

## PCR WITH *Paracoccidioides brasiliensis* SPECIFIC PRIMERS: POTENTIAL USE IN ECOLOGICAL STUDIES

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### SUMMARY

The precise microenvironment of *Paracoccidioides brasiliensis* has not yet been discovered perhaps because the methods used are not sensitive enough. We applied to this purpose the polymerase chain reaction (PCR) using three sets of specific primers corresponding to two *P. brasiliensis* genes. This fungus as well as several other fungi, were grown and their DNA obtained by mechanical disruption and a phenol chloroform isoamylalcohol-based purification method. The DNA served for a PCR reaction that employed specific primers from two *P. brasiliensis* genes that codify for antigenic proteins, namely, the 27 kDa and the 43 kDa. The lowest detection range for the 27 kDa gene was 3 pg. The amplification for both genes was positive only with DNA from *P. brasiliensis*; additionally, the mRNA for the 27 kDa gene was present only in *P. brasiliensis*, as indicated by the Northern analysis. The standardization of PCR technology permitted the amplification of *P. brasiliensis* DNA in artificially contaminated soils and in tissues of armadillos naturally infected with the fungus. These results indicate that PCR technology could play an important role in the search for *P. brasiliensis*' habitat and could also be used in other ecological studies.

**KEYWORDS:** *P. brasiliensis*; PCR detection; Specific primers; Soil and animal detection.

### INTRODUCTION

*Paracoccidioides brasiliensis* is the dimorphic fungus responsible for paracoccidioidomycosis (PCM), a disease highly prevalent in Latin America<sup>6</sup>. Skin testing studies carried out in areas of endemicity have shown a relative high frequency of reactivity ( $\geq 12\%$ ) in healthy persons. However, only a few of these will develop the disease later on. The absence of epidemic outbreaks and the long periods of latency of the mycosis hinder determination of the source of the primary contact<sup>6</sup>. PCM endemic areas have certain ecological characteristics which suggest that the following factors favor the presence of *P. brasiliensis*: temperatures between 17 to 24 °C, high humidity with annual pluviosities ranging from 500 to 2,500 mm, abundant forests and watercourses, short winters and rainy summers<sup>12</sup>.

The precise microenvironment of *P. brasiliensis* has not yet been discovered, although its habitat is considered to be exogenous to man<sup>24</sup>. The fungus has been rarely isolated from natural sources, such as soils; as an example, it was isolated once from Argentinean soil<sup>22</sup>, twice from the same farm in Venezuela<sup>1</sup>, and once from a coffee growing area in Brazil<sup>27</sup>. Additionally the fungus has been isolated from dog food<sup>11</sup>, bats and penguin excreta<sup>14,15</sup>; nonetheless, numerous attempts to repeat these observations have failed<sup>24</sup>.

In the past, the disease had rarely been informed in animals; more recently, however, the presence of the fungus has been repeatedly reported in armadillos (*Dasypus novemcinctus*). These isolations have been confirmed in different paracoccidioidomycosis endemic areas of Brazil. The armadillos become infected in nature and as such, they are enzootic hosts to *P. brasiliensis*<sup>3,9,21</sup>.

The search for the natural habitat of *P. brasiliensis* using conventional techniques has been difficult. The application of molecular biology procedures, specifically, the polymerase chain reaction (PCR) technique may prove more appropriate to detect this pathogenic fungus, as it has high sensitivity and specificity<sup>16</sup>. Additionally, it can also be applied to detection of environmental microorganisms<sup>17,31</sup>; when used to amplify DNA in soil samples, the procedures may be hindered by the presence of natural contaminants such as polysaccharides and/or humic acids, two powerful PCR inhibitors, which interfere with the reaction<sup>2,4,28</sup>.

An essential step for the development of PCR for *P. brasiliensis* is the designing of specific primers. We have approached this step by means of two genes, one that codifies for the 43 kDa glycoprotein, an immunodominant antigen<sup>8</sup> and the other codifying for the 27 kDa protein, also an antigenic protein. The latter has been the second *P. brasiliensis* gene to be cloned, sequenced and characterized<sup>18,19,23</sup>.

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We report here on the attempt to standardize a PCR technique using primers designed to match two specific sequences of *P. brasiliensis*, for amplification in artificially contaminated soils as well as in the tissues of naturally infected armadillos, captured in an endemic area for paracoccidioidomycosis in Colombia.

