

Isolation of Fungi from Nature in the Region of Botucatu, State of São Paulo, Brazil, an Endemic Area of Paracoccidioidomycosis

MR Montenegro⁺, M Miyaji*, M Franco, K Nishimura*, KI Coelho, Y Horie**, RP Mendes***, A Sano*, K Fukushima*, D Fecchio

Departamento de Patologia, Faculdade de Medicina, UNESP, 18618-000 Botucatu, SP, Brasil *Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan **Natural History Museum and Institute, Chiba, Japan ***Departamento de Moléstias Infecciosas e Parasitárias, Faculdade de Medicina, UNESP, Botucatu, SP, Brasil

In an attempt to isolate Paracoccidioides brasiliensis from nature 887 samples of soil from Botucatu, SP, Brazil, were collected cultured in brain heart infusion agar supplemented with dextrose, in potato dextrose agar and in yeast extract starch dextrose agar, all with antibiotics, at 25° and 37°C. Five thermo-dependent dimorphic fungi morphologically resembling P. brasiliensis were isolated; two from armadillo holes; further studies of the biology, antigenicity and genetic features of the five dimorphic fungi are necessary to clarify their taxonomy and their possible relation to P. brasiliensis. In addition, 98 dematiaceous fungi and 581 different species of Aspergillus spp. were also isolated. Our findings emphasize that armadillos and their environment are associated with thermo-dimorphic fungi and confirm the ubiquity of pathogenic dematiaceous fungi and Aspergillus spp.

Key words: ecology - nature - pathogenic fungi - *Paracoccidioides brasiliensis*

There is uncertainty about the natural habitat of *Paracoccidioides brasiliensis* (Pb). It is assumed that the fungus lives saprophytically in nature; conidia, asexual spores of the fungus, may disperse in the air and infect man by the inhalatory route. Pb is capable of surviving in soil (Medina & Bodziak 1949) and in vegetal debris (Lacaz 1949). Is has been isolated from the soil in Brazil (Shome & Batista 1963), Argentina (Negroni 1966) and Venezuela (Albornoz 1971), but results could not be replicated. The fungus has also been isolated from dogfood in Brazil (Ferreira et al. 1990), from intestinal tract of bats (Grose & Tramsitt 1965), and from antarctic penguin feces (Gezuele 1989); again results have not been confirmed.

The epidemiology of Paracoccidioidomycosis (Pb mycosis) that effects mainly rural workers, the high frequency of reactive Pb skin tests in animals living on soil when compared with tree dwelling animals (Costa et al. 1995), the finding of the agent

in internal organs of armadillos (Naiff et al. 1986), the increased rate of positivity to paracoccidioidin in indians after they were exposed to dust from coffee plantations in a deforested area (Wanke et al. 1992), all point to the soil as one of the most likely habitat of the fungus.

In 1993, the Paracoccidioidomycosis Study Group of Botucatu - UNESP Campus and a group of Japanese mycologists from the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan, carried out a cooperative work to study the ecology of Pb in Botucatu and neighbourhood. They also tried to isolate pathogenic dematiaceous fungi which are the causative agents of chromomycosis and *Aspergillus* species which produce mycotoxins. As is well known, chromomycosis has frequently been reported from Latin America and there are several reports on the isolation of pathogenic dematiaceous fungi from nature in the area (Melin & Nannfeldt 1934, Trejos 1954, Salfelder et al. 1968, Gezuele et al. 1972, Mackinon et al. 1973, Nishimura 1994). Furthermore, mycotoxins may be dangerous contaminants of food and aflatoxin a known carcinogen was first extracted in Great Britain from ground nuts imported from Brazil (Asplin & Carnaghan 1961, Sargeant et al. 1961).

The present paper describes the preliminary results of this ecological survey.

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⁺Corresponding author. Fax: +55-14-821.2348

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MATERIALS AND METHODS

Table I describes the localities and number of samples collected. The work was done during winter (July and August 1993), the dry season in Botucatu.

Pro-Verde is a suburb of Botucatu where flowers are cultivated; Pratania is a village surrounded by farms 35 km to the northwest of Botucatu; Edgardia is an experimental farm of the UNESP Agricultural School where several crops are cultivated; Vitoriana is a village around 30 km to the southeast of Botucatu also surrounded by farms. We collected 887 samples of soil and rotten plants.

