

Experimental infections with *Paracoccidioides brasiliensis* Obtained from Armadillos: Comparison to Clinical Isolates

Angela Satie Nishikaku¹, Maria Terezinha S. Peraçoli², Eduardo Bagagli², Maria Fátima Sugizaki² and Alexandrina Sartori²

¹Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP; ²Department of Microbiology and Immunology, Institute of Biosciences, São Paulo State University of Botucatu; Botucatu, SP, Brazil

Paracoccidioides brasiliensis causes paracoccidioidomycosis (PCM) that is one of the most prevalent systemic human mycoses in Latin America. Armadillos show a high incidence of PCM infection and could, therefore, be a natural reservoir for this fungus. In this study we compared the virulence profiles of isolates obtained from nine-banded armadillos (*Dasypus novemcinctus*) (PbT1 and PbT4) and isolates from PCM patients (Pb265 and Bt83). Pathogenicity was evaluated by fungal load and analysis of colony morphology. Immunity against the fungus was tested by delayed type hypersensitivity test (DTH) and antibody quantification by ELISA. The higher virulence of PbT1 and PbT4 was suggested by higher fungal load in spleen and lungs. Armadillo isolates and Bt83 presented a cotton-like surface contrasting with the cerebriform appearance of Pb265. All isolates induced cellular and humoral immune responses in infected BALB/c mice. DTH reactions were similarly induced by the four isolates, however, a great variability was observed in specific antibody levels, being the highest ones induced by Bt83 and PbT4. The present work confirms that armadillos harbor *P. brasiliensis*, whose multiplication and induced immunity in experimentally infected mice are heterogeneous, resembling the behavior of isolates from human PCM. This study reinforces the possibility that armadillos play an important role in the biological cycle of this pathogen.

Key-Words: *Paracoccidioides brasiliensis*, armadillos, infection, BALB/c mice.

Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America [1]. *P. brasiliensis* is a thermally dimorphic fungus, which grows at room temperature as mycelia or develops multiple budding yeast form in human or experimental hosts or when cultured *in vitro* at 35–37°C [2]. Epidemiological studies demonstrated that the majority of the subjects exposed to *P. brasiliensis* develop an asymptomatic infection, indicating that they are resistant hosts. Even though a variety of clinical manifestations can be associated with the disease, two predominant types are described: the acute/subacute or juvenile form that affects mainly young subjects of both sexes and involves the mononuclear-phagocytic system and the chronic or adult form that affects mainly adult males, who frequently present pulmonary and/or mucocutaneous involvement [3].

Several evidences show that propagules of *P. brasiliensis* conidia are the infective form, however, the natural habitat of this fungus is not fully determined. A few reports demonstrated isolation of this fungus from environment (soil, plants and animals). In addition, some studies confirmed the isolation of *P. brasiliensis* from nine-banded armadillos (*Dasypus novemcinctus*) in endemic regions of PCM, suggesting that these animals could be an important primary natural reservoir of this fungus [4–6]. The virulence of *P. brasiliensis* isolates obtained from armadillos has been studied using both, hamster

[7–9] and mouse virulence model systems [10]. These animal models have been extremely valuable to understand many aspects of protective immunity, histopathological alterations and also the host-parasite interaction in this fungal disease [11,12]. Moreover, these studies have also revealed different virulence profiles of *P. brasiliensis* isolates, which could explain the distinct clinical forms of the disease [13,14].

Similarities between fungal isolates from armadillo and clinical isolates have been shown in morphological, antigenic and molecular studies [10,15,16]. However, the real contribution of armadillos to the ecology and pathogenicity of *P. brasiliensis* isolates and to epidemiology of human PCM remains still unsolved.

Identification of antigenic components of *P. brasiliensis* that are able to induce specific immune response has provided useful tools for clinical diagnosis and for better understanding the outcome of the disease [17]. Immunity against *P. brasiliensis* is not completely understood, but many studies showed a strong correlation between high antibody levels and severity of the disease [18,19]. On the other hand, the cellular immune response characterized by persistent delayed-type hypersensitivity (DTH) and by macrophage activation through interferon-gamma and tumour necrosis factor-alpha, has been linked to resistance of human and animal hosts to infection [20,21].

