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Isolation of a *Paracoccidioides brasiliensis* strain from the soil of a coffee plantation in Ibiá, State of Minas Gerais, Brazil

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Paracoccidioides brasiliensis has rarely been isolated from its habitat in rural areas. In order to investigate the hypothesis that human infection with this fungus is linked to coffee plantations (*Coffea arabica*), material was collected monthly over a period of 1 year from farms in the town of Ibiá, State of Minas Gerais, Brazil. A total of 760 samples of soil, coffee leaves and fruits was cultured and inoculated into mice. A fungus isolated from the liver of a mouse inoculated with soil showed temperature-dependent dimorphism and *in vitro* mycelium and yeast phases characteristic of *P. brasiliensis*. Yeast cells of this fungus caused disseminated infection after intraperitoneal inoculation in Wistar rats from which the fungus was re-isolated. An antigen reacting with sera from patients with paracoccidioidomycosis was obtained from this *P. brasiliensis* strain; antigenic identity with strain 339 and with four other *P. brasiliensis* strains was detected by gel immunodiffusion. However, when the exo-antigen was submitted to SDS–PAGE, we observed low gp43 expression in this new strain, which we called Ibiá. The isolation of *P. brasiliensis* from the soil at a coffee plantation suggests that this is one of its habitats and supports the hypothesis of acquisition of paracoccidioidomycosis during agricultural activity in these areas.

Keywords coffee plantation, *Paracoccidioides brasiliensis*, occupational diseases

Introduction

Paracoccidioides brasiliensis is a dimorphic fungus characterized and described for the first time in Brazil [1,2] as the aetiological agent of paracoccidioidomycosis. This mycosis is endemic in tropical and subtropical areas in Latin America where it characteristically occurs in males between the ages of 30 and 50 years. Infection is closely linked to activity and/or residence in rural areas [3–7]. The disease usually has a chronic course with a clinical picture predominantly involving the lungs, skin, mucosa and mononuclear phagocytic system [8–10]. Advances have been made in the understanding of the various aspects of this mycosis and of its aetiological agent [11,12]. However, little is known about its microhabitat, i.e. the ecological niche of *P. brasiliensis* in nature and the time and circumstances of human infection.

The fungus has been isolated twice from soil [13,14], whereas many other attempts have failed [15]. *P. brasiliensis* was isolated from the faeces of frugivorous bats (*Artibeus lituratus*) [16], the viscera of squirrel monkeys (*Saimiri sciureus*) [17], the faeces of Antarctic penguins (*Pygoscelis adeliae*) [18], the viscera of armadilloes (*Dasypus novemcinctus*) [19,20], and from dog food probably contaminated with soil [21]. Nevertheless, significance of these findings within the epidemiological context of paracoccidioidomycosis and the ecological niche of its aetiological agent is still unknown [22].

P. brasiliensis has been linked to the coffee industry since it was isolated at coffee plantations in Venezuela [13] and developed *in vitro* on coffee leaves [23]. Evidence of paracoccidioidomycosis has been reported recently in

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Indians from the Suruí tribe in the Amazon jungle, subsequent to the cultivation of coffee [24]. There is also a considerable superposition of endemic areas of paracoccidioidomycosis in regions where coffee is grown in Brazil, Colombia and Venezuela.

These facts led us to investigate coffee plantations as one of the potential areas of transmission of this fungus, with the aim of isolating it from nature.

Materials and methods

The study was conducted in the town of Ibiá, State of Minas Gerais, Brazil, located 268 km from Belo Horizonte and 640 km from São Paulo (19°28′00″ latitude south, 46°32′30″ longitude) at an altitude of 840 m above sea level, with a dry and mild climate. The town has a regular seasonal cycle with 60% humidity, an average temperature of 19 °C and 1600 mm of rainfall per year. The pH of the soil at the time of the study was 4·5. The economy of the region is mostly based on agriculture, especially coffee, the cattle industry and dairy products. The choice of this area was based on the characteristics described above and on the occurrence of patients having paracoccidioidomycosis.

