

Review article

The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle?

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When trying to understand the pathophysiology of any infectious agent, one key piece of information is the determination of its habitat. In the case of *Paracoccidioides brasiliensis*, the precise location of the fungus' environmental niche remains undefined despite the efforts of various research groups. This review summarizes recent studies on the ecology of *P. brasiliensis* and certain facets of paracoccidioidomycosis. Studies on the juvenile form of paracoccidioidomycosis in children less than 13 years of age, the characterization of the ecological factors in the 'reservarea' where the infection is acquired and the presence of *P. brasiliensis* in the nine-banded armadillo (*Dasypus novemcinctus*), are all helping to pinpoint the microniche of this pathogen. The application of molecular biology techniques based on the amplification of nucleic acids will also hopefully help in establishing the precise habitat of *P. brasiliensis*.

Keywords armadillos, ecological factors, habitat, *Paracoccidioides brasiliensis*

Introduction

Paracoccidioidomycosis, an endemic mycosis caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*, is of singular importance in Latin American countries [1–4]. The endemic area extends from Mexico (23° North) to Argentina (35° South), but the prevalence is higher in South America than in Central America; the disease has not been reported in Nicaragua, Guyana or Chile [5,6]. The disease is observed predominantly in adult males engaged in farming activities; females and children experience the disease less often [1,2,7,8]. Brazil has the largest number of patients [1,3,6] whereas Colombia ranks in third place [9,10]. Since paracoccidioidomycosis is not a notifiable disease, its real prevalence and incidence cannot be calculated. In Brazil, however, estimates indicate an annual incidence rate of 1–3 per 100 000 inhabitants and a mean mortality rate of 0.14 per 100 000 [11,12]. In Colombia, Torrado *et al.* [10]

recorded lower but fluctuating incidence rates (0.05–0.22 per 100 000 inhabitants) during a 30-year period [10]. It should be stressed that in the endemic regions, the infection as measured by skin testing is far more prevalent than the disease. In Colombia, for instance, it is estimated that 9% of the population has had previous contact with *P. brasiliensis* [13].

Almost 16 years have elapsed since the gaps in our knowledge concerning the micro-niche of *P. brasiliensis* were reviewed in this journal [14]. However, despite significant progress, the fungus generally continues to elude the efforts of those searching for its whereabouts. Thus, as an example, *P. brasiliensis* has only been isolated on six occasions from soil, initially in 1963 by Shome and Batista in Brazil [15]. This isolation was regarded as invalid for a time; recently, however, further studies have revalidated its position [16]. Subsequent isolations have been made from Argentina (once) [17], from a Venezuelan farm (three times) [18], and from a Brazilian coffee plantation (once) [19]. Attempts at repeated fungal isolation from soils have met with failure [20,21]. *P. brasiliensis* has also been cultured from a commercial animal forage which was possibly contaminated with soil [22].

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Accordingly, this review aims to present the latest findings on the ecology of *P. brasiliensis*, derived from studies on the disease, on the ecological factors predominating in endemic areas and on its presence in other mammalian hosts. Relevant data will be analyzed and possible connections with the fungus' habitat will be stressed.

Ecology: the issue at stake

Relevant findings in paracoccidioidomycosis patients

In general, childhood paracoccidioidomycosis represents approximately 5% of all reported cases [1]. However, retrospective studies clearly indicate that this presentation is not that infrequent in certain areas of Brazil where rates as high as 13% have recently been identified [23]. Due to the fact that children aged 3–13 years have a restricted migratory profile, the places where they live may furnish valuable data concerning the habitat of *P. brasiliensis*. Based on this premise, Cadavid & Restrepo [24] carried out an ecologically oriented study using skin testing with paracoccidioidin in persons living in places of birth and only residence of children with the mycosis. Logistic regression analysis revealed that when comparing communities with low and high infection rates, several variables were associated with the latter, among them the presence of certain watercourses ($P = 0.001$), contact with armadillos and their burrows ($P = 0.008$) and work in vegetable gardens ($P < 0.01$) [24].

