland et al., 2006; Shubitz et al., 2006) and *C. albicans* (Bromuro et al., 1994). In these fungi, antigenic proteins have been found useful for diagnosis and follow up purposes, and some of them are vaccine candidates due to the protective immunity they elicit through activation of the T-cell response. Protein p27 is one of the major antigenic proteins in *P. brasiliensis* with potential use in PCM diagnosis (Diez et al., 2003; Ortiz et al., 1998, 1996); it has been shown to promote protective immunity in BALB/c mice against infection with *P. brasiliensis* yeast forms (Reis et al., 2008) and has been proposed as a virulence factor (Matute et al., 2008; Ortiz et al., 1996; Reis et al., 2008). The low genetic variability of this protein in non-Pb01 strains together with its confirmed expression in all isolates evaluated, its preferential expression in the yeast forms, and its surface-associated localization reinforce its potential use as a diagnostic tool. Further studies on the expression behavior of p27 during the transition process, expression kinetics and regulation by promoter sequences should allow a more detailed understanding of the variable expression profile observed in this study.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.09.001.

References

Batista Jr., J., De Camargo, Z.P., Fernandes, G.F., Vicentini, A.P., Fontes, C.J.F., Hahn, R.C., 2010. Is the geographical origin of a *Paracoccidioides brasiliensis* isolate important for antigen production for regional diagnosis of paracoccidioidomycosis? Mycoses 53, 176–180.

Berzaghi, R., da Silva, S.H., de Camargo, Z.P., 2005. Variable gp43 secretion by *Paracoccidioides brasiliensis* clones obtained by two different culture methods. J. Clin. Microbiol. 43, 491–493.

Beyer, A., Hollunder, J., Nasheuer, H.P., Wilhelm, T., 2004. Post-transcriptional expression regulation in the yeast *Saccharomyces cerevisiae* on a genomic scale. Mol. Cell. Proteomics 3, 1083–1092.

Biondo, C., Beninati, C., Delfino, D., Oggioni, M., Mancuso, G., Midiri, A., et al., 2002. Identification and cloning of a cryptococcal deacetylase that produces protective immune responses. Infect. Immun. 70, 2383–2391.

Borba Cde, M., Correia, J., Vinhas, E., Martins, A., Alves, B.C., Unkles, S., et al., 2008. Genetic characterization of morphologically variant strains of *Paracoccidioides brasiliensis*. Mem. Inst. Oswaldo Cruz 103, 306–309.

Borges-Walmsley, M.I., Chen, D., Shu, X., Walmsley, A.R., 2002. The pathobiology of *Paracoccidioides brasiliensis*. Trends Microbiol. 10, 80–87.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bromuro, C., Torosantucci, A., Gomez, M.J., Urbani, F., Cassone, A., 1994. Differential release of an immunodominant 65 kDa mannoprotein antigen from yeast and mycelial forms of *Candida albicans*. J. Med. Vet. Mycol. 32, 447–459.

Brummer, E., Castaneda, E., Restrepo, A., 1993. Paracoccidioidomycosis: an update. Clin. Microbiol. Rev. 6, 89–117.

Burger, E., Vaz, C.C., Sano, A., Calich, V.L., Singer-Vermes, L.M., Xidieh, C.F., et al., 1996. *Paracoccidioides brasiliensis* infection in nude mice. studies with isolates differing in virulence and definition of their T cell-dependent and T cell-independent components. Am. J. Trop. Med. Hyg. 55, 391–398.

Camargo, Z.P., Baruzzi, R.G., Maeda, S.M., Floriano, M.C., 1998. Antigenic relationship between Loboa loboi and *Paracoccidioides brasiliensis* as shown by serological methods. Med. Mycol. 36, 413–417.

Camargo, Z.P., Berzaghi, R., Amaral, C.C., Silva, S.H., 2003. Simplified method for producing *Paracoccidioides brasiliensis* exoantigens for use in immunodiffusion tests. Med. Mycol. 41, 539–542.

Carrero, L.L., Nino-Vega, G., Teixeira, M.M., Carvalho, M.J., Soares, C.M., Pereira, M., et al., 2008. New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen. Fungal Genet. Biol. 45, 605–612.

Carvalho, K.C., Ganiko, L., Batista, W.L., Morais, F.V., Marques, E.R., Goldman, G.H., et al., 2005. Virulence of *Paracoccidioides brasiliensis* and gp43 expression in isolates bearing known PbGP43 genotype. Microbes Infect. 7, 55–65.

Chaffin, W.L., Lopez-Ribot, J.L., Casanova, M., Gozalbo, D., Martinez, J.P., 1998. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol. Mol. Biol. Rev. 62, 130–180.

da Silva, S.H., Grosso Dde, M., Lopes, J.D., Colombo, A.L., Blotta, M.H., Queiroz-Telles, F., et al., 2004. Detection of *Paracoccidioides brasiliensis* gp70 circulating antigen and follow-up of patients undergoing antimycotic therapy. J. Clin. Microbiol. 42, 4480–4486.

de Mattos Grosso, D., de Almeida, S.R., Mariano, M., Lopes, J.D., 2003. Characterization of gp70 and anti-gp70 monoclonal antibodies in *Paracoccidioides brasiliensis* pathogenesis. Infect. Immun. 71, 6534–6542.