Aproximately 20 g of soil were collected into a plastic bag with a spoon, from fields, forests, farms, armadillo holes and other sites and transported to the laboratory (Table I). The same procedure was followed for rotten plants.

Isolation of thermo-dependent dimorphic fungi and pathogenic dematiaceous fungi was carried out as follows. One to 2 g of soil were transferred to a sterile 10 ml plastic tube; 5 ml of sterile saline containing antibiotics (50-100 mg of chloramphenicol, 10^6 units of penicillin, 167 mg of streptomycin and 500mg of cyclohexamide per liter) were then added, mixed by agitation and set for 30 min; 0.5 ml was collected from the middle part of the soil suspension and placed on brain heart infusion agar (BHIA, Difco) supplemented with 1% dextrose and the same antibiotics. A drop was spread on the surface of the agar medium and incubated

at 37°C for 10 days. The same procedure was done with two other media: potato dextrose agar (PDA, Difco) and YPSS (yeast extract 1%, soluble starch 1%, dextrose 1% and agar 2%), with the same antibiotics. The plates were incubated at 25°C and 37°C for 10 days.

For isolation of *Apergillus* spp., the same media were used, with no cyclohexamide.

The suspected colonies were subcultured on Sabouraud dextrose agar (SDA), BHIA and PDA at 25°C and 37°C and checked grossly and microscopically. Thermo-tolerance and nitrate utilization tests were also done to further identify the pathogenic dematiaceous fungi.

RESULTS

Isolation of thermo-dependent dimorphic fungus - Five yeast like fungal isolates which resembled Pb were obtained from BHIA and YPSS at 37°C (isolates no. 170, 212, 268, 328-1 and 328-2). The isolates grew slowly and formed wrinkled, folded, glabrous, whitish colonies. Mycelial colonies on PDA at room temperature for a few months showed similarities to those of Pb; they grew slowly as glabrous, leathery, flat, wrinkled, folded and flobose to velvety colonies colored from white to beige and brown, with little tufts of aerial mycelium. They also had central fissures, another common feature of Pb colonies.

Microscopically the five isolates formed chlamydospores on SDA at 25°C for 28 days and converted from mycelial to yeast form at 35°C on

TABLE I
Localities, sites and number of samples collected in a survey to study fungi in nature in Botucatu

Site	Localities					Total
	Pro-Verde	Pratania	Edgardia	Vitoriana	Botanical Garden ^d	
Field ^b	48	74	88	14	0	224
Plantation ^c	9	50	0	52	0	111
Forest ^d	23	78	3	0	0	104
Farm ^e	0	46	0	27	0	73
Water side ^f	7	40	7	5	0	59
Road side ^g	30	21	5	2	0	58
Armadillo hole	21	20	11	3	0	55
Rotten plant	10	22	6	0	0	38
Bush ^h	11	3	1	3	0	18
Ant hole	7	8	0	2	0	17
Others ⁱ	8	47	1	0	74	130
Total	174	409	122	108	74	887

a: Botanical Garden of UNESP Campus; b: an area covered with different types of grass and a few isolated trees; c: cultivated area (coffee, sugar cane, oranges); d: eucalyptus or pine; e: neighborhood of farmers house; f: margin of ponds; g: shoulders of unpaved road; h: area covered with several different types of mixed local small trees; i: insect nests, lawns, egg shells, bird droppings, animal food.

BHIA. Multiple buddings were seen on isolate 268. Table II shows the localities and sites from where they were collected; three of them (isolates 212, 328-1, 328-2) were isolated from soil of Pratania and two (isolates 170 and 268), from armadillo holes also in Pratania.

TABLE II
Thermally dependent dimorphic fungi isolated from soil in Botucatu

Isolate number ^a	Site
170	Armadillo hole
212	Field
268	Armadillo hole
328-1	Eucalyptus forest
328-2	Eucalyptus forest

a: all five thermo-dimorphic fungi were isolated from Pratania.

Isolation of pathogenic dematiaceous fungi - Ninety-eight black colonies were obtained (Table III). They were identified as *Curvularia lunata*: 3, *Exophiala spinifera*: 55, *Fonsecaea pedrosoi*: 2, *F. pedrosoi*-like fungi: 14, *Phialophora verrucosa*: 6, *Exophiala* sp.: 11, *Ramichloridium anceps*: 3, *Rhinochrysiella atrovirens*: 3 and *Veronaea botryosa*: 3.