Prior to this, Pedrosa [25] had conducted a skin testing survey among rural children (6–11 years of age) in the county of Barra Mansa, Rio de Janeiro, where paracoccidioidomycosis had been diagnosed in a 3-year-old child. He found that 34% of the children were positive to paracoccidioidin. When distributed by county, most of the positive children (73%) inhabited the foothills of the Sierra de Mantequiera (altitude 900 m) while the remainder (25%) had their residence near the river Paraíba do Sul (altitude 380 m). He concluded that the exposure rate was higher in places characterized by higher altitude, a colder climate, abundant rain and rich vegetation [25]. Subsequently, Rios-Gonçalves *et al.* [26] analyzed the records of 36 children diagnosed in the state of Rio de Janeiro during the period 1981–1996. Most of these patients (44%) came from the rural counties previously mentioned by Pedrosa [25], where the native vegetation used to be abundant and where gradual deforestation had taken place over the years, as described in Table 1. Over a similar period (1985–1996) Fonseca *et al.* [23] found that 13 children, who represented 12.7% of all cases, had been diagnosed in the Amazonian states of Pará and Tocantins, where paracoccidioidomycosis had been formerly regarded as

Table 1 Ecological characteristics of the residence of children with paracoccidioidomycosis

State and country	Rio de Janeiro, Brazil
Counties	Barra Mansa and Volta Redonda
Characteristics of the land	Hilly upland around the Sul River Valley, which is also rich in streams and watercourses; this area is the settlement of many residents who have profited from the waterways to construct small dams
Climatic conditions	Altitude 300–900 m above sea level; temperature 8–40 °C (mean 23 °C); annual precipitation 1100–1300 mm
Land exploitation	In the past, there were abundant deciduous forests but in 1883 deforestation started and resulted in monoculture (coffee). By 1888, soil depletion was evident and forced a change to pastures and cattle breeding. Since then, native forests are found only along river courses

Adapted from Rios-Gonçalves *et al.* [25].

rare. These children had lived permanently in areas where, for the last three decades, colonization of the Amazonian area had been intense and was followed by gradual removal of the original unexplored forests [23].

Recently, Blotta *et al.* [1] compiled the records of 584 paracoccidioidomycosis patients diagnosed between 1988 and 1996 in the state of São Paulo, 33 (5.6%) of whom were less than 14-years-old [1]. A particular county (Campinas) had 48% of all patients, including most of the childhood cases. This county experiences a tropical climate with high rainfall indices for 8 months of the year and its economy is based mainly on agriculture (coffee, sugar cane, cotton, pastures) and animal husbandry. As such, most of the patients, including children, could have been exposed to *P. brasiliensis* infected soils [1].

Relevant factors in the 'reservarea'

Based on the long periods of latency recorded for paracoccidioidomycosis, Borelli, in 1964 [27], proposed to differentiate the endemic areas where cases are usually diagnosed from those corresponding to the places where the infection is acquired because the latter should correspond to the microniche of the fungus. The term 'reservarea' was coined to describe such areas. Since then, many attempts have been made to define the characteristics of such reservareas [1,9,10,23–26,28–32]. In general the results of these studies, including pioneering work performed between 1953 and 1967, have emphasized the presence of significant rainfall, of abundant forests and watercourses, and of limited

temperature variations (17–24 °C), as well as of certain occupations and particular crops [32–36]. A hypothesis was also enunciated concerning the need for a very humid natural microniche in which the fungus would grow uninhibited [14].

There are other circumstances unrelated to the use of the land for agricultural purposes that may also expose man to the fungus, namely, the abrupt changes introduced into the formerly unexplored forests that constitute the reservareas. For example, paracoccidioidomycosis was considered rare among Amerindians until 1988 when Thalhari *et al.* [37] reported 15 patients among a population of 500 people living in the Amazonian States of Brazil. This finding prompted a skin testing survey of the regions's inhabitants that revealed infection by *P. brasiliensis* in over 30% of the natives tested [38]. Later, in 1991, Valle *et al.* [39] diagnosed paracoccidioidomycosis in a Suruí Indian who had always lived in the midst of the unexplored Brazilian Amazon region. This clustering of cases suggested a point source thought to be associated to the Suruí's adoption of new agricultural practices enhancing the likelihood of exposure to *P. brasiliensis*. The traditional subsistence of these Amerindians is based on slash-and-burn horticulture, complemented by hunting, fishing and gathering of forest products. This scheme was changed to coffee farming that required not only the felling of trees in the forest but also preparation of the land for the coffee crop, both of which are activities that may generate infectious aerosols [39,40].