Deepe Jr., G.S., Gibbons, R., 2001. Protective efficacy of H antigen from *Histoplasma capsulatum* in a murine model of pulmonary histoplasmosis. Infect. Immun. 69, 3128–3134.

Díez, S., 2004. Characterization and Use of Serodiagnostically Important Antigens from Pb. St. John's Institute of Dermatology, The Guy's, King's and St. Thomas' Medical School (KCL), vol. PhD. University of London, London, 2004, pp. 121.

Diez, S., Gomez, B.L., McEwen, J.G., Restrepo, A., Hay, R.J., Hamilton, A.J., 2003. Combined use of *Paracoccidioides brasiliensis* recombinant 27-kilodalton and purified 87-kilodalton antigens in an enzyme-linked immunosorbent assay for serodiagnosis of paracoccidioidomycosis. J. Clin. Microbiol. 41, 1536–1542.

Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinf. 5, 113.

Fernandes, V.C., Coitinho, J.B., Veloso, J.M., Araujo, S.A., Pedroso, E.P., Goes, A.M., 2011a. Combined use of *Paracoccidioides brasiliensis* recombinant rPb27 and rPb40 antigens in an enzyme-linked immunosorbent assay for immunodiagnosis of paracoccidioidomycosis. J. Immunol. Methods 367, 78–84.

Fernandes, V.C., Martins, E.M., Boeloni, J.N., Coitinho, J.B., Serakides, R., Goes, A.M., 2011b. Additive effect of rPb27 immunization and chemotherapy in experimental paracoccidioidomycosis. PLoS ONE 6, e17885.

Franco, M., Bagagli, E., Cunha, M., Chamma, L.G., Fecchio, D., 1996. *Paracoccidioides brasiliensis* antigen batches from the same isolate show immunological and biochemical differences. Mycopathologia 135, 13–19.

Garcia, A.M., Hernandez, O., Aristizabal, B.H., De Souza Bernardes, L.A., Puccia, R., Naranjo, T.W., et al., 2010. Gene expression analysis of *Paracoccidioides brasiliensis* transition from conidium to yeast cell. Med. Mycol. 48, 147–154.

Gil, M.L., Penalver, M.C., Lopez-Ribot, J.L., O'Connor, J.E., Martinez, J.P., 1996. Binding of extracellular matrix proteins to *Aspergillus fumigatus* conidia. Infect. Immun. 64, 5239–5247.

Goldman, G.H., dos Reis Marques, E., Duarte Ribeiro, D.C., de Souza Bernardes, L.A., Quiapin, A.C., Vitorelli, P.M., et al., 2003. Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. Eukaryot. Cell. 2, 34–48.

Gomez, B.L., Figueroa, J.I., Hamilton, A.J., Ortiz, B., Robledo, M.A., Hay, R.J., et al., 1997. Use of monoclonal antibodies in diagnosis of paracoccidioidomycosis: new strategies for detection of circulating antigens. J. Clin. Microbiol. 35, 3278–3283.

Gomez, B.L., Figueroa, J.I., Hamilton, A.J., Diez, S., Rojas, M., Tobon, A.M., et al., 1998. Antigenemia in patients with paracoccidioidomycosis: detection of the 87-kilodalton determinant during and after antifungal therapy. J. Clin. Microbiol. 36, 3309–3316.

Gonzalez, A., Gomez, B.L., Restrepo, A., Hamilton, A.J., Cano, L.E., 2005. Recognition of extracellular matrix proteins by *Paracoccidioides brasiliensis* yeast cells. Med. Mycol. 43, 637–645.

Hanada, K., Shiu, S.H., Li, W.H., 2007. The nonsynonymous/synonymous substitution rate ratio versus the radical/conservative replacement rate ratio in the evolution of mammalian genes. Mol. Biol. Evol. 24, 2235–2241.

Huang, C., Nong, S.H., Mansour, M.K., Specht, C.A., Levitz, S.M., 2002. Purification and characterization of a second immunoreactive mannoprotein from *Cryptococcus neoformans* that stimulates T-Cell responses. Infect. Immun. 70, 5485–5493.

Kirkland, T.N., Raz, E., Datta, S.K., 2006. Molecular and cellular mechanisms of protective immunity to coccidioidomycosis. Vaccine 24, 495–500.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Marques, A.F., da Silva, M.B., Juliano, M.A., Travassos, L.R., Taborda, C.P., 2006. Peptide immunization as an adjuvant to chemotherapy in mice challenged intratracheally with virulent yeast cells of *Paracoccidioides brasiliensis*. Antimicrob. Agents Chemother. 50, 2814–2819.

Matute, D.R., McEwen, J.G., Puccia, R., Montes, B.A., San-Blas, G., Bagagli, E., et al., 2006. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol. Biol. Evol. 23, 65–73.

Matute, D.R., Quesada-Ocampo, L.M., Rauscher, J.T., McEwen, J.G., 2008. Evidence for positive selection in putative virulence factors within the *Paracoccidioides brasiliensis* species complex. PLoS Negl. Trop. Dis. 2, e296.