Three coffee-growing farms were selected in the rural area of Tobati, Ibiá, from which 60–80 samples were collected monthly at random, for a total of 760 samples collected over 1 year. The samples consisted of soil taken from the superficial soil layer under the coffee trees, and coffee leaves (about 50% of each). At harvest time green and ripe coffee fruits were also collected. These materials were aseptically removed with gloves, wrapped in plastic bags and processed the day after collection.

The soil samples were weighed and 50 g was placed in 50 ml of sterilized water, stirred with a glass rod for 60 s, and left to stand for another 60 s. Five ml were then aspirated from the supernatant with a syringe and needle. Amounts of leaves and fruits weighing 70–250 g were washed by shaking in 100 ml of sterilized water, the wash was centrifuged at 3000 rpm for 10 min and the sediment was obtained after dilution in 5 ml of the floating supernatant. Six mg of chloramphenicol was then added to this sample and to those extracted from the soil. All samples were used to inoculate animals and cultured.

From each sample 0·1 ml was inoculated onto Sabouraud glucose agar containing antibiotics (1·5 mg ml^{-1} cycloheximide, 50 μg ml^{-1} chloramphenicol; 1500 U ml^{-1} penicillin G, 40 μg ml^{-1} gentamicin; and 300 μg ml^{-1} imipenem) and incubated at room temperature for 12 weeks. The same amount of each sample was inoculated into Fava–Netto medium [25] containing antibiotics (1500 U ml^{-1} penicillin, 40 μg ml^{-1} gentamicin, 160 μg ml^{-1} chloramphenicol, and 300 μg ml^{-1} imipenem) and incubated at 35 °C for the same period of time.

The same material as that used for the cultures was inoculated intraperitoneally into C57BI6 mice (n = 360) or Swiss mice (n = 400) weighing 15–25 g (one mouse per sample). Once a week during the following 3 weeks these animals received 0·6 ml of the same sample mixed with 0·6 ml of ciprofloxacin (2 mg ml^{-1}). Therefore, each mouse was injected with a total of 1·8 ml of each sample. Three months after the first inoculation the animals were sacrificed and their liver, lungs and spleen were removed, fragmented in a sterilized Petri dish and cultured on Mycosel® and Fava–Netto media at room temperature and at 35 °C, respectively. The cultures were observed for 12 weeks and the isolated fungi were identified.

Results

It was not possible to isolate *P. brasiliensis* by direct culturing of soil, leaf and fruit samples. There was intense growth of yeasts and filamentous fungi. Some of these isolates initially resembled *P. brasiliensis* when grown at 35 °C and at room temperature, but they were identified as *Fusarium* spp.

Among the animals inoculated with samples from coffee substrates, one mouse inoculated with soil was found to have multiple abscesses in the peritoneum and liver at autopsy. Dimorphic fungi producing the typical morphology of *P. brasiliensis in vitro* were isolated from the liver of this mouse after culture on Mycosel® and in Fava–Netto media (Fig. 1). At room temperature the culture showed cottony colonies consisting of thin filaments of mycelium with swollen cells. At 35 °C the colonies had a cerebriform aspect, a yellow-white colour, globose yeasts with a double, refringent wall, and simple or multiple budding forms ranging from 2 to 28 μm in diameter (Fig. 2). Interconvertibility of these forms was demonstrated by subculture at the higher and lower temperatures.

The virulence of this isolate was tested in 10 Wistar rats weighing 50 g which were inoculated intraperitoneally with $3{\cdot}9 \times 10^8$ yeasts. The inoculum was prepared from a culture grown on Fava-Netto medium at 35 °C with a viability greater than 90%. Forty-five days later 50% mortality occurred and the remaining animals were sacrificed. These rats showed deep and disseminated infection involving the abdominal cavity and wall, liver, spleen and lungs (Fig. 3). A granulomatous inflammatory process with large numbers of yeasts similar to *P. brasiliensis* was observed in histological sections (Fig. 4). Fragments of the lungs and livers of these rats were cultured on

