Mycopathologia (2011) 171:197–202
DOI 10.1007/s11046-010-9366-6

# Serological Evidence of *Paracoccidioides brasiliensis* Infection in Chickens from Paraná and Mato Grosso do Sul States, Brazil

**Gabriela G. Oliveira · Luciane H. Silveira · Eiko N. Itano · Rodrigo M. Soares · Roberta L. Freire · Maria A. E. Watanabe · Zoilo P. Camargo · Mario A. Ono**

Received: 11 February 2010 / Accepted: 7 September 2010 / Published online: 19 September 2010


**Abstract** The objective of this study was to detect antibodies against *Paracoccidioides brasiliensis* in free-range and caged chickens *Gallus domesticus*. Initially, the humoral immune response of two chickens immunized with *P. brasiliensis* was evaluated. Both animals showed the production of antibodies to gp43, the major *P. brasiliensis* antigen. The seroepidemiological survey was conducted in chickens from the Pantanal region in Mato Grosso do Sul State (free-range $n = 40$) and from northern region of Paraná State (free-range $n = 100$, caged $n = 43$). The serum samples were analyzed by indirect ELISA using gp43 as antigen. The positivity observed in free-range chickens from Mato Grosso do Sul (55%) was significantly higher ($P = 0.0001$) than in free-range chickens from Paraná State (16%). In contrast to the free-range chickens, no positivity was observed in the caged chickens ($P = 0.003$). This is the first report showing serological evidence of *P. brasiliensis* infection in chickens. The results suggest that free-range chickens are more frequently infected by *P. brasiliensis*, probably due to the constant contact with soil than caged chickens and could be useful as epidemiological markers of paracoccidioidomycosis.

**Keywords** Paracoccidioidomycosis · Epidemiology · Free-range chickens · Pantanal

G. G. Oliveira · E. N. Itano · M. A. E. Watanabe · M. A. Ono (✉)
Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Campus Universitário, Londrina, Paraná 86051-980, Brazil
e-mail: marioono@uel.br

L. H. Silveira
Departamento de Patologia Geral, Faculdades Luiz Meneghel, Bandeirantes, Paraná, Brazil

R. M. Soares
Departamento de Medicina Veterinária Preventiva e Saúde Animal Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil

R. L. Freire
Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Londrina, Paraná, Brazil

Z. P. Camargo
Disciplina de Biologia Celular, Universidade Federal de São Paulo, São Paulo, Brazil

## Introduction

*Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis, a systemic mycosis that affects mainly rural workers in Latin American countries. This fungus is thermodimorphic, growing as yeast in the host tissue or when cultured at 37°C and as mycelium when cultured at lower temperatures [1].

Despite the attempts of several researchers to disclose the *P. brasiliensis* habitat, it remains undefined to date. The soil is considered the probable fungus habitat [2–8], and the infection occurs by the respiratory route [9].

Epidemiological studies have demonstrated that domestic (dogs, cows, horses and sheep) and wild animals (armadillos and monkeys) may be infected by *P. brasiliensis* [10–19]. The fungus has also been isolated from armadillos in Brazil and Colombia, bats in Colombia, a penguin in Uruguay and a dog in Brazil [20–25]. Recently, the infection by *P. brasiliensis* was detected by nested-PCR in tissues from road-killed wild animals such as armadillos, a guinea pig, a porcupine, raccoons and grisons [26].

High positivity to *P. brasiliensis* infection has been described by our group in dogs from Paraná and Mato Grosso do Sul States [11, 12], which are endemic areas for human paracoccidioidomycosis [27, 28].

Taking into account that the animals exposed to soil are at risk of *P. brasiliensis* infection, the objective of this study was to evaluate the infection by *P. brasiliensis* in free-range chickens, which are in constant contact with soil and caged chickens that have no contact with the soil.

## Materials and Methods

### Studied Area

This study was carried out in five municipalities in Paraná State and two municipalities in the Pantanal region of Mato Grosso do Sul State (Fig. 1).

