

Molecular detection of *Paracoccidioides brasiliensis* in road-killed wild animals

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Paracoccidioides brasiliensis infections have been little studied in wild and/or domestic animals, which may represent an important indicator of the presence of the pathogen in nature. Road-killed wild animals have been used for surveillance of vectors of zoonotic pathogens and may offer new opportunities for eco-epidemiological studies of paracoccidioidomycosis (PCM). The presence of *P. brasiliensis* infection was evaluated by Nested-PCR in tissue samples collected from 19 road-killed animals; 3 *Cavia aperea* (guinea pig), 5 *Cerdocyon thous* (crab-eating-fox), 1 *Dasypus novemcinctus* (nine-banded armadillo), 1 *Dasypus septemcinctus* (seven-banded armadillo), 2 *Didelphis albiventris* (white-eared opossum), 1 *Eira barbara* (tayra), 2 *Gallitaxis vittata* (grison), 2 *Procyon cancrivorus* (raccoon) and 2 *Sphiggurus spinosus* (porcupine). Specific *P. brasiliensis* amplicons were detected in (a) several organs of the two armadillos and one guinea pig, (b) the lung and liver of the porcupine, and (c) the lungs of raccoons and grisons. *P. brasiliensis* infection in wild animals from endemic areas might be more common than initially postulated. Molecular techniques can be used for detecting new hosts and mapping 'hot spot' areas of PCM.

Keywords *Paracoccidioides brasiliensis*, Paracoccidioidomycosis, road-killed animals, molecular epidemiology

Introduction

Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM), the most important and prevalent systemic mycosis in Latin America, mainly in Brazil, Colombia and Venezuela [1]. The fungus is thermo-dimorphic, growing as multi-budding

yeast cells in the hosts or when cultured at 35–37°C and as a mold under saprobic conditions of 28–30°C. It is in the latter phase that the fungus produces its infective propagula [2]. Since the recovery of *P. brasiliensis* from environmental sources is a rare event and since the disease has a prolonged latency period (with no outbreaks), its exact niche in nature remains a mystery. The observation that the nine-banded armadillo *Dasypus novemcinctus*, a primitive mammal that evolved in the same geographic area as *P. brasiliensis*, is naturally infected by the fungus opened up new research opportunities. The animal has been used as both a sentinel for locating risk areas of the disease and for supplying insights about the

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pathogen's evolution [3]. Could the pathogen's association with animal hosts be a primary strategy for the fungus to survive in nature or are such infections just a blind alley? These questions point to the necessity of looking for the fungus in several wild mammals. Wild animals, by living outdoors all the time and being constantly exposed to airborne pathogens, have been considered better environmental indicators of human risk than companion animals [4]. Since preservation of wildlife is a real concern, we are proposing herein to use highly sensitive molecular techniques with road-killed wild animals to detect the fungus. This approach, which has already been applied in parasitological studies, could also be useful to elucidate PCM eco-epidemiology. The use of sensitive and specific molecular tools would overcome the difficulties encountered in detecting the pathogen in host tissue through either culture or histopathological analysis. PCR reaction with panfungal primers from rDNA genomic region can be considered more sensitive because they usually target a multicopy gene [5]. The combined use of specific primers derived from this genomic region with Nested-PCR may increase the specificity of molecular detection of *P. brasiliensis* [6]. The present work aimed to describe possible new hosts of PCM by using molecular tools to detect *P. brasiliensis* in tissues of road-killed animals that had lived in endemic areas of the disease.

Materials and methods

Study area and animals

The road-killed animals were collected in the Botucatu endemic PCM area by the Departamento de Estradas de Rodagem do Estado de São Paulo (DER) team that routinely patrol the roads. Only animals that appeared to have been recently killed (1–7 hours) and had not completely disfigured were placed into plastic bags, labelled as to date, hour and geographic location and sent to the laboratory where they were necropsied. The animal organs were collected and processed for DNA extraction at necropsy or preserved at -80°C . The taxonomical data of the animals, including their home ranges, sex and the tissues analysed are summarized in Table 1.

The geographic positions of the road-killed animals, established through GPS (Global Positioning System), were plotted on a digital map using a geographic database by the IDRISI32 GIS and Surfer (Fig. 1).

This study was developed after receiving authorization from the Brazilian Protection Agency (IBAMA) and Animal Ethics Committees (CEEA) at the Institute of Biosciences/UNESP-Botucatu, SP, Brazil.