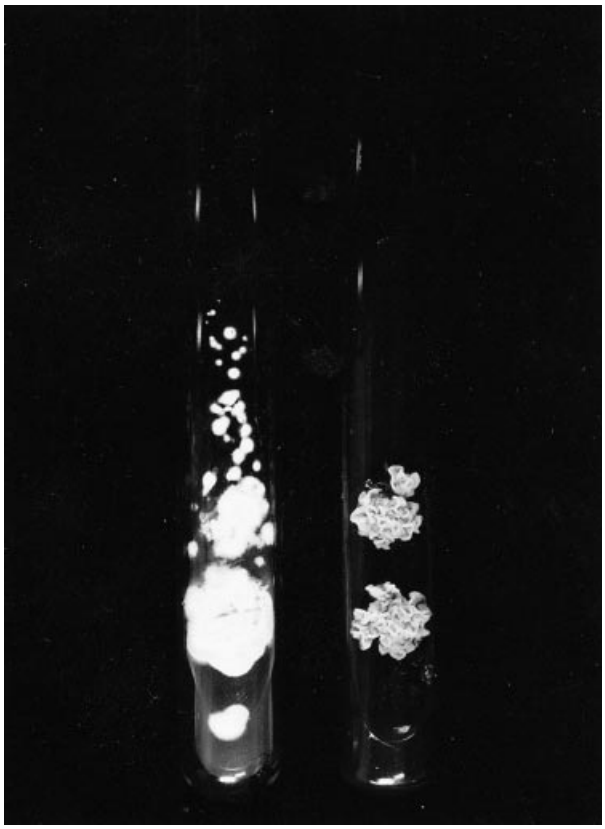

Fig. 1 *P. brasiliensis* isolated from soil. Left: white cotton-like colonies (25 °C). Right: cerebriform colonies (35 °C).

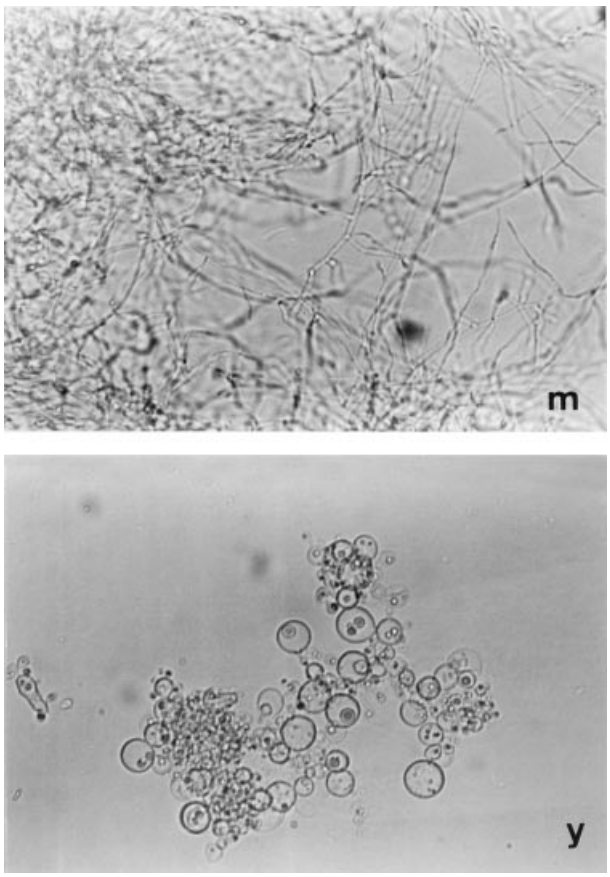

Fig. 2 Micromorphology of the new strain. m: fine mycelial filaments and swollen cells (25 °C); y: yeast cells with refringent walls and multiple buds (35 °C). (× 250).

Sabouraud glucose agar and a dimorphic fungus was again isolated, identical to the culture used as inoculum. The virulence of the fungus isolated from the soil was also evaluated in two guinea pigs whose right testes were inoculated with 0·5 ml of a suspension containing 1×10^6 yeast cells. Local erythema and enlargement of the testicles was observed 25 days later. The orchitis process due to the new *P. brasiliensis* isolated was confirmed by histological examination.