The municipalities of Bandeirantes (latitude 23°06′36″ S, longitude 50°27′28″ W, altitude 420 m), Itambaracá (latitude 23°03′45″S, longitude 50°26′15″W, altitude 402 m), Andirá (latitude 23°03′02″S, longitude 50°13′44″W, altitude 479 m) Cambará (latitude 20°02′00″S, longitude 50°06′00″, altitude 450 m), Santa Mariana (latitude 23°11′15″S, longitude 50°33′45″W, altitude 484 m) are located in north Paraná State. The climate is humid subtropical with mean annual temperatures of 22°C and annual rainfall of 1,500 mm to 2,000 mm. The rainy season is from December to February, and oxisol is the predominant soil. The municipalities of Aquidauana (latitude 20° 28′ 15″ S, longitude 55° 47′ 13″ W, altitude 174 m) and Rio Verde (latitude 18° 55′ 4″ S, longitude 54° 50′ 38″ W, altitude 162 m) are located in Pantanal region of Mato Grosso do Sul State, Midwestern Brazil. The climate of both municipalities is humid subtropical with mean annual temperatures of 27°C, annual rainfall of 1,100 mm, and the rainy season is from November and April. Sandy soil is predominant in the Pantanal region.


**Fig. 1** Map showing the municipalities of Aquidauana (*a*) and Rio Verde (*b*) in Mato Grosso do Sul State, and Santa Mariana (*c*), Bandeirantes (*d*), Itambaracá (*e*), Andirá (*f*), Cambará (*g*) in Paraná State

### Animals

The serum samples for the seroepidemiologic study were collected from the northern region of Paraná State and the Pantanal region in Mato Grosso do Sul by convenience sampling, as follows: 100 free-range chickens (88 female and 12 male) and 43 caged chickens (all females) from north Paraná State, 40 free-range chickens (23 female and 17 male) from the Pantanal region of Mato Grosso do Sul State.

### *P. brasiliensis* Antigens

The cellular antigen for chicken immunization was obtained from *P. brasiliensis* B-339 isolate as previously described [11]. The exoantigen was obtained from culture of *P. brasiliensis* B-339 as described by Camargo et al. [29], and the gp43 antigen was purified from *P. brasiliensis* exoantigen by immunoaffinity chromatography as described by Puccia and Travassos [30].

### Chicken Immunization

Two chickens, maintained in individual cages with water and commercial feed *ad libitum*, were inoculated with *P. brasiliensis* B-339 ($1 \times 10^6$ inactivated yeast cells) in Incomplete Freund Adjuvant (Sigma, Saint Louis, MO, USA). Each animal received three doses intramuscularly into the breast muscle at two weekly intervals. The antibody production was evaluated in egg yolk samples by indirect ELISA using gp43 as antigen. The yolk antibody extracts were obtained by dilution of yolk 1:1 (vol/vol) in distilled water and heating at 62°C for 15 min in order to separate the yolk lipids from the proteins. The samples were centrifuged at 11,500*g* for 10 min, and the supernatants were used for the indirect ELISA [31].

### Indirect ELISA with gp43

The egg yolk samples from immunized chickens and 183 serum samples from free-range and caged chickens were analyzed by indirect ELISA using gp43 as antigen. Flat bottom microtiter polystyrene plates (Costar Corporation, Corning, NY, USA) were coated at 4°C overnight with 100 µl gp43 (250 ng/well) in carbonate buffer 0.1 M, pH 9.6. After washing with PBS-T (PBS with 0.05% Tween 20), the wells were blocked with 5% skim milk in PBS for 1 h at 25°C. After washing with PBS-T, the egg yolk samples diluted 1:200, or serum samples diluted 1:100 in PBS-1% skim milk (100 µl/well), were incubated at 25°C for 1 h. The plates were washed with PBS-T, and 100 µl/well anti-chicken IgY-peroxidase conjugate (Sigma, St Louis, MO, USA) was added followed by incubation for 1 h at 25°C. After washing with PBS-T, 100 µl substrate-chromogen ($H_2O_2$/TMB) was added. The reaction was stopped by the addition of 4 N $H_2SO_4$ (50 µl/well). The absorbance at 450 nm was measured in a Microplate Reader (Biotek Instruments Inc., Winooski, VT, USA). All the samples were analyzed twice. The positive and negative controls used in the seroepidemiological study were serum from a chicken immunized with *P. brasiliensis* and a pool of young caged chickens, respectively. The serum samples with twofold absorbance of the negative control were considered positive.

### Data Analysis

The statistical analysis was performed with the program Epi Info 3.5.1, and data were analyzed by the Pearson chi-square and Fisher's exact test. The values of $P < 0.05$ were considered statistically significant.

## Results

### Humoral Immune Response of Caged Chickens Immunized with *P. brasiliensis*

The two chickens immunized with *P. brasiliensis* yeast cells produced antibodies to gp43, showing a similar reactivity with a higher response after the third dose of antigen (Fig. 2).

### Seroepidemiology of *P. brasiliensis* Infection in Chickens

The free-range chickens from Mato Grosso do Sul State showed a significantly higher positivity to gp43 in the ELISA test (55%) than free-range chickens from Paraná State (16.0%) (Table 1).