Molecular analyses

The DNA extraction was performed by grinding the liquid-nitrogen frozen tissue sample with mortar and pestle as described by Corredor et al. [8]. The DNA pellet was suspended in 100 μl of ultra-pure water and the quality was checked by 1% agarose gel electrophoresis using Low Mass DNA Ladder (Invitrogen) as molecular marker. The molecular detection was carried out by Nested-PCR reactions, using as outer primers the panfungal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAACG-3'), annealing temperature of 60°C [5] and inner primers PbITSE (5'-GAGCTTTGACGTCTGAGACC-3') and PbITSR (5'-AAGGGTGTTCGATC GAGAGAG-3'), annealing temperature of 62°C [6], and matching from 162 to 548 nucleotides at GenBank (AY374339) access. The specificity of the Nested-PCR was evaluated in a blind test against a panel of 16 DNA samples from *Emmonsia parva*, *Histoplasma capsulatum*, *P. brasiliensis*, *Renispora* spp. and *Sporothrix schenckii*, provided by Setor de Imunodiagnóstico do Serviço de Micologia (IPEC/FIOCRUZ). The samples code numbers and identities were revealed only after amplifications. The Nested-PCR amplicons were purified by the commercial kit GFX PCR DNA and Gel Band Purification (Amersham Biosciences) and the sequencing reactions were carried out in both strands in a MegaBaceTM 1000 (Amersham Biosciences). The sequences were compared to the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>).

Results

Area of discovery of animals

Fig. 1 illustrates the geographic location of all the road-killed animals evaluated.

DNA amplification

The specificity of the PbITSE/R primers were successfully tested against a panel of DNA samples from several fungi such as *S. schenckii*, *Renispora* spp., *H. capsulatum*, *E. parva* as well as from *P. brasiliensis*. Overall, the panfungal PCR with ITS4/ITS5 primers amplified a 650bp DNA fragment in all fungi tested, but the PbITSE/R primers in the PCR showed an amplicon of 387bp only in DNA samples obtained from *P. brasiliensis* (Fig. 2). With respect to animal samples, predictive specific amplicons of *P. brasiliensis* were detected by Nested-PCR reactions in tissue fragments from: (i) several organs of the two armadillo species (Dn1 and Ds1) and a guinea pig (animal

Table 1 Taxonomical data of the road-killed animals, including the species home range, sex, evaluated tissues and Nested-PCR results.

Order	Family	Species	Home range* (ha)	Animal	Sex	Tissue/Nested-PCR (+ or -)			
Carnivora	Canidae	<i>Cerdocyon thous</i>	0.1	Ct1	Male	lu (-), s (-), l (-), k (-),h (-), mln (-)			
				Ct2	Male	lu (-), s (-), l (-), k (-),h (-), mln (-)			
				Ct3	Male	lu (-), s (-), l (-), k (-),h (-), mln (-)			
				Ct4	Na	lu (-), s (-), l (-), k (-), h (-)			
				Ct5	Male	lu (-), s (-), h (-), mln (-)			
	Mustelidae	<i>Eira barbara</i>	2.44	Eb1	Male	lu (-), s (-), l (-), h (-), mln (-)			
				Gv1	Male	lu (+) , s (-), l (-), k (-), h (-)			
				Gv2	Male	lu (-), s (-), l (-), h (-)			
				Procyonidae	<i>Procyon cancrivorus</i>	Na	Pc1	Male	lu (+) , l (-), k (-), h (-), mln (-)
							Pc2	Male	lu (-), s (-), l (-), k (-), h (-), mln (-)
Didelphimorphia	Didelphidae	<i>Didelphis albiventris</i>	0.57	Da1	Female	lu (-), s(-), l (-), mln (-)			
				Da2	Female	lu (-), s (-), l (-), mln (-)			
Rodentia	Cavidae	<i>Cavia aperea</i>	0.1	Ca1	Male	lu (+) , s (+) , l (-), k (+) , mln (+) ,h (-), ag (+)			
				Ca2	Male	lu (-), s (-), h (-), mln (-)			
	Erethizontidae	<i>Sphiggurus spinosus</i>	15-20	Ca3	Female	lu (-), s (-), l (-), k (-), h (-), mln (-)			
				Ss1	Female	lu (-), s (-), l (+) , k (-), h (-)			
				Ss2	Male	lu (+) , s (-), k (-)			
Xenartha	Dasypodidae	<i>Dasypus novemcinctus</i>	3.4-15	Dn1	Female	lu (+) , s (+) , l (+) , k (+) , h (-), mln (+)			
		<i>Dasypus septemcinctus</i>	Na	Ds1	Male	lu (+) , s (+) , l (+) , k (-), h (-), mln (+)			

lu, lung; s, spleen; l, liver; k, kidney; h, heart; mln,mesenteric lymph node; ag, adrenal gland.

