Mycopathologia (2014) 177:91–95
DOI 10.1007/s11046-013-9724-2

Detection of Antibodies Against *Paracoccidioides brasiliensis* in Free-Range Domestic Pigs (*Sus scrofa*)

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Received: 29 November 2013 / Accepted: 21 December 2013 / Published online: 17 January 2014

Abstract Paracoccidioidomycosis, caused by the thermodimorphic fungus *Paracoccidioides brasiliensis*, is a human systemic mycosis prevalent in Latin America. Paracoccidioidomycosis affects mainly male rural workers, causing granulomatous lesions in several organs such as the lungs, liver and spleen. The participation of other animal species in the fungus epidemiology is not well understood. The objective of this study was to evaluate the infection of free-range domestic pigs by *P. brasiliensis*. Serum samples from 106 pigs were analyzed by ELISA and the immunodiffusion test, using *P. brasiliensis* gp43 and exoantigen as antigens, respectively. The overall positivity to gp43 in ELISA was 37.7 %, although no reactivity was observed in the immunodiffusion test and nor was *P. brasiliensis* detected in tissue samples (spleen, lung, liver and lymph nodes) from slaughtered animals submitted to culture, histopathological examination and PCR analysis. Five pigs seronegative to gp43 were exposed to natural infection by *P. brasiliensis,* and all animals seroconverted 3 months after exposure. The results suggest that free-range pigs are frequently infected with *P. brasiliensis* but are resistant to disease development. This is the first report of paracoccidioidomycosis in pigs.

Keywords *Paracoccidioides brasiliensis* · *Sus scrofa* · Epidemiology

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Introduction

Paracoccidioidomycosis is a systemic mycosis described for the first time in 1908 in Brazil [1]. The disease is endemic in most Latin American countries and affects mainly rural workers.

The etiologic agent is the thermodimorphic fungus *Paracoccidioides brasiliensis* [2], and infection occurs by inhalation of fungus propagules. The lungs are primarily affected followed by dissemination to other tissues such as the liver, spleen, lymph nodes and skin [3]. Paracoccidioidomycosis can be classified as paracoccidioidomycosis disease, when lesions occur in one or more organs, and paracoccidioidomycosis infection that occurs in asymptomatic individuals with positive reaction to *P. brasiliensis* antigens [4].

Despite the advances in the pathology and diagnosis of paracoccidioidomycosis, the fungus habitat remains unknown. Isolation of *P. brasiliensis* from soil samples and armadillos that live in close contact

with the soil suggests that the fungus lives as a saprobe in soil such as other pathogenic fungi [5–13].

Infection by *P. brasiliensis* has been reported in epidemiological studies with domestic and wild animals such as dogs [14–19], cats [20], cattle [21], horses [22], chickens [23], sheep [24], goats [25], monkeys [26] and a sloth [27]. Reproducible isolations of the fungus have been obtained until now only from armadillos in Brazil and Colombia, reinforcing the fact that close contact with soil is an important risk factor for infection [6–12].

Taking into account that free-range pigs are in close contact with the ground, the objective of this study was to evaluate the infection of free-range pigs by *P. brasiliensis* in an endemic area for human paracoccidioidomycosis.

Materials and Methods

Study Area

The study was carried out on five farms located in the municipalities of Londrina (latitude 23°51′10″S, longitude 51°14′35″W, altitude 551 m) and Cambé (latitude 23°16′33″S, longitude 51°16′42″, altitude 650 m), Northern Paraná State. The average temperature of the warmest month is usually higher than 25.5 °C and the coldest month, less than 16.4 °C and the annual average relative humidity is 69 %. The average annual rainfall is 1,566 mm, and January, July and December are the rainiest months and June, September and August are the driest months.