To test the above assumptions, a cross-sectional epidemiological survey that included skin testing with paracoccidioidin and histoplasmin, physical examination and lung X-rays, was carried out among Amerindians residing in the Aripuaná Indian Park, Southwestern Amazonia, on the border between the states of Rondônia and Mato Grosso (60–61° W, 10–12° S) [40]. Of the population tested, approximately 500 individuals belonging to the same tribe, the Tupí-Mondé, which are organized in different societies with the following three tribes being studied: the Suruí, among whom paracoccidioidomycosis had been diagnosed, and their neighbors, the Gavião and the Zoró. Although the results did not reveal the presence of active mycotic infections, there were important differences in the rate of paracoccidioidomycosis infection, as judged by reactive skin tests. Thus, 43.8% of the Suruí proved reactive to paracoccidioidin while only 6.4% and 14.9% of the Gavião and the Zoró, respectively, recognized this antigen. On the other hand, histoplasmin reactions were more frequently positive in the latter two tribes. Multiple logistic regression analysis confirmed the significance of the findings, supporting the connection between increased

incidence rates of paracoccidioidomycosis and higher paracoccidioidin sensitivity with the environmental changes brought about by a different type of soil exploitation [40].

The same authors [40] further examined the environmental conditions recorded for the Aripuaná Indian Park, and indicated that several of the characteristics previously mentioned as favorable to *P. brasiliensis* were present. These included the average temperature (24–27 °C), the annual rainfall (1750–2500 mm), the presence of upland rainforests and the existence of numerous water courses [41]. These data indicate that the Brazilian Amazon river basin constitutes a reservarea and that the anthropogenic changes conditioned the exposure of the Amerindians to the *P. brasiliensis* microniche [39].

Along the same lines, an important, although preliminary finding, was made by Mangiaterra *et al.* [42] in the Corrientes province, northeastern Argentina. This province lies in the vicinity of the Brazilian Paraná's river dam, where building of the Yacyreta hydroelectric plant is being accompanied by important environmental changes. A skin testing study in 455 individuals residing in this province, where paracoccidioidomycosis was unknown, revealed that 11.4% of individuals reacted to paracoccidioidin. This important index of human infection with *P. brasiliensis* also suggested a connection with the anthropogenic changes taking place in the area [42]. However, it is of note that the extent of the Amerindian problem can not be compared directly to the one reported by Mangiaterra *et al.* [42]. In the case of the Suruí Indians, the environmental changes were introduced manually, via the use of unsophisticated tools, which curtailed the exposure rate [39,40]. On the contrary, at the Paraná river dam project [42], the impact of unintended exposure on a large number of residents and workers, the latter provided with modern excavating and demolishing machines capable of generating powerful aerosols, is of great concern.

Two recent Colombian studies have reviewed the same patient register, although they have used different approaches. Calle *et al.* [9], who examined 1016 Colombian municipalities, established the association between certain ecological variables and the incidence of paracoccidioidomycosis. The records of 940 patients served as the basis for the study as well for determining the association of the ecological variables to the amount of patients per total rural population in each municipality. Such association was established through multivariate analysis. All patients were found to belong to only 216 municipalities (20.3%) of the country, 93 of which were mentioned by 121 patients as being their birth and long-term residence places. The incidence rate ratio (IRR) was determined for the latter municipalities

where the infection should have been acquired and developed into overt disease some time afterwards. The following ecological variables fitted the model: altitude 1000–1499 m above sea level (IRR = 6.37), rainfall 2000–2999 mm (IRR = 2.15), presence of humid forests according to Holdridge [43] (IRR = 1.79), and coffee (IRR = 1.95) and tobacco (IRR = 3.59) crops. These results indicated that the above municipalities constitute reservareas for *P. brasiliensis* [9].