*According to Eisenberg & Redford 1999 [7].

Na, not available.

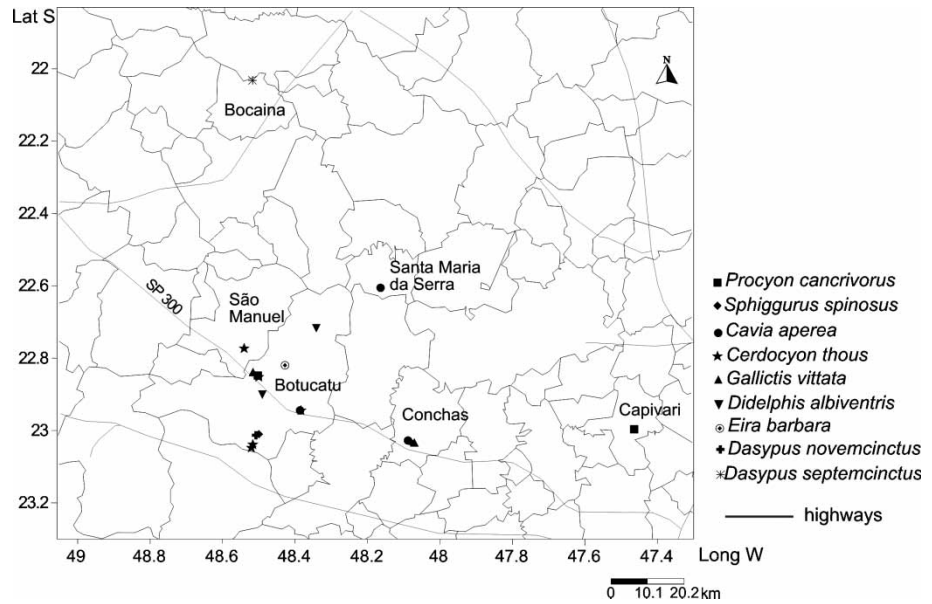


Fig. 1 Geographic location of the road-killed animals employed for *Paracoccidioides brasiliensis* molecular detection.

Ca1), (ii) porcupine liver (Ss1) and (iii) the lungs of raccoon (Pc1), grison (Gv1) and porcupine (Ss2) (Table 1). The molecular identities of the amplicons from the guinea pig (Ca1), raccoon (Pc1) and porcupine (Ss1) were confirmed by direct double-strand sequencing. The latter studies produced unambiguous fragments, varying from 332 to 340 bp, which showed 100% similarity with *P. brasiliensis* DNA sequences deposited at Gen Bank, which included 44 different accession numbers, obtained both from armadillo (AY374339) and human isolates (AF416745, ATCC 32069).

Discussion

PCM in both in domestic and wild animals has been reported in the literature based on the use of intradermal reactions with paracoccidioidin. It was shown that some of these animals showed high rates of

infection, especially those whose habitats are related to soil [9,10]. Serological surveys have also been employed to determine PCM in dogs [11], equines [12], bovines [13], free-living monkeys [14] and armadillos [15], thus showing that a wide variety of mammals can be infected by *P. brasiliensis*.

The systematic isolation of *P. brasiliensis* in armadillo tissues demonstrated the importance of this animal as a natural reservoir of the etiologic agent in endemic areas. It has been suggested that armadillos were the only animals that could acquire PCM [16,17]. However, the natural occurrence of PCM was confirmed in two dogs with generalized lymphadenitis by the recovery of *P. brasiliensis* in culture, as well as by histopathological, immunohistochemical and molecular detection of the gp43 gene [18,19].

In order to increase our knowledge on the ecology of *P. brasiliensis* and the epidemiology of PCM, we developed a new approach that combined molecular

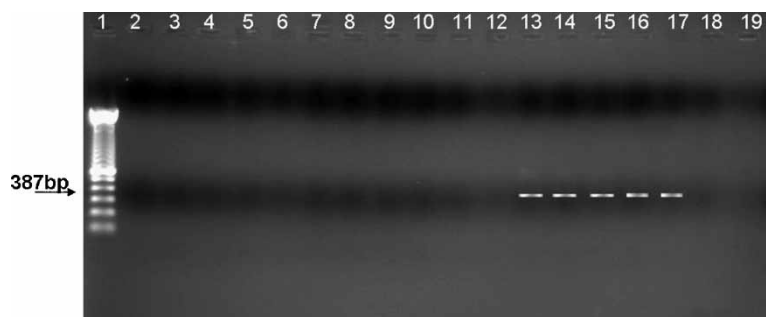


Fig. 2 Specificity testing of the Nested-PCR using DNA from several fungi: Lane 1–19: (1) 100pb DNA ladder (Invitrogen), (2–6) *S. schenckii*, (7–11) *H. capsulatum*, (12) *Renispora* sp. (13–17) *P. brasiliensis*, (18) *Emmonsia* sp., and (19) negative control.

tools to identify *P. brasiliensis* in other hosts by demonstrating fungal DNA in animal tissue.