## MATERIALS AND METHODS

The different fungi that were studied were grown and their total RNA and genomic DNA extracted and analyzed by Northern-blot and PCR, respectively. Additionally, DNA was extracted both from negative soil samples artificially contaminated with *P. brasiliensis* and tissues obtained from armadillos captured in an endemic area. These materials were used as the template for a PCR reaction that used *P. brasiliensis* specific primers (Table 1).

### Culture conditions

*P. brasiliensis* isolates B 339 (ATCC 32069) and Gar (ATCC 60855), *Candida albicans* (ATCC 90028), *Saccharomyces cerevisiae* (ATCC 9763), *Cryptococcus neoformans* (ATCC 90112), *Histoplasma capsulatum* var *capsulatum* Hc1980, *Sporothrix schenckii* CIB 37405, *Aspergillus fumigatus* CIB 214 were obtained from the culture collection of the CIB, and *Blastomyces dermatitidis* was obtained from the MAMH 3559, Alberta University, Canada. *P. brasiliensis* and *H. capsulatum* were grown for 10-12 days in a gyratory shaker set at 120 rpm (Gallenkamp, Leicester, England) both in the mycelial and yeast phases, in the modified liquid synthetic medium of McVeigh and Morton<sup>25</sup>, at their optimal growth temperatures (26 °C and 36 °C, respectively). *C. albicans* and *A. fumigatus* were grown in Sabouraud liquid medium supplemented with L-asparagine (0.14%) and thiamine (0.01%), at 22 °C ( $\pm$  4 °C) in a gyratory shaker for 8 days. *B. dermatitidis*, *S. schenckii*, *S. cerevisiae*, and *C. neoformans*, were grown in the same medium but at 37 °C.

### Armadillos

As previously described<sup>9</sup> the area where the armadillos were captured was selected, on the basis of the records corresponding to patients with paracoccidioidomycosis, in the Department of Caldas, Colombia. Particular attention was given to those cases that have lived and worked within the same locality; we selected a coffee growing terrain. This place has ecological conditions favorable to the growth of *P. brasiliensis* and it corresponds simultaneously, to the habitat of the nine-banded armadillo<sup>9</sup>. Permission for capture of armadillos was obtained from the wild-life preservation authorities (Instituto Colombiano Agropecuario - ICA, Corpocaldas branch of ICA). Four male adults and one female adult *D. novemcinctus* were captured within a period of 6 months. The animals were sacrificed by prolonged anesthesia<sup>9</sup>. Samples from the liver, the spleen, the lungs, and the mesenteric lymph nodes were cultured, used for animal inoculation and PCR.

### Processing of tissues from armadillos for culture and inoculation

The technique used for the study of animal tissues can be summarized as follows<sup>9</sup>: biopsies were minced and/or homogenized in tissue grinders and split in three portions, one of which was then plated in Sabouraud dextrose agar and Mycosel agar (BBL, Beckton Dickinson Microbiology

Systems, Cockeysville, MD, USA) and incubated under appropriate conditions. When growth occurred, microscopic observations were carried out and the mycelial growth sub-cultured at 36 °C in Sabouraud glucose agar with thiamine and asparagine, both at 0.1%, with incubation at 36 °C, in order to facilitate conversion to the yeast phase. Colonies developing here were subjected to microscopic observation. Other portion of the homogenized tissues was used for the intraperitoneal inoculation of adult, male BALB/c mice, 4 per tissue. These mice were sacrificed after 8 weeks and the lungs, spleen, liver and mesenteric lymph nodes, cultured as indicated above<sup>9</sup>. The remaining tissue homogenates were subjected to molecular biology procedures, as described below.

### Artificial contamination of soils with *P. brasiliensis*

Fresh garden soil (1g) was contaminated with different quantities of *P. brasiliensis* isolate B339 in the mycelial phase ( $1.5 \times 10^5$ - $20 \times 10^6$  cells). The quantification of the inoculum was done as described by ESPINEL-INGROFF & KERKERING<sup>10</sup>. The inoculated soils were resuspended in 6 volumes of EDTA 0.125 M and 6 volumes of extraction buffer (2% triton x-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1mM EDTA). Prior to DNA extraction, these soils were shaken at 100 rpm overnight (Gallenkamp, Leicester, England) at room temperature.