The caged chickens from Paraná showed a significantly lower positivity to gp43 (0%) than free-range chickens (16%) from the same region (Table 2).

The positivity to gp43 observed in free-range chickens from both regions showed no significant difference in relation to sex (Table 3).

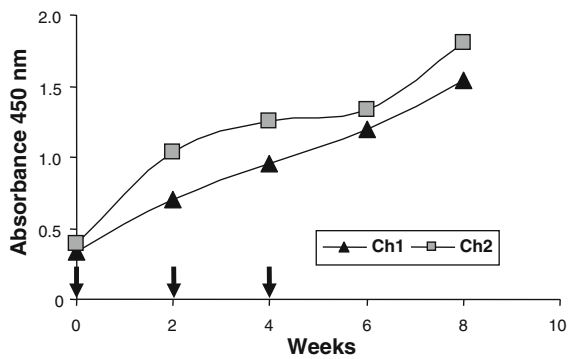


**Fig. 2** Antibody production against gp43 determined by ELISA in two chickens immunized with three doses (*arrows*) of inactivated *P. brasiliensis* yeast cells

**Table 1** Reactivity to *P. brasiliensis* gp43 antigen evaluated by indirect ELISA in serum samples from free-range chickens from Paraná and Mato Grosso do Sul States

| Free-range chickens | Positive *n* (%) | Negative *n* (%) |
|---|---|---|
| Paraná | 16 (16) | 84 (84) |
| Mato Grosso do Sul | 22 (55) | 18 (45) |

*P* value = 0.0001

**Table 2** Reactivity to *P. brasiliensis* gp43 antigen evaluated by indirect ELISA in serum samples from free-range and caged chickens from Paraná State

| Chickens | Positive *n* (%) | Negative *n* (%) |
|---|---|---|
| Free-range | 16 (16) | 84 (84) |
| Caged | 0 (0) | 43 (100) |

*P* value = 0.003

## Discussion

*P. brasiliensis* infection has been reported in several species of domestic and wild animals initially by means of skin tests and more recently by the ELISA test with purified gp43.

This study evaluated initially the humoral immune response against gp43 in chickens immunized with *P. brasiliensis*, the main antigen used for immunodiagnosis and seroepidemiology of paracoccidioidomycosis. The high response observed in both animals suggested that gp43 antigen is also immunogenic to chickens, as observed by our group in dogs and cattle [11, 13], and consequently can be used for seroepidemiology of *P. brasiliensis* infection in chickens.

The higher positivity observed in free-range chickens from the Pantanal region in Mato Grosso do Sul State was probably due to the regional characteristics that could be more favorable for *P. brasiliensis* development. In a study carried out by our group to evaluate paracoccidioidomycosis infection in dairy cattle from Mato Grosso do Sul, a significantly higher positivity to paracoccidioidomycosis was observed in animals from the Pantanal region [13]. Terçarioli et al. 2007 [7] showed that *P. brasiliensis* may grow and produce infectious conidia both in sand and clay soils with high humidity, as occurs in the Pantanal region that is usually humid and hot, with periods of flood [32]. Taking into account that the favorable range of altitude for *P. brasiliensis* is very variable (47–1,300 m above the sea level) [8] and the means of annual temperature and rainfall are similar in both regions studied, it is possible that the high humidity in Pantanal region is a more relevant factor for the production of infective propagules by *P. brasiliensis*.

The higher positivity observed in free-range chickens when compared to caged chickens reinforce that frequent contact with soil can be considered an important risk factor for *P. brasiliensis* infection.

As observed in other studies with humans and other animal species, the difference in positivity was not statistically significant in relation to sex, suggesting that this is not a risk factor for paracoccidioidomycosis infection [11, 19].

Free-range chickens, due to their constant contact with soil, are more exposed to infectious diseases and can be used as epidemiological indicators of several pathogens.

Although gp43 had been successfully used in serodiagnosis and seroepidemiology of paracoccidioidomycosis, some cross-reactivity with other pathogen antigens may be occurring due to sharing of common epitopes with other pathogenic fungi as *Lacazia loboi* (syn. *Loboa loboi*) [33]. Therefore, more studies are required in order to evaluate whether chickens can develop paracoccidioidomycosis disease. This is the first report showing serological evidence of *P. brasiliensis* infection in chickens.