In this context, the aim of the present work was to compare the virulence profile of two isolates from armadillos (PbT1 and PbT4) with two clinical isolates (Pb265 and Bt83) and also to evaluate the cellular and humoral immune responses induced by these isolates in BALB/c mice.

Material and Methods

Animals

Four to six-week-old male BALB/c mice were used. Animals were maintained at the Animal Facility of the Department of

Received on 19 August 2007; revised 21 January 2008.

Address for correspondence: Dr. Alexandrina Sartori, M.D., Ph.D., Departamento de Microbiologia e Imunologia, Instituto de Biociências, UNESP, Distrito de Rubião Júnior, s/n, Botucatu, SP. Zip code CEP: 18618-000. Phone: 55 (14) 3811-6240; Fax: (14) 3815-3747. E-mail: sartori@ibb.unesp.br.

The Brazilian Journal of Infectious Diseases 2008;12(1):57-62.
© 2008 by The Brazilian Journal of Infectious Diseases and Contexto Publishing. All rights reserved.

Microbiology and Immunology, São Paulo State University (UNESP), Botucatu, Brazil, supplied with filtered water and conventionally fed (Nuvital-CR1) *ad libitum*. Room temperature was set at 22°C and lighting consisted of alternate 12 h light/dark cycles.

Fungal Isolates and Inoculum Preparation

Two *P. brasiliensis* isolates (PbT1 and PbT4) were originally obtained from armadillos living in an endemic area for PCM (Botucatu, SP), as described previously [5] and then maintained in the laboratory by weekly cultivation. Two human isolates (Pb265 and Bt83) were also included in this study aiming to a comparative analysis. Bt83 was isolated from the sputum of a patient from Clinical Hospital of the School of Medicine, São Paulo State University in 1994, and Pb265 was obtained from the fungal culture collection of the Department of Microbiology, University of São Paulo, Brazil, and has been maintained in culture for more than 30 years, with no record of the precise date of isolation. *P. brasiliensis* isolates Pb265 and Bt83, respectively, are slightly and highly virulent for mice [10,14,22]. For inoculum preparation, the yeast form of the four isolates were grown in semi-solid medium containing 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar (GPY) at 36°C for six days. Yeast cell suspensions were washed in phosphate buffered saline (PBS), pH 7.2 and viability was determined by phase contrast microscopy [23]. After being homogenized and agitated in a vortex for 5-10s, the suspensions were adjusted to 25.10^6 viable yeasts/mL. Mice were infected with $5.10^6/0.2$ mL by intraperitoneal route. Fungal suspensions containing more than 90% viable cells were used for the experiments.

Colony Morphology

Colony morphology of the four isolates was evaluated by observation of giant colonies in their mycelial form, as described by Sano et al. [24]. Each isolate obtained from a six day-old culture in GPY medium at 35°C was plated on the central area of three 220x100 mm Petri dishes with Potato Dextrose Agar (PDA) with a platinum loop that allows an inoculation area of 3 mm in diameter. After 40 days of incubation at 25-28°C, the colonies were examined for shape, size, texture and pigment production.

Quantification of Colony Forming Units (CFU)

Virulence was quantified by counting the number of viable fungi recovered from tissues of infected mice. Thirty and ninety days after infection, spleen and lungs were aseptically removed, weighed and individually homogenized in 2 mL of sterile PBS. Aliquots of 100 μ L of each homogenate were plated by triplicate onto Petri dishes, with brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) supplemented with 4% normal horse serum and 5% Pb192 culture filtrate as a source of growth-promoting factor [25]. Plates were incubated at 35°C for three weeks and colonies were counted. The results were expressed as logarithmic values of the CFU counts per g of tissue.

Cell-Free Antigen Preparation

Cell-free antigen (CFag) was obtained by the methodology described by Camargo et al. [26]. Briefly, Pb265 was grown in GPY medium at 35°C during four days. The fungal growth (about 300 mg) was collected by gently scraping the agarose surface. The cell mass was then resuspended in 1 mL of PBS, mixed for 30 s on a vortex-mixer and immediately centrifuged at 10,000 g during 1 min. The supernatant was sterilized by filtration through a 0.22 μ m membrane. Protein concentration was determined by the method of Lowry et al. [27]. Aliquots were kept at -20°C until use but storage did not exceeded 15 days. The same procedure was used to obtain CFag from the other isolates, however, pilot experiments with the autologous CFag to evaluate antibody levels showed only basal levels of specific antibodies. Therefore, CFag from Pb265 was used as the antigen to evaluate humoral and also cellular immunity.