In addition, Torrado *et al.* [10] have carried out a systematic analysis of over 1000 paracoccidioidomycosis cases diagnosed in Colombia over a 50-year period (1949–1999). The results allowed the establishment of the endemicity of paracoccidioidomycosis in the various departments based on both the total number of cases diagnosed and the annual reporting of new cases. Nine such departments were classified as endemic and from these eight belonged to the Andean region. The latter region is characterized by altitudes between 1000 and

2000 m above sea level and corresponds to the country's coffee growing areas. The ninth department corresponded to a mountainous area isolated from the Andes (Sierra Nevada de Santa Marta) but which also includes lands dedicated to agriculture, including coffee growing.

Despite taking different approaches, these studies have pinpointed the Colombian regions/municipalities that possess the ecological variables most probably required for the development of *P. brasiliensis* in nature [9,10]. Thus, the Colombian reservareas are restricted to regions of high altitude belonging to the humid forest and as such, are characterized by substantial rainfall. In turn, these circumstances favor certain crops. Consequently, men working/living in these reservareas would encounter *P. brasiliensis* infectious propagules during their daily laboring and become infected as a result. Anecdotally, such occupational/leisure exposure has been inferred by other workers in the past [7,27,29,44,45]. Table 2 presents a summary of the

Table 2 Chronology of most relevant *Paracoccidioides brasiliensis* ecological findings

First author (Country)	Year	Ecological finding	[Ref]
Mackinnon (Uruguay)	1953	Most cases of the mycosis occur in forestry laborers working alongside rivers	[33]
Borelli (Venezuela)	1961	All paracoccidioidomycosis patients have lived in tropical and sub-tropical areas located between parallels 23° N and 30° S, where median annual temperature oscillates 17–24 °C	[34]
Borelli (Venezuela)	1964	Definition of the term 'reservarea' and characterization of its most notorious characteristics	[27]
Chirife (Argentina)	1965	The mycosis prefers warm, moderately humid climates with rainy summers and mild winters, avoiding excess cold and permanent dryness	[35]
Bopp (Brazil)	1967	Observation of uneven distribution of the mycosis in Rio Grande do Sul where the higher prevalence rates are recorded in areas with dense forests, intense agriculture and abundant rainfall	[36]
Restrepo (Colombia)	1968	Determination of the ecological factors, prevailing in endemic areas; the latter were classified according to Holdridge's scheme	[30]
Borelli (Venezuela)	1972	Absence of essential indicators has hindered the discovery of the microniche. The latter would need an animal host ('protector'), preferably living underground	[32]
Restrepo (Colombia)	1972	Most paracoccidioidomycosis patients have lived in areas corresponding to Holdridge's subtropical and tropical humid forests	[31]
Londero (Brazil)	1972	Delimitation of the reservareas to forested regions distributed along waterways in the slopes of major hills in the State of Rio Grande do Sul, Brazil	[29]
Pedrosa (Brazil)	1976	In particular counties of Rio de Janeiro, most of the paracoccidioidin-positive children (73%) inhabited the foothill of a Sierra while the remaining (25%) lived near a river. The exposure rate was then higher in places characterized by a significant altitude, a colder climate, abundant rain and rich vegetation	[24]
Cadavid (Colombia)	1993	Logistic regression analysis revealed that several variables were significantly associated with higher risk of infection, including altitude over 1300 m, temperatures 18–24 °C, presence of numerous watercourses and armadillo burrows in the patients' vicinity, as well as work in vegetable gardens	[23]
Coimbra (Brazil)	1994	Finding paracoccidioidomycosis patients among natives of the Amazonian region suggested that changes in agricultural practices connected with deforestation were related to both increased exposure to the fungus, as shown by positive skin tests, and higher rates of disease in the indigenous population	[40]
Torrado (Colombia)	2000	Endemic areas defined on the bases of incidence rates and years of reporting the mycosis, centered around the Andean region of the country	[18]
Calle (Colombia)	2001	Definition of ecological variables fitting a multivariate model. Most significant were altitude 1000–1499 m above sea level; annual rainfall 2000–2999 mm; presence of humid forests (Holdridge); coffee and tobacco crops	[17]

ecological aspects that have been emphasized by several previous authors.