Animals

Blood samples were collected from 106 free-range pigs (55 males and 51 females, 2–6 months of age, crossbred) housed in fenced areas in close contact with the ground and fed with crops and food waste. After slaughtering, tissue samples were collected (liver, spleen, lymph nodes and lungs) and divided into three portions. One portion was cultured in Mycosel and Sabouraud dextrose agar and incubated at 35 °C for 8 weeks, and the other two portions were submitted to histopathological examination (hematoxylin–eosin and Grocott staining) and Nested PCR analysis as previously described by Richini-Pereira et al. [28]. This study was approved by the Animal Ethics Committee of the State University of Londrina.

Use of Pigs as Sentinel Animals of Paracoccidioidomycosis

Five crossbred pigs (one male and four females) seronegative to gp43, maintained on the farm with higher seropositivity to *P. brasiliensis* (Londrina), were followed for 4 months to evaluate seroconversion in the ELISA using gp43 as antigen. At the end of 4 months, the animals were slaughtered and tissue samples of the lungs, liver, spleen and lymph nodes were collected for histopathological examination and PCR analysis.

P. brasiliensis Exoantigen and gp43

The exoantigen was obtained from culture of *P. brasiliensis* B-339 as previously described [29], and the gp43 antigen was purified from exoantigen by affinity chromatography [30]. The protein concentration was analyzed according to Bradford [31].

ELISA with gp43

The serum samples were analyzed by indirect ELISA using gp43 as antigen. Microtiter polystyrene plates were coated with 100 µl gp43 (250 ng/well), and after washing with PBS-T (PBS with 0.05 % Tween-20), the wells were blocked with 5 % skim milk in PBS for 1 h. After washing with PBS-T, pig serum samples diluted 1:100 in PBS/1 % skim milk were incubated for 1 h. The plates were washed with PBS-T, and anti-pig IgG-peroxidase conjugate was added followed by incubation for 1 h. After washing with PBS-T, chromogen/substrate solution (H_2O_2/TMB) was added. The reaction was stopped with 4 NH_2SO_4, and the absorbance at 450 nm was analyzed in a microplate reader. All samples were analyzed twice. Positive and negative controls were a serum sample from a pig immunized with *P. brasiliensis* and a pool of sera from young pigs, respectively. Serum samples with twofold or more the absorbance of the negative control were considered positive.

Immunodiffusion Test

The immunodiffusion test was performed according to Camargo et al. [29] using *P. brasiliensis* exoantigen as reagent.

Table 1 Reactivity of 106 serum samples from free-range pigs to *P. brasiliensis* evaluated by ELISA (gp43) and immunodiffusion (exoantigen) according to sex and farms

Sex	ELISA		Immunodiffusion	
	Positive *n* (%)	Negative *n* (%)	Positive *n* (%)	Negative *n* (%)
Male	23 (41.8)	32 (58.2)	0	55 (100)
Female	17 (33.3)	34 (66.7)	0	51 (100)
Farms				
A (Londrina)	23 (69.7)*	10 (30.3)	0	33 (100)
B (Cambé)	14 (28.0)	36 (72.0)	0	50 (100)
C (Irerê District)	3 (13.0)	20 (87.0)	0	23 (100)
Total	40 (37.7)	66 (62.3)	0	106 (100)

* $P = 0.001$

Statistical Analysis

The statistical analysis was performed with the program BioStat 2009 Professional Package Analyst Soft, and data were analyzed by the Pearson chi-square test and the Tukey test. The values of $P < 0.05$ were considered statistically significant.

Results

Seroepidemiological Study

The analysis of serum samples from 106 free-range pigs by ELISA using gp43 as antigen showed 37.7 % positivity, and no significant difference was observed between males (41.8 %) and females (33.3 %). No reactivity was observed in the immunodiffusion test (Table 1).

The reactivity of pigs to *P. brasiliensis* from one of the farms (69.7 %) was significantly higher than the two other farms (13 and 28 %) (Table 1).