Delayed Type Hypersensitivity Test

The delayed type hypersensitivity (DTH) reaction was evaluated by the footpad swelling test as described previously [28]. Briefly, healthy or infected mice were inoculated, in one of the back footpads, with 50 μ g/50 μ L of CFag from Pb265. The footpad thickness was measured immediately before and 24 hours after antigen inoculation using a dial caliper (Mitutoyo, Tokyo, Japan) and was expressed in mm. Optimal antigen concentration for DTH assays was determined in preliminary experiments (not shown).

Humoral Immunity

Specific antibody levels to *P. brasiliensis* were evaluated by indirect enzyme-linked immunosorbent assay (ELISA) in blood samples collected after 15, 30 and 70 days of infection. Plastic 96-well microtitration plates (MaxiSorp, Nunc, International Corp., Rochester, NY, USA) were coated with cell-free antigen prepared from Pb265 previously diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (40 μ g/mL) and incubated for 60 min at 37°C followed by an overnight incubation at 4°C. The plates were washed with PBS containing 0.05% of Tween 20 (PBS/T). The remaining binding sites were blocked by addition of 5% non fat dry milk diluted in PBS/T during 30 min at 37°C. Serum samples in two-fold dilutions were added after plate washing, and after 60 min incubation at 37°C, the plates were washed again and a goat anti-mouse total immunoglobulin peroxidase conjugate (Sigma Chemical Co., St Louis, Mo, USA) diluted to 1/5,000 was added. After an additional incubation at 37°C for 60 min, the plates were washed and covered with a mixture containing *o*-phenylenediamine (10 mg) plus 30 μ L of 30% H₂O₂ diluted in 12.5 mL of citrate-phosphate buffer. Optical densities (OD) were read in an ELISA reader (Multiskan EX, Manchester, UK) after reaction interruption with 4 N H₂SO₄. A cut-off value was obtained by using 1:2 diluted sera from non-infected mice housed under identical conditions as the infected animals. Antibody titers were expressed as the reciprocal of the highest dilution whose OD was higher than the cut-off value.

Statistical Analysis

The number of CFU/g of tissue, DTH reactions and antibody titers were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparisons by the Tukey test. A *p* value of < 0.05 was considered significant.

Results

High Fungal Loads are Recovered from Mice Infected with *P. brasiliensis* Isolated from Armadillos

Thirty and 90 days after infection by intraperitoneal route, mice were sacrificed and the fungal load determined by the number of CFU in spleen and lungs. As can be observed in Figure 1a for the lungs and Figure 1b for the spleen, higher amounts of fungal cells were recovered from animals infected with PbT1 and PbT4 in comparison to the clinical isolates. The number of CFU detected in lungs of mice infected with PbT1 was significantly higher in relation to the animals inoculated with Pb265 at 30 and 90 days of infection. CFU recovered from the spleen of the animals infected with PbT1 and PbT4 at 30 and 90 days of infection were significantly higher than those obtained from mice inoculated with Pb265 at the same periods and higher than in animals inoculated with Bt83 at 30 days of infection. Interestingly, at 90 days of infection the fungi disappeared from lungs in animals infected with PbT4, but these animals still presented high amounts of fungi in their spleens.

P. brasiliensis Isolated from Armadillos Develop a Cottony Phenotype

Phenotypes of *P. brasiliensis* isolates were distinguished mainly by differences in colony size, shape, texture and pigment production. Both Pb isolates from armadillos developed larger colonies than the clinical isolates. They presented a typical cotton-like surface that varied in color from white to beige. Also, a central fissure was observed in PbT4 (Figure 2a) but not PbT1 (Figure 2b). Bt83 morphology was more similar to armadillo isolates. In this case, the colony presented an intense wrinkled appearance and a predominant white cotton-like surface (Figure 2c). Also, a very pronounced central fissure was observed presenting a brownish color. On the other hand, Pb265 presented a very distinct glabrous phenotype characterized by an irregular format and a cerebriform surface (Figure 2d).