Finally, it is worth noting that back in 1972, Campbell [46] anticipated that the environmental changes being brought about by man's 'progress', such as deforestation of primitive sylvan areas with the corresponding disturbance of fungal microniches, was going to result in the exposure of larger numbers of individuals to these until now, uncommon pathogens [46]. Is her prediction becoming a reality?

Relevant findings in a new mammalian host, the armadillo

In the past, *P. brasiliensis* had been infrequently observed in animal's tissues or excrement, originating from animals as diverse as the bat, *Artibeus lituratus* [47], the squirrel monkey, *Saimiri sciureus* [48], the penguin, *Pygoscelis adeliae* [49] and more recently, as described below, in the nine banded armadillo, *Dasypos novemcinctus* [50–55]. Concerning bats, additional studies and experimental models were not confirmatory of the initial findings [56–57]. Table 3 summarizes the most pertinent findings. Additionally, several studies have indicated that domestic animals might have been exposed to the fungus as they recognize *P. brasiliensis* antigens [58,59]; however, the fungus has never been observed in or isolated from these 'infected' animals.

Consistent information has recently been gathered concerning *P. brasiliensis* infection in the nine-banded armadillo *D. novemcinctus* [60]. Quite fortuitously while searching for *Leishmania* in sylvan reservoirs in the Pará state of the Brazilian Amazonia, Naiff *et al.* [50] found that several nine banded armadillos (4/20), harbored *P. brasiliensis* in their spleens and/or livers. Initially, this was verified by positive cultures and subsequently by histopathological observations of various organs and inoculation into hamsters [50]. Shortly thereafter, these authors repeated the isolation of *P. brasiliensis* from 10 of 18 armadillos captured in a different region of the same state [51]. Bagagli *et al.* [52] in the area of Botucatú, Sao Paulo State, Silva-Vergara & Martinez [55] in Ibiá, State of Minas Gerais and Macedo *et al.* [54] in Serra da Mesa, Goiás State confirmed the presence of natural *P. brasiliensis* infections in armadillos from different regions of Brazil. Overall, as reported in the literature [53,60], a total of 81 nine-banded armadillos have been studied and of these, 29 (35.8%) have harbored *P. brasiliensis* in their lungs, spleens, livers and/or mesenteric lymph nodes [52–55,60]. Thus, Bagagli and coworkers [52,60] found that over 60% of the nine-banded armadillos studied were infected with the fungus. Bagagli *et al.* [52] and Silva-Vergara & Martinez [55] also described the presence of granulomas formed around *P. brasiliensis* yeast cells in the spleens or the lung of four

Table 3 Animals and *Paracoccidioides brasiliensis*: cases defined on the basis of direct histologic observation and/or isolation in culture

Animal species (number positive)	<i>P. brasiliensis</i> demonstrated by			First author [Ref]
	Histology	Culture	Other methods	
The frugivorous bat <i>Artibeus lituratus</i> (3)		Guano*		Grosse [47]
The squirrel monkey <i>Saimiri sciureus</i> (1)	Liver†			Johnson [48]
The penguin <i>Pygoscelis adeliae</i> (1)		Guano		Gezuele [49]
The nine-banded armadillo <i>Dasypos novemcinctus</i> (4)‡	Liver, spleen	Same organs	Inoculation (hamster)	Naiff [50]
<i>D. novemcinctus</i> (18)	Liver, spleen, lung	Same organs	Inoculation (hamster)	Naiff [51]
<i>D. novemcinctus</i> (10)	Liver, spleen, lung mesenteric lymph nodes	Same organs	Inoculation (hamster)	Bagagli [52]
<i>D. novemcinctus</i> (2)		Spleen		Macedo [54]
<i>D. novemcinctus</i> (1)	Lung			Silva [55]
<i>D. novemcinctus</i> (1)	Lung, spleen, liver mesenteric lymph nodes	Lymph nodes	Same organs (PCR)	Corredor [53]