Culture, Histopathological Examination and PCR Analysis of Tissue Samples

The fungus *P. brasiliensis* was not detected in samples of the spleen, lungs, liver, lymph nodes from slaughtered pigs ($n = 40$) by culture, histopathological examination and PCR analysis.

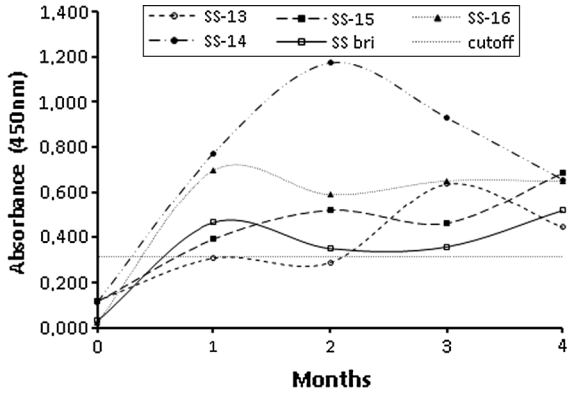

Fig. 1 Levels of IgG anti-gp43 evaluated by ELISA in serum samples from sentinel pigs exposed to natural infection with *P. brasiliensis*

Use of Pigs as Sentinel Animals for Paracoccidioidomycosis

A seroconversion of 80 % was observed in five pigs in the first month after exposure to natural infection with *P. brasiliensis,* and all animals were positive to gp43 in the third month (Fig. 1).

Discussion

ELISA with gp43, because it is highly sensitive, has been used in the immunodiagnosis of paracoccidioidomycosis in humans [32] and also in seroepidemiological studies to evaluate *P. brasiliensis* infection in domestic [16–19, 21–25] and wild animals [26].

Despite the high frequency of infection by *P. brasiliensis* in domestic and wild animals, the development of the disease has only been reported in armadillos [7], two dogs in Brazil [14, 15], a cat in Chile [20] and a two-toed sloth in Mexico [27].

In this study, the free-range pigs showed an overall positivity of 37.7 % to *P. brasiliensis* infection, and the animals from one of the farms showed a significantly higher positivity frequency (69.7 %). A higher infection frequency (89.5 %) was observed by our group in dogs from the same region [19], probably because the pigs observed in this study were very young (2–6 months). Age was considered a risk factor for *P. brasiliensis* infection in dogs and cattle [18, 21]. Dogs under one and cattle under 2 years of age showed a significantly lower positivity than older animals [18, 21].

The free-range pigs exposed to natural infection with *P. brasiliensis* showed a seroconversion of 80 and 100 % in the first and third month, respectively, although the fungus was not detected by histopathological and PCR analysis in tissue samples. These results suggest that despite the high infection frequency, the free-range pigs are resistant to development of the disease. An 83.3 % seroconversion rate was observed in free-range rabbits exposed for 6 months to natural infection with *P. brasiliensis* in the same region as this study and one animal was positive in histopathological and PCR analysis [33]. The pigs showed higher seroconversion in a shorter period of time than rabbits probably due to their soil-eating behavior [34].

The failure to detect *P. brasiliensis* in tissues from free-range pigs suggests that after infection, the fungus is cleared from tissues by the animals' immune response. Although in this study the animals apparently have shown only infection, it is not possible rule out that the disease would develop in a longer follow-up period.

Sentinel animals may be useful as indicators of *P. brasiliensis* presence in the environment and consequently contribute to elucidating the fungus habitat.

The veterinarians from paracoccidioidomycosis endemic areas must be alert for the possibility of the development of this mycosis in pigs and other production animals. This is the first study reporting paracoccidioidomycosis in pigs.

Acknowledgments The authors thank the farmers Jose Quinteiro, Geraldo Gomes Guerreiro, José Crespin, João Baptista da Silva and Geraldo Nicodemos da Silva who participated in the study, the CNPq and Araucária Foundation for financial support and the CNPq for the productivity fellowship granted to Z. P. Camargo and M. A. Ono.

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