P. brasiliensis Isolated from Armadillos Induce Cellular Immunity in Mice

Cellular immunity evaluated by delayed-type hypersensitivity (DTH) test was similarly induced by the four isolates and was characterized by a significant increase in the footpad thickness in comparison to non-infected controls, as depicted in Figure 3. As can be observed, some variation was associated with the different isolates. The higher increases in the footpad thickness were detected in animals infected with Pb265, the highest value being found at the 70th day of infection. The DTH reactions found in mice infected with Bt83, PbT1

and PbT4 were similar in intensity, except at the 70th day of infection when the footpad increase was not significant in the group infected with PbT4.

P. brasiliensis from Armadillos Induce Humoral Immunity in Mice

All four isolates were able to induce specific antibody production after intraperitoneal infection in BALB/c mice when the sera were tested with CFag obtained from Pb265 (Figure 4). Antibody levels detected by using the homologous CFag (Bt83, PbT1 and PbT4) were very low and similar to basal levels found in non-infected mice (not shown). All Pb isolates induced lower antibody levels at 15 than at 30 and 70 days after infection. Antibody levels induced by Pb265, PbT1 and PbT4 were similar at 30 and 70 days after infection, with no statistical difference among them. Only the Bt83 isolate elicited a very distinct serological profile characterized by a significantly higher antibody titer in comparison to the three other isolates.

Discussion

In this study we evaluated the ability of *P. brasiliensis* isolated from armadillos to cause disease in mice and we also studied their ability to induce immune response in this experimental model. These characteristics were also compared to the ones of clinical *P. brasiliensis* isolates. Mice were chosen as animal models because they have been very useful to study host-parasite interactions in PCM. This model indicated that distinct *P. brasiliensis* isolates vary in their virulence, which could explain the diversity found in clinical manifestations of this disease [13, 29, 30].

Two parameters of virulence were analyzed: amounts of fungi in spleen and lungs and the morphology of giant colonies from the fungal mycelial form. Higher fungal loads were recovered from mice infected with armadillo isolates in comparison with the two clinical isolates, Bt83 and Pb265, suggesting, therefore, that armadillo isolates were more virulent. According to Singer-Vermes et al. [19], estimation of the number of viable fungi in infected mouse organs by CFUs counts is one of the most direct and trustworthy methods to assess severity of this disease. The results observed in mice infected with Bt83 differed from those previously reported, i.e., we expected the recovery of higher amounts of fungi in this case. Lower fungal loads of Bt83 isolate obtained from organs of infected mice could be attributed to the phenomenon known as virulence attenuation that has been described for *P. brasiliensis* [9,24] and also for other fungi that cause deep mycosis, such as *Blastomyces dermatitidis* [31] and *Cryptococcus neoformans* [32]. Spleen colonization was similar between the two armadillo Pb isolates, however, at 90 days, more fungi were recovered from the lung tissue in PbT1 infected mice. It is tempting to speculate that this isolate has a higher tropism or ability to disseminate to lungs than PbT4. Some support to this hypothesis is given by the finding of Singer-Vermes et al. [14] that showed that distinct *P.*

Figure 1. Fungi recovery (CFU) from mice infected with *P. brasiliensis* isolates. Lungs (A) and spleens (B) were collected from infected mice after 30 and 90 days of infection (5.10^6 yeasts/0.2 mL/i.p. route) and the number of CFU was determined by plating in BHI media enriched with horse serum and fungi growth factor. Each bar represents the mean \pm SEM CFU per gram of the tissue from six animals.