*, This isolation remains unconfirmed as experimental studies and further testing failed to reveal the fungus in bat excreta [56,57];

†, the fungal structures depicted are not confirmatory of *P. brasiliensis*;

‡, total number of armadillos studied was 36.

armadillos. These findings indicate that *D. novemcinctus* may not be a simple carrier but may also develop overt paracoccidioidomycosis, as no gross pathology had been previously observed in these mammals. A new isolation has also been reported in Manizales, Colombia [53]. In this study, the fungus was cultured from various internal organs and its DNA detected in mesenteric lymph nodes, as well as in hamsters inoculated with the armadillos' tissues [53,61] (Table 3).

Bagagli *et al.* [52,60] also focused on the Brazilian counties where 15 adult nine-banded-armadillos had been captured under the appropriate governmental license. Four different counties (Botucatu, Manduri, Pardino and Prata) served as capture areas. The distribution of *P. brasiliensis* positive and negative animals varied according to the county; for instance, in Manduri four armadillos were positive while in Pardino, the three animals studied proved negative. Positive armadillos were associated more often with places located near water sources and where the vegetation had been highly disturbed [52]. *P. brasiliensis*-positive animals had been captured at sites where there was a variety of plants and trees, among them the non-native *Pinus* and *Eucalyptus*. There were also forests, savanna lands, and both semideciduous tropical and riparian forests. The altitudes were below 800 m and the medium temperature fluctuated between 14.8 and 25.8 °C. Soils differed in their composition, pH and fertility. By contrast, in the county where armadillos had tested negative, the altitude was higher (950 m), the vegetation, a semideciduous tropical forest, was preserved and water sources were scarce [52,60]. Surprisingly, soil samples collected around the burrows of the positive armadillos proved negative when inoculated into hamsters, an animal susceptible to *P. brasiliensis* [50,55].

P. brasiliensis isolates from armadillos have been analyzed in terms of virulence [62,63], antigens [64] and molecular aspects [64,65], which has provided strong evidence that both human and animal isolates are quite similar. It is interesting that, according to Sano *et al.* [64], individual armadillos may be infected not with one but with various different genotypes of *P. brasiliensis*. In addition to *D. novemcinctus*, two genera and three other different species of armadillos have been studied, among them two *D. kapplari* [51], one *D. septemcinctus* and four *Euphractus sexcinctus* [54]; however none of them has proven positive for *P. brasiliensis*. This is peculiar as all animals share similar life styles within the reservareas. Preliminary studies by Silva-Vergara *et al.* [66], in several arboreal marsupials of the genus *Didelphis*, captured in rural areas of Brazil, have also proved negative. Immunologic tests also failed to demonstrate circulating

antibodies to *P. brasiliensis* in this marsupial, as well as in other mammals captured in Colombia [67].

It is of note that armadillos are widely distributed in Latin America and *D. novemcinctus*' habitat spans from the south central USA down to Argentina, along the west of the Andean ridge [68–70]. In Colombia, the genus *Dasyus* is amply distributed in the territory where its existence was initially recorded in 1783 [71]. The global distribution partially coincides with that of human paracoccidioidomycosis; furthermore, armadillos have been captured in areas where this disease has been previously reported in man [5,51,52,54]. These areas share a certain number of ecological factors such as high precipitation rates, presence of forests, abundant water-courses, short winters, rainy summers, as well as temperatures between 14 and 27 °C [5,28,52,68–71]. Armadillos are heterothermic mammals, capable of regulating their metabolism accordingly; furthermore, they are long-lived and as such may develop chronic infections [68–70]. They have a deficient immune system and have served as models for human disease [72]. Their capacity to excavate the soil at great speed and to depths of 3.5–7 m [68–71] probably results in their exposure to infected aerosols. Alternatively, their feeding habits include disturbing ant colonies, uprooting grasses and collecting foliage, which may also facilitate and increase such exposure [52,55,70]. As previously mentioned, in three isolated areas of Colombia, surveyed because they were the place of birth and of lifetime residence of children with paracoccidioidomycosis, regression analysis revealed that those inhabitants recalling contact with armadillos, had a significantly higher rate of positive reactions to paracoccidioidin [24].