### Northern blot analysis of the p27 gene

Total RNA was extracted using Trizol™ (GIBCO BRL, Gaithersburg, MD), from the fungi described above, except for *A. fumigatus* and *S. schenckii* which were not used in the Northern blot analysis. These RNAs were run in agarose gel, transferred to nylon membranes and visualized with ethidium bromide. The probe corresponding to a 536 bp from the gene's sequence that codifies for the 27 kDa protein<sup>19</sup> was obtained by PCR, using primers LO/UP (Table 1) and labelled using the Fluorescein Gene Images™ from Amersham (Buckinghamshire, UK). The probes were then exposed in the nylon membrane (Probe to X-ray film) for 2 hours<sup>26</sup>.

### DNA Fungal extraction

The DNA was extracted by mechanical disruption as reported by VAN BURIK *et al.* (method 1)<sup>30</sup>, with minor modifications, as follows: fungal growth was transferred to micro-centrifuge tubes and suspended in 400 µl of extraction buffer as described above; mechanical disruption of the fungal suspension was achieved by means of glass beads; a extraction with 400 µl phenol/chloroform/iso-amyl alcohol (PHE/CHL/IAA) (25:24:1) was done with vortexing for 30 minutes, (Genie 2, Fisher Scientific, New York, USA). The aqueous layer was re-extracted twice with an equal volume of PHE/CHL/IAA (25:24:1), and once with equal volume of (chloroform/iso-amyl alcohol) CHL/IAA (24:1), then precipitated with ammonium acetate and ethanol<sup>26</sup>. The DNA pellet was resuspended in 100 µL of TE buffer (10 mM tris [pH 8.0], 1 mM EDTA); RNase treatment was done with 2 µl (2mg/ml) ribonuclease A (Sigma, St Louis, USA) at 37 °C for one hour. To remove residual cellular debris, an additional extraction with PHE/CHL/IAA and CHL/IAA was done and the DNA pellet finally resuspended in 100 µl of TE buffer. The DNA obtained was measured by spectrophotometry at 260nm, and its purity was determined by the ratio  $A_{260/280}$ . In order to determine the minimal concentration that could be detected by PCR, the extracted DNA was serially diluted (1µg-1pg per reaction).

**Table 1**  
Primers used for PCR amplifications

Primers	Sequence	Amplification conditions Temperature(°C) & time (m,s)	Specific amplification products (bp)			
ITS 1 ITS 4	5' TCCGTAGGTGAACCTGCGG 5' TCCTCCGCTTATTGATATGC	1 cycle at 95 x 5 m 30 cycles at <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>95 x 50 s</td></tr><tr><td>50 x 50 s</td></tr><tr><td>72 x 50 s</td></tr></table> 1 cycle at 72 x 7 m	95 x 50 s	50 x 50 s	72 x 50 s	620
95 x 50 s						
50 x 50 s						
72 x 50 s						
gp43-1 gp43-2	5' ATGAATTTTAGTTCTCTTAACCTGGCTCTT 5' CCTGCATCCACCATACTTCCTAGCCCA	1 cycle at 95 x 5 m 30 cycles at <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>95 x 1 m</td></tr><tr><td>40 x 1 m</td></tr><tr><td>72 x 2 m</td></tr></table> 1 cycle at 72 x 7 m	95 x 1 m	40 x 1 m	72 x 2 m	1303
95 x 1 m						
40 x 1 m						
72 x 2 m						
gp2 gpM (nested)	5' TCACCTGTGACACCATACTTCCTAGCCCAAATC 5' GCCATGGTCGACAATGGGCGTGGCATAGGTTTCG	1 cycle at 95 x 5 m 30 cycles at <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>95 x 1 m</td></tr><tr><td>55 x 1 m</td></tr><tr><td>72 x 1 m</td></tr></table> 1 cycle at 72 x 7 m	95 x 1 m	55 x 1 m	72 x 1 m	300
95 x 1 m						
55 x 1 m						
72 x 1 m						
LO UP	5' CACTCTTGGCTTTGGTTGAAG 5' CTGTTGTTTCCGTCCTTGCGC	1 cycle at 95 x 5 m 30 cycles at <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>95 x 1 m</td></tr><tr><td>55 x 1 m</td></tr><tr><td>72 x 1 m</td></tr></table> 1 cycle at 72 x 7 m	95 x 1 m	55 x 1 m	72 x 1 m	536
95 x 1 m						
55 x 1 m						
72 x 1 m						