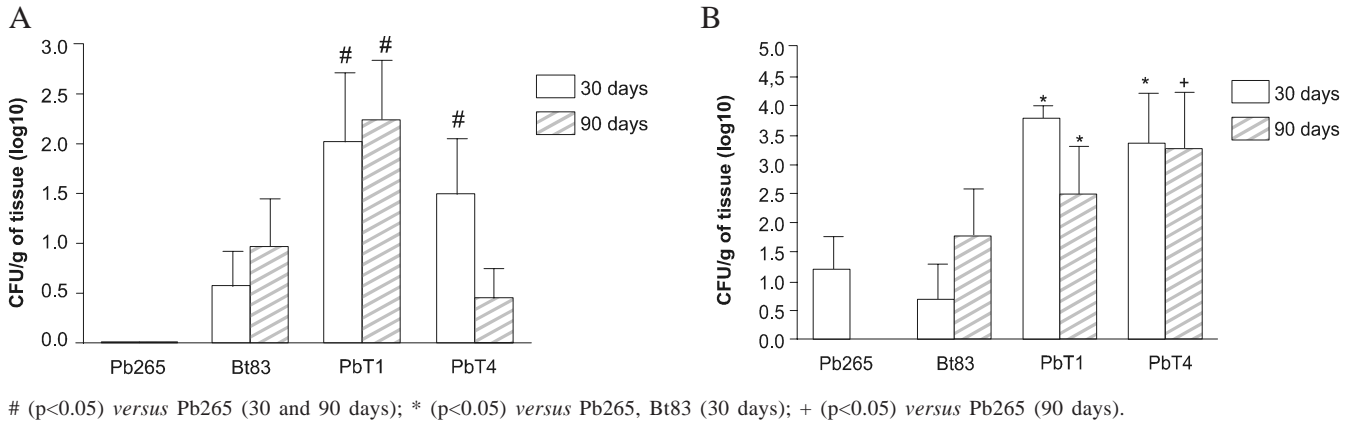


Figure 2. Colony morphology: comparison among the four isolates. Giant colonies from *Paracoccidioides brasiliensis* isolates, cultured at 25°C on potato dextrose agar medium. a) PbT4; b) PbT1; c) Bt83; d) Pb265.

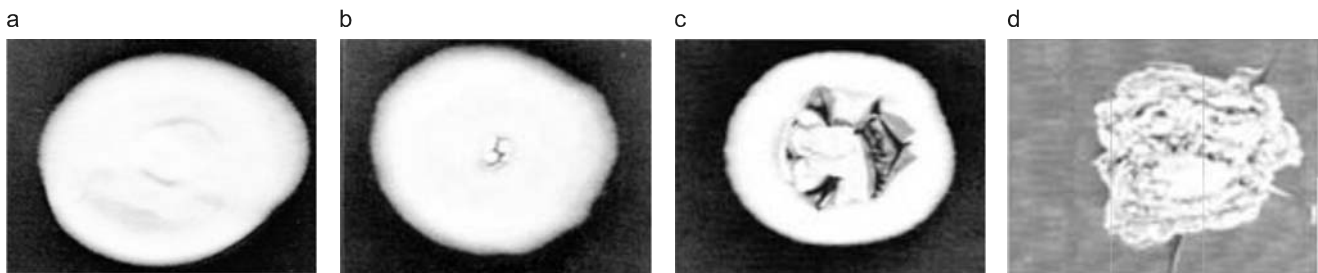


Figure 3. Delayed type hypersensitivity (DTH) reactions in mice infected with *Paracoccidioides brasiliensis* isolates. Results are expressed as mean \pm SEM of footpad swelling obtained from five animals per period of infection. BALB/c male mice were infected by intraperitoneal route with 5.10^6 yeasts/0.2 mL and then (15, 30 and 70 days after infection) they were challenged with 50 σ g/50 σ L of *P. brasiliensis* 265 CFAg in the footpad. Twenty-four hours later the increase of the footpads was measured by using a caliper.

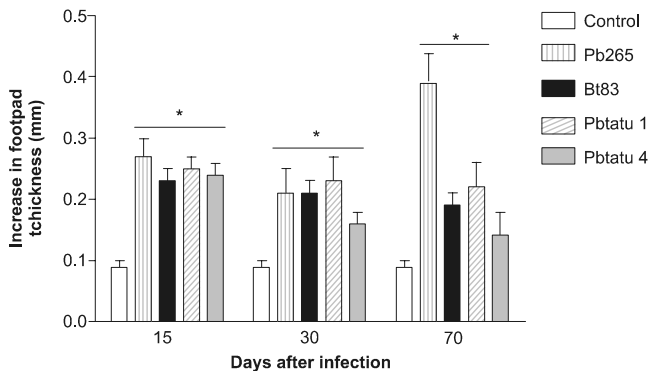
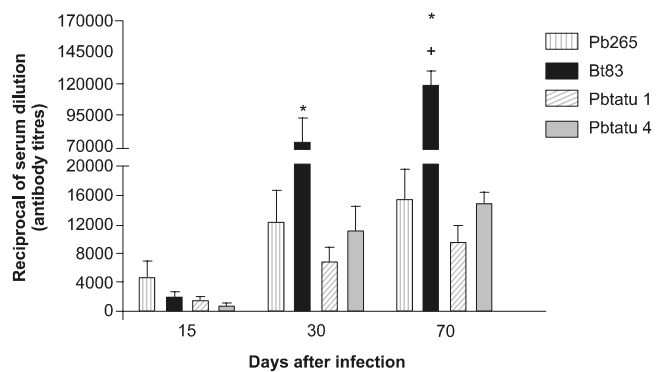