The antigenicity of the new strain was evaluated by an immunodiffusion test in agarose gel using an antigen prepared with sonicated disrupted fungal suspensions (Labsonic R 90w for 15 min). Serum from 20 patients with paracoccidioidomycosis produced one or two precipitin bands with this antigen, whereas serum samples from patients with histoplamosis or aspergillosis did not react with it (Fig. 5).

Two rabbits were immunized with the new isolate of *P. brasiliensis* by daily intravenous injection of $1{\cdot}5 \times 10^6$ yeasts for 14 days. The antiserum was submitted to immunodiffusion in the presence of the corresponding sonicated antigen and of sonicated antigens of strain 339 and of four other strains of *P. brasiliensis* isolated from patients. The precipitin bands were suggestive of identity of the antigens of the different strains (Fig. 6). The yeast exoproteins of the new strain of *P. brasiliensis* secreted in liquid medium (dialysed neopeptone) were analysed by SDS–PAGE together with the exoproteins of the BAT strain of *P. brasiliensis* recovered from a patient with the acute clinical form of paracoccidioidomycosis. The two strains showed several bands in common but the fungus isolated from soil showed a low expression of 43 kDa glycoprotein (Fig. 7).

Discussion

The isolation of *P. brasiliensis* from soil has been achieved only by Negroni in Argentina in 1966 [14], and by Albornoz in Venezuela in 1971 [13], with the latter isolation being more universally accepted by the scientific community [7].

The new isolation of *P. brasiliensis* reported here was obtained by collecting the surface soil from beneath the coffee tree, an environment which receives less sunlight

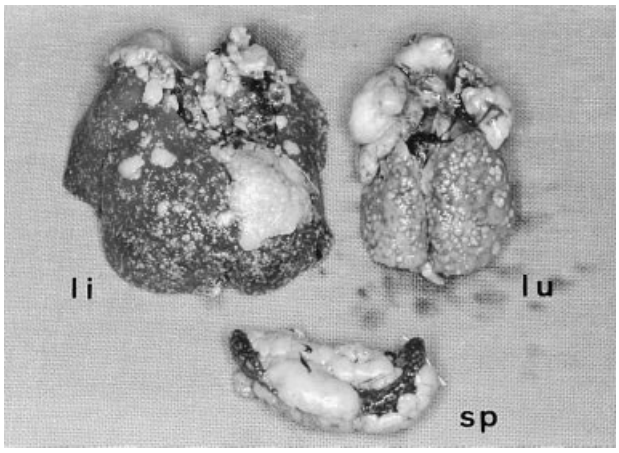

Fig. 3 Multiple subcapsular nodules in the liver (li), lung (lu) and spleen (sp.) of a Wistar rat inoculated with the new *P. brasiliensis* strain.