#### Extraction and purification of *P. brasiliensis* DNA from artificially contaminated soils

After overnight shaking the corresponding supernatants were transferred to different tubes. These were centrifuged at 7,000 x g for 20 min. DNA from these pellets was extracted using the same procedure described above. In order to eliminate soil inhibitors, we employed the procedure reported by MOREIRA with some modifications<sup>20</sup>, as follows: the pellet containing DNA was resuspended in TE buffer and 1 volume of melted 1.6% low melting point agarose (GIBCO BRL, Grand Island, N.Y., USA) was added and mixed. The mixture was poured into sterile 200 µL moulds prior to solidification. The agarose blocks were dialyzed against 15 ml of TE buffer for 24 hours. This procedure was repeated using new TE buffer. PCR was done using the 2 µL obtained from melted (60 °C) agarose blocks.

#### Extraction of *P. brasiliensis* DNA from animal tissues

Homogenized tissues (lymph nodes, liver, spleen) from both the armadillos and the mice inoculated with their organs were subjected to PCR<sup>5</sup>. The DNA extraction was done following the first of the 5 techniques reported by VAN BURIK *et al.*<sup>30</sup> with a modification, namely, the initial extraction step, as we employed grinding the liquid-nitrogen

frozen sample in mortar with pestle. The DNA pellet was suspended in 10 µL of TE buffer.

#### PCR amplifications

10ng of DNA from culture and/or from artificially contaminated soils and/or tissues of armadillos were used as the template in an PCR reaction. The final volume was 50 µL. The mixture used for all reactions contained 10mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1%, triton x-100, 1.5 mM MgCl<sub>2</sub>, 0.2 mM (each) deoxynucleoside triphosphate, 25pM of the primers, and 1U of taq DNA polymerase (Promega, Madison, USA). Amplification was done in a thermocycler (PTC-100, MJ Reseach, INC, Watertown, USA).

The primers used in this study and the amplification conditions are shown in Table 1. The following primers were employed; ITS1/ITS4<sup>5,16</sup>, the fungal specific universal primers used as amplification controls, the 43 kDa *P. brasiliensis* antigenic protein gp43-1/gp43-2 and the nested primers for this protein gp2/gpM<sup>8</sup>, as well as *P. brasiliensis* primers LO/UP<sup>19</sup>, obtained from the gene that codifies for the 27 kDa antigenic protein. The PCR products were visualized by ethidium bromide staining after electrophoresis in agarose (GIBCO BRL, Grand Island, N.Y., USA).

## RESULTS

Northern blot analysis with the p27 probe, revealed that this gene was only transcribed by *P. brasiliensis*, with a positive signal both in the mycelium and the yeast phases of the fungus; no signals were obtained with the mRNAs from other fungi (Figure 1).

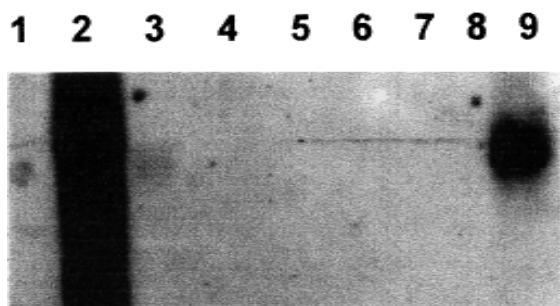
The DNA extracted from fungal cells by means of glass beads plus the extraction buffer and phenol, showed to be of high quality and purity, as determined by the ratio  $A_{260/280}$  which was  $> 1.8$ .

PCR amplification was obtained with all the primers used; thus the ITS1/ITS4 set which corresponds to universal specific fungal primers gave a positive band of 620 bp with all the fungi evaluated (data not shown). The gp43-1/gp43-2, the gp2 gpM (nested) and the LO/UP primers were specific for *P. brasiliensis* and they only amplified with material originating from this fungus but not with those from the remaining fungi tested. Figure 2 shows the specificity obtained with the LO/UP primers. The latter primers showed to be highly sensitive, allowing amplification of low amounts (3pg) of *P. brasiliensis* (approximately 90 cells).

Soil samples artificially contaminated with *P. brasiliensis*, permitted amplification after elimination of the naturally contaminating PCR inhibitors. The addition of  $5.0 \times 10^6$  mycelial cells of *P. brasiliensis* to 1 g of non-sterile sediments resulted in a larger quantity of amplified DNA. The amount of *P. brasiliensis* DNA detected by means of PCR using the species-specific primer (LO/UP) was 40 ng (approximately  $1.2 \times 10^6$  cells), as shown in Figure 3.