Figure 4. Specific antibodies in mice infected with *Paracoccidioides brasiliensis* isolates. BALB/c male mice were infected by intraperitoneal route with 5.10^6 yeasts/0.2 mL and then (15, 30 and 70 days after infection) they were sacrificed and serum samples were obtained by retro-orbital puncture. Results are expressed as mean \pm SEM of the highest reciprocal serum dilution from six animals.



brasiliensis strains showed ability to colonize distinct organs and tissues.

The morphological characteristics of the giant colonies were also evaluated and compared. Both isolates from armadillos presented a very similar appearance: they were rounded showing a white cotton-like surface. The Bt83 colony was similar, being also rounded and showing a predominant white cotton-like surface. As the isolates from armadillos were recovered in higher amounts from infected mice than Bt83, our results are in accordance with previous reports. Kurokawa et al. [10] and Sano et al. [24] showed that *P. brasiliensis* isolates with low *in vitro* subculture time and cottony morphology frequently present higher virulence, while isolates with cottony phenotype, but longer *in vitro* subculture period present lower or intermediate virulence. It is well known that long *in vitro* subculture period of isolates are associated with virulence attenuation [7,29]. Thus, as expected, Pb265 colony presented a very distinctive appearance, being glabrous and characterized by an irregular format and a beige cerebriform surface. These characteristics are in accordance with its low dissemination; only a few colonies were detected in the spleen after 30 days of infection and no fungi were found in the lungs.

Specific antibodies are not protective in PCM, however, its measurement is useful for monitoring the progress of the disease in patients [33]. In this context, higher antibody levels were expected in mice infected with PbT1 and PbT4, due to the higher dissemination observed in organs of these infected mice. However, the highest antibody levels were only detected throughout the infection with Bt83. On the other hand, both armadillo isolates were able to induce significant antibody levels, similarly to the one induced by Pb265. One of the possible explanations for these seemingly unexpected results could be the origin of the antigen used for ELISA. Initially, cell-free antigen from each isolate was used in ELISA. However, only very low antibody titers were detected. Since the use of cell-free antigen from Pb265 detected much higher antibody levels, this antigen source was chosen for antibody and DTH evaluation. Cellular immunity has been described as the most important defense against *P. brasiliensis*. Macrophages activated by cytokines produced by itself and derived from T helper 1 cells are fundamental to eliminate the fungi [20,21]. In this scenario, a differential cellular immunity was expected in mice infected with the four isolates. However, by using a DTH assay, we observed that all four isolates were able to induce a significant increase in the footpad thickness.

Some general comments could be derived from this data. First of all, PbT1 and PbT4 showed some characteristics that are commonly found in the originally more virulent strains including higher multiplication in the murine host and also the cottony colony morphology. On the other hand, their ability to induce antibody production seemed more similar to Pb265 that determines a much milder disease in mice. In addition, all four isolates were able to induce cellular immunity in mice experimentally infected.

Variation in the virulence pattern of *P. brasiliensis* isolated from armadillos was previously reported in hamster and mouse models of PCM and a comparative analysis was done with the clinical isolates. The armadillo *P. brasiliensis* isolates were classified as highly to intermediately virulent to hamsters and lowly virulent to mouse in comparison to clinical isolates [7,8,10]. These findings suggest that *P. brasiliensis* samples obtained from armadillos presented a heterogeneous behavior, being difficult to define then as virulent or not. However, this complex behavior is similarly observed in isolates obtained from human PCM. Thus, the data demonstrated in the present work reinforce the possibility that armadillos play an important role in the biological cycle of this fungal pathogen, acting as a natural reservoir of *P. brasiliensis* isolates, which could be able to infect and cause disease in human and animal hosts. The data also agree with some previous molecular and virulence studies indicating that armadillos isolates can encompass a significant biodiversity, that might reflect the different patterns of host-pathogen interactions [8,34]. It was already suggested that armadillos, by having a constant contact with soil in habitats supporting *P. brasiliensis*, in combination with its low body temperature and a weak cell-mediated immunity, could be infected by and disseminate a wider genotype spectrum of the pathogen than that found in clinical isolates.