**Table 3** Reactivity to *P. brasiliensis* gp43 antigen evaluated by indirect ELISA in serum samples from free-range chickens from Paraná and Mato Grosso do Sul States, according to sex

| Sex | Paraná | | Mato Grosso do Sul | |
|---|---|---|---|---|
| | Positive *n* (%) | Negative *n* (%) | Positive *n* (%) | Negative *n* (%) |
| Female | 15 (17.1) | 73 (82.9) | 14 (60.9) | 9 (39.1) |
| Male | 1 (8.3) | 11 (91.6) | 7 (41.2) | 10 (58.8) |

**Acknowledgments** The authors thank the CNPq, CAPES and the Araucária Foundation for financial support and the CNPq for the productivity fellowship granted to MA Ono, EN Itano, RM Soares, MAE. Watanabe.

## References


1. Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: an update. Clin Microbiol Rev. 1993;6:89–117.
2. Negroni P. El *Paracoccidioides brasiliensis* vive saprofiticamente en el suelo argentino. Prensa Méd Argent. 1966;53:2831–2.
3. Albornoz MB. Isolation of *Paracoccidioides brasiliensis* from rural soil in Venezuela. Sabouraudia. 1971;9:248–53.
4. Silva-Vergara ML, Martinez R, Chadu A, Madeira M, Freitas-Silva G, Maffei CML. Isolation of *Paracoccidioides brasiliensis* strain from the soil of a coffee plantation in Ibiá, State of Minas Gerais, Brazil. Med Mycol. 1998;36:37–42.
5. Conti-Diaz IA, Rilla FD. Hipótesis sobre el nicho ecológico de *Paracoccidioides brasiliensis*. Rev Méd Urug. 1989;5:97–103.
6. McEwen JG, Garcia AM, Ortiz BL, Botero S, Restrepo A. In search of the natural habitat of *Paracoccidioides brasiliensis*. Arch Med Res. 1995;26:305–6.
7. Terçarioli GR, Bagagli E, Reis GM, Theodoro RC, Bosco SMG, Macoris SAG, Pereira VBR. Ecological study of *Paracoccidioides brasiliensis* in soil: growth ability, conidia production and molecular detection. BMC Microbiol. 2007;7:92.
8. Restrepo A. The ecology of *Paracoccidioides brasiliensis*: a puzzle still unsolved. Sabouraudia. 1985;23:323–34.
9. Camargo ZP, Franco MF. Current knowledge on pathogenesis and immunodiagnosis of paracoccidioidomycosis. Rev Iberoam Micol. 2000;17:41–8.
10. Mós EN, Fava-Netto C. Contribuição ao estudo da paracoccidoidomicose I—Possível papel dos cães: Estudo sorológico e anatomo-patológico. Rev Inst Med Trop São Paulo. 1974;16:154–9.
11. Ono MA, Bracarense APFRL, Morais HSA, Trapp SM, Belitardo DR, Camargo ZP. Canine paracoccidioidomycosis: a seroepidemiologic study. Med Mycol. 2001;39:277–82.
12. Silveira LH, Domingos IH, Kouchi K, Itano EN, Silva EA, Landgraf VO, Werneck SM, Camargo ZP, Ono MA. Serological detection of antibodies against *Paracoccidioides brasiliensis* in dogs with leishmaniasis. Mycopathologia. 2006;162:325–9.
13. Silveira LH, Paes RCS, Medeiros EV, Itano EM, Camargo ZP, Ono MA. Occurrence of antibodies to *Paracoccidioides brasiliensis* in dairy cattle from Mato Grosso do Sul, Brazil. Mycopathologia. 2008;165:367–71.
14. Fontana FF, Santos CTB, Esteves FM, Rocha A, Fernandes GF, Amaral CC, Domingues MA, Camargo ZP, Silva-Vergara ML. Seroepidemiological survey of paracoccidioidomycosis infection among urban and rural dogs from Uberaba, Minas Gerais, Brazil. Mycopathologia. 2010;169:159–65.
15. Conti-Diaz IA, Alvarez BJ, Gezuele E, Gonzalez MH, Duarte J, Falcon J. Intradermal reaction survey with paracoccidioidin and histoplasmin in horses. Rev Inst Med Trop São Paulo. 1972;14:372–6.
16. Corte AC, Itano EN, Freire RL, Camargo ZP, Ono MA. Detection of antibodies to *Paracoccidioides brasiliensis* in horses from northern Region of Paraná State. Semina-Ciências Agrárias. 2009;30:441–6.