As it concerns mycological studies, primary agar cultures done with minced lymph node tissues from the first armadillo, showed growth of several colonies of a mold. These were dimorphic as demonstrated when colonies grown at room-temperature were transferred to new media and incubated at 36° C. The corresponding cultures exhibited the multiple budding cells that characterize *P. brasiliensis*<sup>9</sup>. The products of amplification of the lymph node and liver of this first armadillo, are shown in Figure 4.

The results of mycological studies and PCR amplifications from animal tissues are shown in the Table 2.



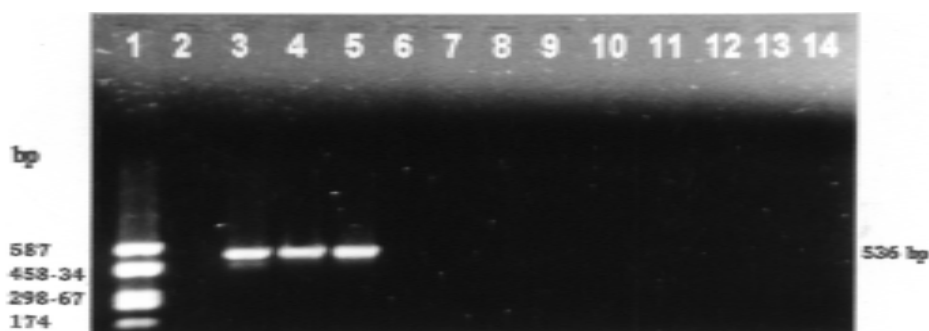
**Fig. 1** - Northern-blot analysis of the p 27 gene. Lane 1. Negative control pBluescript without insert, 2. Positive control (pBluescript with gene of p27), 3. *P. brasiliensis* ATCC 32069 (mycelial), 4. *S. cerevisiae*, ATCC 9763, 5. *B. dermatitidis*, 6. *H. capsulatum*, 7. *C. albicans*, ATCC 90028, 8. *C. neoformans*, ATCC 90112, 9. *P. brasiliensis* (yeast)

**Table 2**

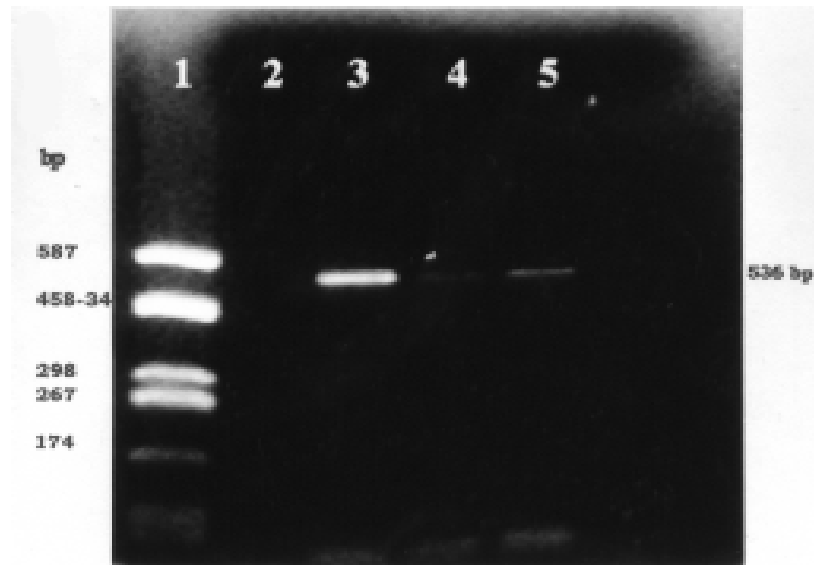
Results of cultures and DNA amplification in animal tissues

Armadillo	Isolation in N°	Amplification by PCR			
		Lungs	Lymph node	Liver	Spleen
1*	+	-	-	+	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	+	+	+	-