Acknowledgement

We are thankful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), for the fellowship (97/10446-1) to Angela Nishikaku.

References

1. Wanke B., Londero A.T. Epidemiology and paracoccidioidomycosis infection. In: Franco M.F., Lacaz C.S., Restrepo A., Del Negro G. eds. Paracoccidioidomycosis. Boca Raton, FL., CRC Press, 1994.
2. McEwen J.G., Bedoya V., Patiño M.M., et al. Experimental murine paracoccidioidomycosis induced by the inhalation of conidia. *J Med Vet Mycol* 1987;25:165-75.
3. Brummer E., Castañeda E., Restrepo A. Paracoccidioidomycosis: an update. *Clin Microbiol Rev* 1993;6:89-117.
4. Naiff R.D., Ferreira L.G.L., Barret T.V., et al. Paracoccidioidomycose enzoótica em tatus (*Dasybus novemcinctus*), no Estado do Pará. *Rev Inst Med Trop São Paulo* 1986;28:19-27.
5. Bagagli E., Sano A., Coelho K.I.R., et al. Isolation of *Paracoccidioides brasiliensis* from armadillos (*Dasybus novemcinctus*) captured in an endemic area of paracoccidioidomycosis. *Am J Trop Med Hyg* 1998;58:505-12.
6. Corredor G.G., Castaño J.H., Peralta L.A., et al. Isolation of *Paracoccidioides brasiliensis* from the nine-banded-armadillo *Dasybus novemcinctus*, in an endemic area for paracoccidioidomycosis in Colombia. *Rev. Iberoam. Micol* 1999;16:216-20.
7. Peraçoli M.T.S., Sugizaki M.F., Mendes R.P., et al. *Paracoccidioides brasiliensis* isolated from armadillos is virulent to Syrian hamsters. *Mycopathologia (Den Haag)* 1999;148:123-30.
8. Hebler-Barbosa F., Montenegro M.R., Bagagli E. Virulence profiles of ten *Paracoccidioides brasiliensis* isolates obtained from armadillos (*Dasybus novemcinctus*). *Med Mycol* 2003a;41:89-96.