In summary, the above findings have revealed several important connections between man, armadillo and *P. brasiliensis*, as follows:

1. Both mammals share activities around the as yet unknown fungal habitat, which when disturbed may lead to infection via the aerosol route;
2. In areas of Brazil and Colombia where paracoccidioidomycosis is endemic, the nine-banded armadillos are frequently infected with *P. brasiliensis*;
3. *P. brasiliensis* might have found in the armadillo a host offering the required protection against environmental hardships [34,52];
4. *D. novemcinctus* may be considered as a sentinel of the habitat of the fungus;
5. Disturbance of the native vegetation around the burrows of positive armadillos coincides with the observation of an increased rate of exposure in Amerindians living in deforested Amazonian areas.

P. brasiliensis and its well-hidden microniche

Paracoccidioidomycosis has a number of peculiar characteristics, including its long period of latency recorded for those cases diagnosed in non-endemic countries [73], the absence of disease outbreaks, and the paucity of acute cases and/or of confirmed subclinical infections [7]. All these circumstances, described previously by Borelli [34], have tended to hinder determination of the possible source of infection. *P. brasiliensis*, in turn, is endowed with certain capacities, all of which tend to eliminate 'fingerprints'. To give only one example, in the laboratory, conidia production is scarce and results only when there is paucity of nutrients [1,34]; correspondingly, infecting propagules may also be produced in small numbers in nature. In the reservarea, exposure of the host may well consist only of a light inoculum evoking no acute symptoms that may lead to diagnosis of the primary infection. Consequently, the infected individual would not be able to remember the infecting moment nor the activity being conducted [27,34]. This being so, techniques regularly used to recover pathogens from environmental samples, such as cultures and animal inoculations, may well prove inadequate. This appears to be demonstrated by the studies of Silva-Vergara *et al.* [19] and Montenegro *et al.* [21], who processed 760 and 885 environmental samples, respectively. The former isolated the fungus once (0.13%) while the latter failed to do so at all.

However, newer molecular biological techniques, such as polymerase chain reaction (PCR), may serve to detect low DNA concentrations, and hence small numbers of fungi. Thus Diez *et al.* [61] have already obtained promising results with this method. The procedure employed allowed detection of the 27 kDa specific gene at a level of 3 pg, but when used with artificially contaminated soil samples, the sensitivity decreased to 40 ng of DNA. This technique has already demonstrated its utility in corroborating the findings in armadillos from which *P. brasiliensis* had been isolated [61]. Improvements in the level of test sensitivity are being assayed. Variations in the DNA extraction methods are also under study with the aim of avoiding inhibition of PCR amplification by agents such as humic acids present in soils. Several methods such as 'touch-down' and Southern blot hybridization are also being developed. By the same token, new sets of primers have been designed for use in a nested PCR for the p27 gene, so as to increase the level of detection of the fungal DNA. These preliminary data indicate that PCR may indeed be a much more reliable and sensitive method to approach the problem of defining the *P. brasiliensis* habitat.

Four different links belonging to the same chain?

From the above data, four solid clues have emerged which appear to point the way to defining the habitat of *P. brasiliensis*:

1. The occurrence of childhood paracoccidioidomycosis indicating early exposure to the fungus in deforested Brazilian regions;
2. The increased infection and disease rates in Amerindians disturbing sylvan areas;
3. The finding of *P. brasiliensis* infection in soil-digging armadillos found in regions where human paracoccidioidomycosis is regularly diagnosed;
4. The more precise characterization of the ecological factors prevailing in the reservareas.

The first three observations all merge into one, namely, disturbance of the reservarea. The fourth characterizes more precisely the ecological factors present therein. Accordingly, never before have we been so close to solving the riddle. We now know where to search for *P. brasiliensis*' microniche. We are now certain that soil disturbances in sylvan forests are conducive to paracoccidioid infection. We presently know that the nine-banded armadillos dig in soils where the fungus has its habitat. We are about to refine a technique sensitive enough to detect minimal quantities of fungal DNA in soils. The next few years should see the conundrum finally solved.

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