In the present study, panfungal primers ITS4/5 amplified an amplicon of around 650 bp in lungs of *C. aperea* (guinea pig), *D. albiventris* (white-eared opossum), *P. cancrivorus* (raccoon) and *S. spinosus* (porcupine), thus demonstrating previous contact and the present existence of fungi, but not necessarily *P. brasiliensis*. This data corroborates that the air-borne route is the major means of transmission of several pathogenic and non-pathogenic fungi in animals [20].

A serious limitation for any molecular protocol is the occurrence of nonspecific annealing when a common DNA sequence is present. Since it has been estimated that there are approximately 1.5 million fungal species [21], it is possible that DNA sequences of phylogenetically closely related fungi, mainly environmental ones, have not yet been deposited in the Gen Bank. *P. brasiliensis* has been recently classified as a member of Ajellomycetaceae, a new family of saprobic and pathogenic vertebrate-associated fungi, which includes *Histoplasma*, *Blastomyces*, *Emmonsia* and *Paracoccidioides* [22]. Up to now, the use of Nested-PCR with PbITSE/R primers has proven to be specific for the detection of only *P. brasiliensis* since any amplification occurs for the several other related genera.

Concerning the members of the Carnivora order evaluated, which are biologically related to the domestic dog, *P. brasiliensis* DNA was detected only in the lung of *P. cancrivorus* (raccoon) and *G. vittata* (grison), while all organs of *C. thous* (crab-eating-fox) and *E. barbara* (tayra) remained negative. It is known that high temperatures are limiting factors for the growth of major fungal species and that domestic dogs have a body temperature around 37.5–38.5°C [23]. This might be a factor contributing to the limited number of cases involving *P. brasiliensis* in Carnivora members. It appears that the members of the Didelphidae family are also not frequently infected by fungi as already reported by Silva-Vergara *et al.* [24].

P. brasiliensis infection does not necessarily indicate systemic PCM. The histopathological studies of armadillo tissue showed that if the disease occurred it was a mild form [25]. The molecular detection of *P. brasiliensis* DNA in organs of *C. aperea* (guinea pig) demonstrated that the fungus indeed disseminated from the lung. We also detected the pathogen in the liver and lungs of *S. spinosus* (porcupine). The importance of rodents as a reservoir of other fungal pathogens such as in *Coccidioides immitis* [26], *Emmonsia* spp [27] and *Penicillium marneffeii* [28,29] has previously been demonstrated. As expected, the

two armadillos evaluated in this investigation provided positive amplification in several organs.

The molecular detection of *P. brasiliensis* in tissues from organs such as the adrenal gland, liver, spleen, kidney and mesenteric lymph node also might exclude the possibility of the presence of non-pathogenic fungi because the latter do not have the capacity to disseminate to extra-pulmonary organs. Since in all evaluated animals the integrity of the organs was preserved we believe that the risk of cross-contamination in this investigation was low. While problems with PCR sensitivity and inhibition can not be excluded completely, we have observed a DNA detection limit of 1.0 µg. In addition, the specific amplification of the target sequence of *P. brasiliensis* was not inhibited even when using only one µg of fungal DNA mixed with a relatively large amount of animal DNA (around 100–200 µg, data not shown).

The detection of *P. brasiliensis* in different organs from different species show that the fungus could have different dissemination profiles. This in turn may indicate different interactions of *P. brasiliensis* with several host species studied and possibly different genotypes of this etiologic agent. It is known that *P. brasiliensis* presents at least three cryptic species [30]. This divergence must be studied in order to evaluate how some genetic differences may indicate distinct host-pathogen interactions as well as distinct ecological niches.

Without the necessity of applying a laborious sampling effort, it was possible to evaluate nine different wild species, belonging to seven different taxonomic families. In fact, the numbers and diversity of road-killed animals are considerably higher and, in general, they are killed in their own habitat, because the roads invade their natural habitats [31]. In this manner, the geographic coordinates of the places where the infected animals might be are well-integrated in databases that use the Geographical Information Systems (GIS), thus contributing to a better understanding of pathogen distribution and the associated biotic and abiotic factors. In summary, our results show that road-killed animals can be important in the eco-epidemiological study of *P. brasiliensis*.

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