9. Macoris S.A.G., Sugizaki M.F., Peraçoli, M.T.S., et al. Virulence attenuation and phenotypic variation of *Paracoccidioides brasiliensis* isolates obtained from armadillos and patients. Mem Inst Oswaldo Cruz **2006**;101:331-4.
10. Kurokawa C.S., Lopes C.R., Sugizaki M.F., et al. Virulence of profile of ten *Paracoccidioides brasiliensis* isolates. Association with morphologic and genetic patterns. Rev Inst Med Trop S Paulo **2005**;47:257-62.
11. Calich V.L.G., Singer-Vermes L.M., Russo M., et al. Immunogenetics in Paracoccidioidomycosis. In: Franco M.F., Lacaz C.S., Restrepo A., Del Negro G. eds. Paracoccidioidomycosis. Boca Raton, FL., CRC Press, **1994**.
12. Coelho K.I., Defaveri J., Rezkallah-Iwasso M.T., Peraçoli M.T.S. Experimental paracoccidioidomycosis. In: Franco M.F., Lacaz C.S., Restrepo A., Del Negro G. eds. Paracoccidioidomycosis. Boca Raton, Fla., CRC Press, **1994**.
13. Kashino S.S., Calich V.L.G., Burger E., Singer-Vermes L.M. *In vivo* and *in vitro* characteristics of six *Paracoccidioides brasiliensis* strains. Mycopathologia (Den Haag) **1985**;92:173-8.
14. Singer-Vermes L.M., Burger E., Franco M.F., et al. Evaluation of the pathogenicity and immunogenicity of seven *Paracoccidioides brasiliensis* isolates in susceptible inbred mice. J Med Vet Mycol **1989**;27:71-82.
15. Vidal M.S., De Melo N.T., Garcia N.M., et al. *Paracoccidioides brasiliensis*. A mycological and immunochemical study of a sample isolate from an armadillo (*Dasyus novemcinctus*). Rev Inst Med Trop Sao Paulo **1995**; 37:43-9.
16. Sano A., Tanaka R., Yokoyama K., et al. Comparison between human and armadillo *Paracoccidioides brasiliensis* isolates by random amplified polymorphic DNA analysis. Mycopathologia (Den Haag) **1999**;143:165-9.
17. Camargo Z.P., Franco M.F. Current knowledge on pathogenesis and immunodiagnosis of paracoccidioidomycosis. Rev Iberoam Micol **2000**;17:41-48.
18. Biagioni L., Souza M.J., Chamma L.G., et al. Serology of paracoccidioidomycosis. II. Correlation between class-specific antibodies and clinical forms of the disease. Trans R Soc Trop Med Hyg **1984**;78:617-21.
19. Singer-Vermes L.M., Caldeira C.B., Burger E., Calich V.L.G., Experimental murine paracoccidioidomycosis: relationship among the dissemination of the infection, humoral and cellular immune responses. Clin Exp Immunol **1993**;94:75-9.
20. Moscardi-Bacchi M., Brummer E., Stevens D.A. Support of *Paracoccidioides brasiliensis* multiplication by human monocytes or macrophages: inhibition by activated phagocytes. J Med Microbiol **1994**;40:159-64.
21. Calich V.L.G., Vaz C.A.C., Burger E. Immunity to *Paracoccidioides brasiliensis* infection. Res Immunol **1998**;149:407-17.
22. Zacharias D., Ueda A., Moscardi-Bacchi M., Franco M., San-Blás G. A comparative histopathological, immunological and biochemical study of experimental intravenous paracoccidioidomycosis induced in mice by three *Paracoccidioides brasiliensis* isolates. J Med Vet Mycol **1986**;24:445-54.
23. Soares A.M.V.C., Calvi S.A., Peraçoli M.T.S., et al. Modulatory effect of prostaglandins on human monocyte activation for killing of high- and low- virulence strains of *Paracoccidioides brasiliensis*. Immunol **2001**;102:480-5.
24. Sano A., Tanaka R., Nishimura K., et al. Characteristics of 17 *Paracoccidioides brasiliensis* isolates. Mycoscience **1997**;38:117-22.
25. Singer-Vermes L.M., Ciavaglia M.C., Kashino S.S., et al. The source of the growth-promoting factor(s) affects the plating efficiency of *Paracoccidioides brasiliensis*. J Med Vet Mycol **1992**;30:261-4.
26. Camargo Z.P., Taborda C.P., Rodrigues E.G., Travassos L.R. The use of cell-free antigens of *Paracoccidioides brasiliensis* in serological tests. J Med Vet Mycol **1991**;29:31-8.
27. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the folin phenol reagent. J Biol Chem **1951**;193:265-75.
28. Fazioli R.A., Singer-Vermes L.M., Kashino S.S., et al. Delayed-type hypersensitivity response in an isogenic murine model of paracoccidioidomycosis. Mycopathologia (Den Haag) **1994**;126:137-46.
29. Brummer E., Restrepo A., Hanson L.H., Stevens D.A. Virulence of *Paracoccidioides brasiliensis*: the influence of *in vitro* passage and storage. Mycopathologia (Den Haag) **1990**;109:13-7.
30. Singer-Vermes L.M., Burger E., Calich V.L.G., et al. Pathogenicity and immunogenicity of *Paracoccidioides brasiliensis* in the human disease and in an experimental murine model. Clin Exp Immunol **1994**; 97:113-9.
31. Brass C., Volkmann C.M., Philpott D.E., et al. Spontaneous mutant of *Blastomyces dermatitidis* attenuated in virulence for mice. Sabouradia **1982**;20:145-58.
32. Franzot S.P., Murkherjee J., Cherniak R., et al. Microevolution of a standard strain of *Cryptococcus neoformans* resulting in differences in virulence and other phenotypes. Infect Immun **1998**;66:89-97.
33. Fava-Netto C. The serology of paracoccidioidomycosis: present and future trends. Pan Am Health Organ **1972**;254:209-13.
34. Hebel-Barbosa F., Morais F.V., Montenegro M.R., et al. Comparison of the sequences of the internal transcribed spacer regions and Pbgp43 genes of *Paracoccidioides brasiliensis* from patients and armadillos (*Dasyus novemcinctus*). J Clin Microbiol **2003b**;41:5735-7.