Isolation of Aspergillus spp. - Five hundred and eighty one of the 831 samples (56 of the 887 samples were not cultivated for technical reasons) had *Aspergillus* spp. They were found in all localities and sites studied. Table IV summarizes the findings.

Table V shows numbers and percentage of *Aspergillus* spp. and black fungi collected from soil and from rotten plants. The majority (80.7%) of our samples were collected from soil and we did not collect rotten plants in two localities.

TABLE III
Pathogenic dematiaceous fungi isolated from nature in Botucatu

Fungal isolate	Localities					Total
	Pro-Verde	Pratania	Edgardia	Viroriana	B. Garden ^a	
<i>Curvularia lunata</i>	1	1	1	0	0	2
<i>Exophiala spinifera</i>	11	18	13	5	8	55
<i>Exophiala</i> sp.	2	5	2	1	1	11
<i>Fonsecaea pedrosoi</i>	1	1	0	0	0	2
<i>F. pedrosoi</i> -like	7	5	1	0	1	14
<i>Phialophora verrucosa</i>	2	1	2	0	1	6
<i>Ramichloridium anceps</i>	1	0	1	0	0	3
<i>Rhinochrysiella atrovirens</i>	0	2	1	0	0	3
<i>Veronaea botryosa</i>	1	0	2	0	0	3
Total	26	33	22	6	11	98

a: Botanical Garden of UNESP Campus.

TABLE IV
Number of *Aspergillus* spp. isolated^a from nature in Botucatu

	Localities					Total 831 ^c
	Pró-Verde n-174	Pratania n-353	Edgardia n-122	Vitoriana n-108	B. Garden ^b n-74	
<i>A. fumigatus</i>	102	221	88	64	58	533
<i>A. niger</i>	83	130	45	45	51	354
<i>A. terreus</i>	19	63	48	38	29	197
<i>A. neoelli</i>	12	13	4	6	8	43
<i>A. flavus</i>	6	32	6	9	15	68
<i>Emericella</i> ^d sp.	26	34	10	6	21	97
<i>Neosartorya</i> ^e sp.	5	12	1	4	25	47
<i>Thielavia</i> ^f sp.	2	16	5	5	11	39
Others ^g	6	5	0	2	2	15

a: more than one species was isolated from the same sample; b: Botanical Garden of UNESP Campus; c: for technical reasons 56 of the 887 samples collected were not cultivated for *Aspergillus*; d: teleomorph of *A. nidulans*; e: Ascomyceta, Plectomyces, Eurotiales; its anamorph is *A. fumigatus*; f: teleomorph genus of Eurotiales; g: already published (Horie et al. 1995a, b).

TABLE V
Number of samples (n) and number of positive *Aspergillus* and black fungi isolates obtained from soil and rotten plants by locality

	Localities					Total
	Pró-Verde	Pratania	Edgardia	Vitoriana	B. Garden ^a	
Soil	n-164	n-331	n-1162	n-108	n-74	n-793
<i>Aspergillus</i> spp.	105	227	94	79	63	568 (71.6%)
Black fungi	15	25	17	6	9	72 (9.1%)
Rotten plants	n-10	n-22	n-6	0	0	n-38
<i>Aspergillus</i> spp.	2	10	1	0	0	38 (34.2%)
Black fungi	4 (40.0%)	3 (13.6%)	1 (16.7%)	0	0	7 (18.4%)

a: Botanical Garden of UNESP Campus.

DISCUSSION

Botucatu is located around 800 m above sea level, has 1,463 mm of rain fall per year and an average temperature of 19°C (Marques et al. 1983), which are the environmental features of the endemic areas of Pbmycosis (Restrepo-Moreno 1994). Indeed, the disease is frequent in the area and a large proportion of the rural population is reactive to paracoccidioidin (Carandina & Magaldi 1974). The knowledge that Pb has been isolated from soil and plants as well as the finding of the fungus in armadillos, a common animal in Brazil, led us to look for the agent in nature in the rural area of Botucatu to better understand how man is infected and to try to define the fungus natural habitat. The methodology used provided the opportunity to isolate several other possibly pathogenic fungi as dematiaceous black fungi and *Aspergillus*.