McEwen, J.G., Ortiz, B.L., Garcia, A.M., Florez, A.M., Botero, S., Restrepo, A., 1996. Molecular cloning, nucleotide sequencing, and characterization of a 27-kDa antigenic protein from *Paracoccidioides brasiliensis*. Fungal Genet. Biol. 20, 125–131.

Molinari-Madlum, E.E., Felipe, M.S., Soares, C.M., 1999. Virulence of *Paracoccidioides brasiliensis* isolates can be correlated to groups defined by random amplified polymorphic DNA analysis. Med. Mycol. 37, 269–276.

Morais, F.V., Barros, T.F., Fukada, M.K., Cisalpino, P.S., Puccia, R., 2000. Polymorphism in the gene coding for the immunodominant antigen gp43 from the pathogenic fungus *Paracoccidioides brasiliensis*. J. Clin. Microbiol. 38, 3960–3966.

Muller, P.Y., Janovjak, H., Miserez, A.R., Dobbie, Z., 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32, 1372–1374, 1376, 1378–1379.

Nunes, L.R., Costa de Oliveira, R., Leite, D.B., da Silva, V.S., dos Reis Marques, E., da Silva Ferreira, M.E., et al., 2005. Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition. Eukaryot. Cell. 4, 2115–2128.

Ortiz, B.L., Garcia, A.M., Restrepo, A., McEwen, J.G., 1996. Immunological characterization of a recombinant 27-kilodalton antigenic protein from *Paracoccidioides brasiliensis*. Clin. Diagn. Lab. Immunol. 3, 239–241.

Ortiz, B.L., Diez, S., Uran, M.E., Rivas, J.M., Romero, M., Caicedo, V., et al., 1998. Use of the 27-kilodalton recombinant protein from *Paracoccidioides brasiliensis* in serodiagnosis of paracoccidioidomycosis. Clin. Diagn. Lab. Immunol. 5, 826–830.

Penalver, M.C., O'Connor, J.E., Martinez, J.P., Gil, M.L., 1996. Binding of human fibronectin to *Aspergillus fumigatus* conidia. Infect. Immun. 64, 1146–1153.

Pinto, A.R., Puccia, R., Diniz, S.N., Franco, M.F., Travassos, L.R., 2000. DNA-based vaccination against murine paracoccidioidomycosis using the gp43 gene from *paracoccidioides brasiliensis*. Vaccine 18, 3050–3058.

Pitarch, A., Nombela, C., Gil, C., 2006. *Candida albicans* biology and pathogenicity: insights from proteomics. Methods Biochem. Anal. 49, 285–330.

Pitarch, A., Nombela, C., Gil, C., 2008. Cell wall fractionation for yeast and fungal proteomics. Methods Mol. Biol. 425, 217–239.

Puccia, R., Travassos, L.R., 1991. 43-kilodalton glycoprotein from *Paracoccidioides brasiliensis*: immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease. J. Clin. Microbiol. 29, 1610–1615.

Puccia, R., Schenkman, S., Gorin, P.A., Travassos, L.R., 1986. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect. Immun. 53, 199–206.

Rappleye, C.A., Goldman, W.E., 2006. Defining virulence genes in the dimorphic fungi. Annu. Rev. Microbiol. 60, 281–303.

Reis, B.S., Fernandes, V.C., Martins, E.M., Serakides, R., Goes, A.M., 2008. Protective immunity induced by rPb27 of *Paracoccidioides brasiliensis*. Vaccine 26, 5461–5469.

Restrepo, A., Jimenez, B.E., 1980. Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined culture medium. J. Clin. Microbiol. 12, 279–281.

Restrepo, A., McEwen, J.G., Castaneda, E., 2001. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Med. Mycol. 39, 233–241.

Rocha, A.A., Malavazi, I., Goldman, G.H., Puccia, R., 2009. Transcription regulation of the Pbgp43 gene by nitrogen in the human pathogen *Paracoccidioides brasiliensis*. Fungal Genet. Biol. 46, 85–93.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shubitz, L.F., Yu, J.J., Hung, C.Y., Kirkland, T.N., Peng, T., Perrill, R., et al., 2006. Improved protection of mice against lethal respiratory infection with *Coccidioides posadasii* using two recombinant antigens expressed as a single protein. Vaccine 24, 5904–5911.

Simon, P., 2003. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19, 1439–1440.

Taborda, C.P., Juliano, M.A., Puccia, R., Franco, M., Travassos, L.R., 1998. Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in BALB/c mice. Infect. Immun. 66, 786–793.

Teixeira, M.M., Theodoro, R.C., de Carvalho, M.J., Fernandes, L., Paes, H.C., Hahn, R.C., et al., 2009. Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus. Mol. Phylogen. Evol. 52, 273–283.

van Burik, J.A., Schreckhise, R.W., White, T.C., Bowden, R.A., Myerson, D., 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med. Mycol. 36, 299–303.

Van Noorden, S., 1986. Tissue preparation and immunostaining techniques for light microscopy. Immunocytochemistry: modern methods and applications, pp. 26–53.