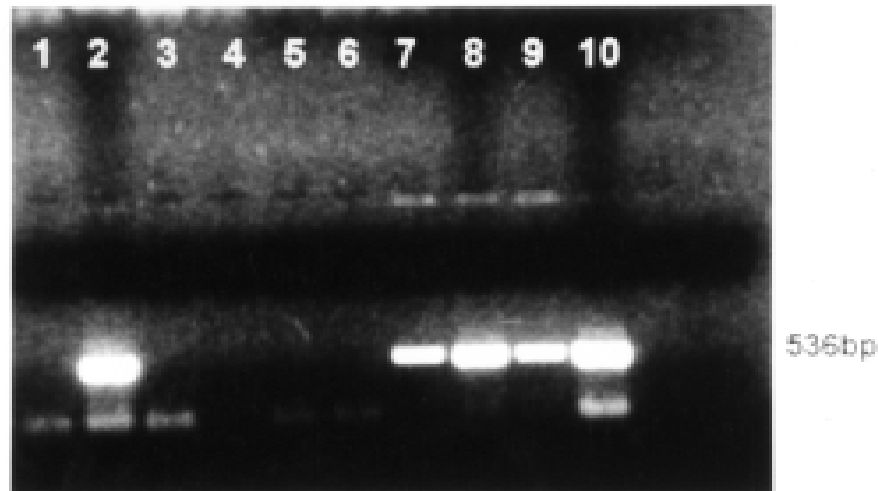
\*Mice inoculated with lymph node and liver from this armadillo, were also positive by PCR (Mouse lungs and liver).



**Fig. 2** - DNA amplification by PCR with specific primers LO/UP in agarose gel electrophoresis stained with ethidium bromide. The following DNA templates were used for PCR amplification (from line 3 on): Lanes: 1. Molecular weight markers (pUC18 *Hae* III digested), 2. Negative control, 3. Positive control *P. brasiliensis*, ATCC 32069 (mycelial phase), 4., 5. *P. brasiliensis* ATCC 60855 (mycelial and yeast phases), 6., 7. *H. capsulatum* (mycelial and yeast phases respectively), 8. *B. dermatitidis*, 9. *S. cerevisiae*; ATCC 9763, 10. *C. albicans*; ATCC 90028, 11. *C. neoformans*; ATCC 90112, 12. *S. schenckii*, 13. *A. fumigatus*, 14. Negative control.



**Fig. 3** - DNA amplification by PCR with specific primers LO/UP in agarose gel electrophoresis stained with ethidium bromide. Lanes: 1. Molecular weight markers (pUC18 *Hae* III digested), 2. Negative control, 3. Positive control *P. brasiliensis*, 4. & 5. Products of amplification from *P. brasiliensis* DNA extracted from soil.



**Fig. 4** - DNA amplification by PCR with specific primers LO/UP in agarose gel electrophoresis stained with ethidium bromide. The following DNA templates were used for PCR amplification: Lanes: 1. Negative control, 2. Positive control, 3 & 4. Lung of first and fourth armadillos, 5 & 6. Spleen of first and five armadillos, 7. Lymph node of first armadillo, 8. Liver of first armadillo, 9. Lung of mouse inoculated with lymph node from the same armadillo, 10. Liver of mouse inoculated with armadillo's liver.

## DISCUSSION

This report describes a method for amplification of *P. brasiliensis* genes through the use of PCR. This technology has previously been employed in the identification of other clinically important pathogens<sup>13,29</sup>, as well as of *P. brasiliensis* in an animal model<sup>13</sup>. In the later study, authors inoculated  $5.8 \times 10^6$  yeast cells and obtained positive amplicons from the animals' sera 8 weeks afterwards; the level of sensitivity was 10 pg. PCR can also be similarly applied to the search of environmental microorganisms<sup>2,4</sup>.

The ability to extract DNA from fungal cells through mechanical disruption techniques and the use of SDS buffer and phenol for nucleic acid extraction, proves adequate for biochemical and/or genetic analyses and is also effective when obtaining DNA products of high quality<sup>30</sup>.

The gene codifying for the main *P. brasiliensis* antigenic protein of 43kDa was the first to be cloned and characterized<sup>8</sup>. This protein albeit not the recombinant one, has been widely employed as an antigen in the diagnosis of PCM<sup>7</sup>. The primers obtained from this gene sequence (gp43-1/gp43-2) have been shown to be highly specific for the amplification of

*P. brasiliensis* DNA, and they were used to corroborate the positive findings in the armadillo's studies<sup>8</sup>. Similar results have been obtained using the gene that codifies for the 27 kDa antigen protein which was cloned and characterized in our laboratory<sup>19,23</sup>. With the use of these primers it has been possible to detect low concentrations of fungal DNA (3pg, equivalent to 90 cells). We have also tested the mRNA levels of this 27kDa protein in different fungal cultures by Northern blot analysis and demonstrated that this protein mRNA is exclusively transcribed in *P. brasiliensis* (Figure 1).