The thermo-dependent fungi that in culture had gross and microscopic similarities with Pb were isolated from Pratania an area in which more than 10% of the population is paracoccidioidin positive (Marques et al. 1983). Furthermore, several patients with PCM seen at the UNESP University Hospital live in Pratania. Two of these isolates were collected from soil of armadillo's holes (Table II), an interesting finding that correlates with the isolation of the agent in Amazonian armadillos (Naiff et al. 1986). We are in the process of studying the biology, antigenicity and virulence of these isolates as well as expanding the ecological survey to collect further soil samples from other armadillo holes. The results of these studies will be published in the future.

The fact that only five thermo-dependent dimorphic fungi were isolated from 887 samples indicates their scarcity and may explain the difficulties to isolate from nature the causative agent of Pbmycosis. If the morphological likeness to Pb of these dimorphic fungi is associated with antigenic similarities, exposure to them may interfere with the immune status of the local population leading to alterations on host-parasite relationship with consequences in the evolution of Pbmycosis.

Potentially pathogenic dematiaceous fungi of nine different species were also isolated (Table III). Among them, *F. pedrosoi*, *P. verrucosa* and *E. spinifera* are important causative agents of chromomycosis and phaeophycomycosis. As these diseases usually occur by the penetration of the causative agent through skin wounds, it is relevant to clarify their habitat in nature and the environmental circumstances in which they may infect man.

Trejos, in 1954, first succeeded in isolating *F. pedrosoi* from soil in Costa Rica; later on the fungus was isolated from soil and plant debris in Venezuela, Uruguay and Japan (Salfelder et al. 1968, Gesuele et al. 1972, Iwatsu et al. 1981, Okeke & Gugnani 1986). Nishimura (1994) and Nishimura et al. (1989) investigated the ecology of pathogenic fungi in natural and living environments in Colombia, Venezuela, Brazil, China and Japan and succeeded in isolating various species of pathogenic dematiaceous fungi including *F. pedrosoi*, *P. verrucosa* and *E. spinifera*. We also succeeded in isolating two strains of *E. pedrosoi* and 14 strains of *F. pedrosoi*-like fungi.

P. verrucosa is found around the world, on decayed wooden materials. The species was first

isolated from wood pulp by Melin and Nannfeldt (1934). Since then many successful isolations have been reported (Emmons 1954, Wang 1965, Klite et al. 1965, Udagawa & Horie 1971, Gesuele et al. 1972, Dixon et al. 1980, Iwatsu et al. 1981, Okeke & Gugnani 1986, Nishimura et al. 1989). We isolated six strains of *P. verrucosa* in Botucatu.

E. spinifera was isolated from nature by Mackinnon et al. (1973), by Dixon et al. (1980) and Nishimura (1994). According to these surveys, *E. spinifera* prevails in South America and China. We isolated 55 strains of *E. spinifera*. Our findings indicate that *E. spinifera* also prevails in Botucatu and neighbourhood.

Another interesting result is the isolation of *V. botryosa* from nature in Botucatu. This is the first report from South America; up to now, the species has only been successfully isolated in New Guinea and China (Nishimura et al. 1989).

Five hundred and eighty-one *Aspergillus* spp. were isolated from 831 culture samples (Table IV). As is well known, several species of *Aspergillus* produce mycotoxins particularly aflatoxin, one of the dangerous carcinogenic mycotoxin (Asplin & Carnaghan 1961, Lancaster et al. 1961, Sargeant et al. 1961). A serious accident with chickens due to aflatoxin occurred in Great Britain caused by groundnuts imported from Brazil. Therefore it is important to investigate the ecology of *Aspergillus* spp. in Brazil.

Horie et al. (1989, 1991) studied the distribution of *Aspergillus* species in Colombia, Venezuela and Brazil. In our survey various species of *Aspergillus* were isolated from nature. Studies to evaluate their ability to yield mycotoxins are under way.

The data on Table V indicate that *Aspergillus* spp. were isolated from 71.6% of the samples of soil and were present in all the localities studied. Black fungi, however were more frequently isolated from rotten plants. The small number of samples from these plant debris do not permit any valid comparison or conclusion. Furthermore plant debris were not collected from all of the localities studied.

We are now studying more samples collected from pasture grass, ant holes, earth worms and plant debris as well as from armadillos. Our preliminary results confirm our above described findings and are very suggestive of the close association of Pb and armadillos.

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