17. Costa EO, Fava-Netto C. Contribution to the epidemiology of paracoccidioidomycosis and histoplasmosis in the State of São Paulo, Brazil. Paracoccidioidin and Histoplasmin intradermic tests in domestic animals. Sabouraudia. 1978;16:93–101.
18. Fernandes GF, Deps P, Tomimori-Yamashita J, Camargo ZP. IgM and IgG antibody response to *Paracoccidioides brasiliensis* in naturally infected wild armadillos (*Dasypus novemcinctus*). Med Mycol. 2004;42:363–8.
19. Corte AC, Svoboda WK, Navarro IT, Freire RL, Malanski LS, Shiozawa MM, Ludwig G, Aguiar LM, Passos FC, Maron A, Camargo ZP, Itano EN, Ono MA. Paracoccidioidomycosis in wild monkeys from Paraná State, Brazil. Mycopathologia. 2007;64:225–8.
20. Naiff RD, Ferreira LCL, Barret TV, Naif MF, Arias JR. Paracoccidioidomicose enzoótica em tatus (*Dasypus novemcinctus*) no estado do Pará. Rev Inst Med Trop São Paulo. 1986;28:19–27.
21. Bagagli E, Sano A, Coelho KI, Alquati S, Miyaji M, Camargo ZP, Gomes GM, Franco M, Montenegro MR. Isolation of *Paracoccidioides brasiliensis* from armadillos *(Dasypus novemcinctus)* captured in an area of paracoccidioidomycosis. Am J Trop Med Hyg. 1998;58:505–12.
22. Corredor GG, Castaño JH, Peralta LA, Díez S, Arango M, McEwen J, Restrepo A. Isolation of *Paracoccidioides brasiliensis* from the nine-banded armadillo *Dasypus novemcinctus*, in an endemic area for paracoccidioidomycosis in Colombia. Rev Iberoam Micol. 1999;16:216–20.
23. Grose E, Tamsitt JR. *Paracoccidioides brasiliensis* recovered from intestinal tract of three bats (*Artibeus lituratus*) in Colombia, S.A. Sabouraudia. 1965;4:124–5.
24. Gezuele E. Aislamiento de *Paracoccidioides brasiliensis* de heces de pinguino de la Antardida. IV Encuentro Internacional sobre Paracoccidioidomicosis, Caracas. Venezuela, Instituto Venezuelano de Investigaciones Cientificas (IVIC): 1989, Abstract B-2.
25. Bosco SMG, Theodoro RC, Macoris SAG, Farias MR, Muro M, Ribeiro MG, Bagagli E. Morphological and molecular characterization of the first isolate of *Paracoccidioides brasiliensis* from dog (*Canis familiaris*). Rev Inst Med Trop São Paulo. 2005;47(Suppl.14):62–3.
26. Richini-Pereira VB, Bosco SM, Griese J, Theodoro RC, Macoris SA, Silva RJ, Barrozo L, Tavares PM, Zancopé-Oliveira RM, Bagagli E. Molecular detection of *Paracoccidioides brasiliensis* in road-killed wild animals. Med Mycol. 2008;46:35–40.
27. Paniago AM, Aguiar JI, Aguiar ES, Cunha RV, Pereira GR, Londero AT, Wanke B. Paracoccidioidomycosis: a clinical and epidemiological study of 422 cases observed in Mato Grosso do Sul. Rev Soc Bras Med Trop. 2003;36:455–9.
28. Bittencourt JI, de Oliveira RM, Coutinho ZF. Paracoccidioidomycosis mortality in the State of Paraná, Brazil, 1980/1998. Cad Saude Publica. 2005;21:1856–64.
29. Camargo ZP, Unterkircher CS, Campoy SP, Travassos LR. Production of *Paracoccidioides brasiliensis* exoantigens

for immunodiffusion tests. J Clin Microbiol. 1988;26: 2147–51.
30. Puccia R, Travassos LR. The 43-kDa glycoprotein from the human pathogen *Paracoccidioides brasiliensis* and its deglycosylated form: excretion and susceptibility to proteolysis. Arch Biochem Biophys. 1991;289:298–302.
31. Holt PS, Stone HD, Gast RK, Greene CR. Application of the agar gel precipitin test to detect antibodies to *Salmonella enterica* serovar enteritidis in serum and egg yolks from infected hens. Poult Sci. 2000;79:1246–50.
32. Alho CJ. Biodiversity of the Pantanal: response to seasonal flooding regime and to environmental degradation. Braz J Biol. 2008;68:957–66.
33. Camargo ZP, Baruzzi RG, Maeda SM, Floriano MC. Antigenic relationship between *Loboa loboi* and *Paracoccidioides brasiliensis* as shown by serological methods. Med Mycol. 1998;36:413–7.