Isolation of *P. brasiliensis* from soils by means of conventional techniques such as culture and animal inoculation, has low reproducibility<sup>6</sup>. In order to improve these conditions, we standardized the PCR methodology in artificially *P. brasiliensis* contaminated soils samples, an important step in the search of its precise ecological niche and its possible use for future epidemiological studies. The main problem in the search of fungal DNA in soils by PCR, is the presence of inhibitors contained in these samples. Soils with high organic contents have humic acids with phenolic groups, which exhibit similar solubility to that of the DNA. As a consequence, they are not totally removed during classical extraction protocols and remain as contaminants in the final DNA preparation<sup>4,20</sup>. An alternative to eliminate the presence of inhibitors is to consider that there are important differences in the size of DNA macromolecules and most common soluble PCR inhibitors; these inhibitors are effectively removed from the agarose blocks by diffusion during the washing steps, whereas genomic DNA remains trapped<sup>20</sup>. In our study, the PCR detection limit for *P. brasiliensis* species-specific sequences from soils, proved to be 40 ng of DNA extracted from 1 g of soil (approximately  $1.2 \times 10^6$  cells).

The infection with *P. brasiliensis* has been established in the nine-banded armadillo *D. novemcinctus*, an animal amply distributed in Latin America where it occupies areas that coincide, at least partly, with the paracoccidioidomycosis endemic regions. Additionally, in the armadillo's burrows, the environmental conditions would increase the possibilities for a stable fungal microniche<sup>12</sup>.

The positive fungal amplification obtained in some organs of a culture-positive armadillo, as well from tissues of another three culture-negative armadillos, suggest that PCR is not only a more sensitive technique than the conventional ones used with the same purpose<sup>9</sup>, but has also some advantages over culture of non sterile organs, such as lungs, usually heavily contaminated with various microorganisms. In one of the armadillos, the results obtained by PCR was confirmed by isolation of the agent in culture, the gold standard technique. The latter supports previous findings by other authors<sup>3,9,21</sup>, indicating that *P. brasiliensis*, can be isolated not only from humans samples, but also from armadillos, such as the one captured in an endemic area of Colombia<sup>9</sup>. The fact that only one armadillo from the 5 studied, was positive for culture while 3 revealed to be infected by DNA amplification, tends to suggest that PCR is indeed a more sensitive technique<sup>16</sup>.

The results reported here suggest that the PCR amplification of *P. brasiliensis* DNA by the use of species-specific primers, may play an important role in the search of this fungus habitat and also for future epidemiological studies. At present analysis of soil samples obtained from different endemic areas in Colombia and Brazil is being pursued. Several questions arise from this study, such as: how do armadillos get

infected?. Once the microniche is detected, what environmental measures could be taken to avoid human infection?. Answers to these and other questions would require further explorations.

## RESUMO

### PCR com «primers» específicos de *Paracoccidioides brasiliensis*: uso potencial em estudos ecológicos

O microambiente adequado do *Paracoccidioides brasiliensis* não foi ainda bem esclarecido, talvez porque os métodos utilizados não sejam suficientemente sensíveis. Aplicamos com este propósito, a reação em cadeia da polimerase (PCR) usando três jogos de primers específicos do *P. brasiliensis*, correspondendo a dois dos genes do *P. brasiliensis*. Este fungo, assim como outros fungos, foram cultivados e seus DNAs obtidos por ruptura mecânica e purificados com mistura de fenol-clorofórmio com álcool isoamílico. Os DNAs serviram para a reação de PCR utilizando-se primers específicos para dois dos genes do *P. brasiliensis* que codificam para as proteínas antigênicas, denominadas, 27 kDa e 43 kDa. O limite mínimo de detecção para o gene 27 kDa foi de 3 pg. A amplificação para os dois genes foi positiva só com o DNA do *P. brasiliensis*; além disso, o mRNA para o gene de 27kDa estava presente apenas no material do *P. brasiliensis*, como mostrado pela análise por Northern-blot. A padronização da técnica do PCR permitiu a amplificação do DNA do *P. brasiliensis* em solos contaminados artificialmente com o fungo e em tecidos de tatus infectados na natureza. Estes resultados indicam que a técnica do PCR poderia ter um papel muito importante na pesquisa do habitat do *P. brasiliensis* e, além disso, poderia ser utilizada em outros estudos ecológicos.

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