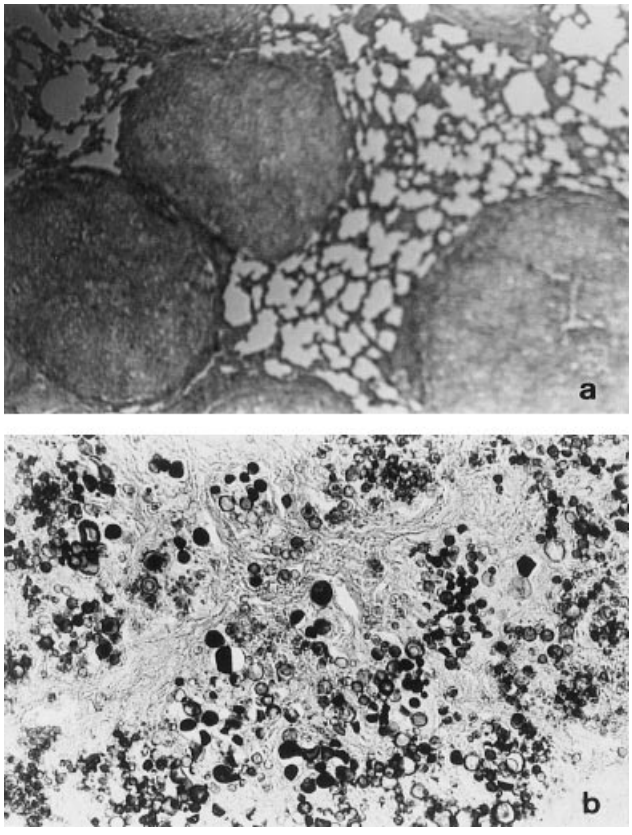

Fig. 4 Histological examination of the viscera of a rat infected with the new isolate: (a) confluent granulomatous nodules in the lung (H&E stain, × 63); (b) yeast cells, some of them with multiple buds, dispersed through the spleen (GMS stain, × 250).

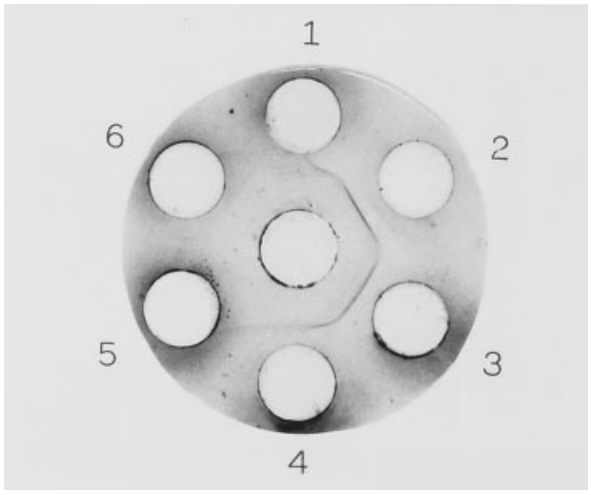

Fig. 5 Agarose gel immunodiffusion. Central well: antigen of sonicated yeast cells of the new *P. brasiliensis* strain; wells 1–4: sera from patients with paracoccidioidomycosis; well 5: serum from a patient with histoplasmosis; well 6: serum from a patient with aspergillosis.

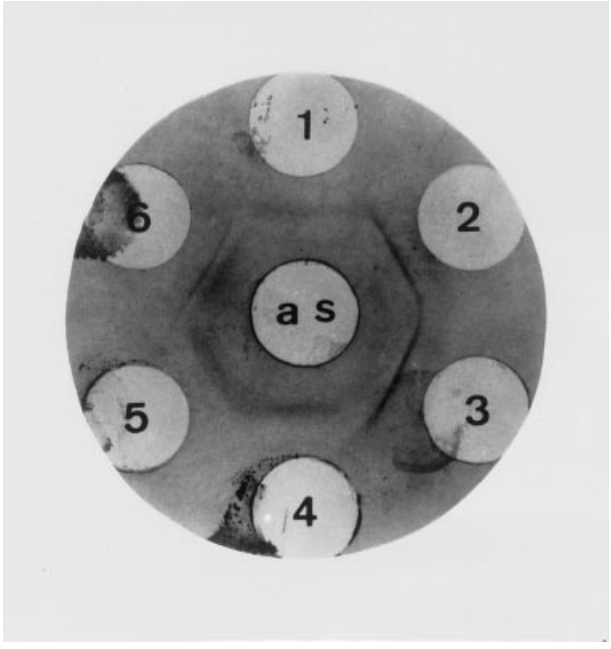

Fig. 6 Agarose gel immunodiffusion. Serum from a rabbit immunized with yeast cells of the new *P. brasiliensis* strain (as) formed precipitin lines with an antigen of this strain (well 1), with strain 339 (well 6) and with four other strains isolated from patients (wells 2–5).

and therefore is more humid. Injection of a suspension of this soil into a mouse permitted isolation of the fungus from fragments of the animal's liver 3 months after inoculation. The soil sample was collected in July 1994, which corresponds to the winter harvest period in Brazil when the weather is cool and dry. In contrast to other times of the year, fertilizer and antifungal products are

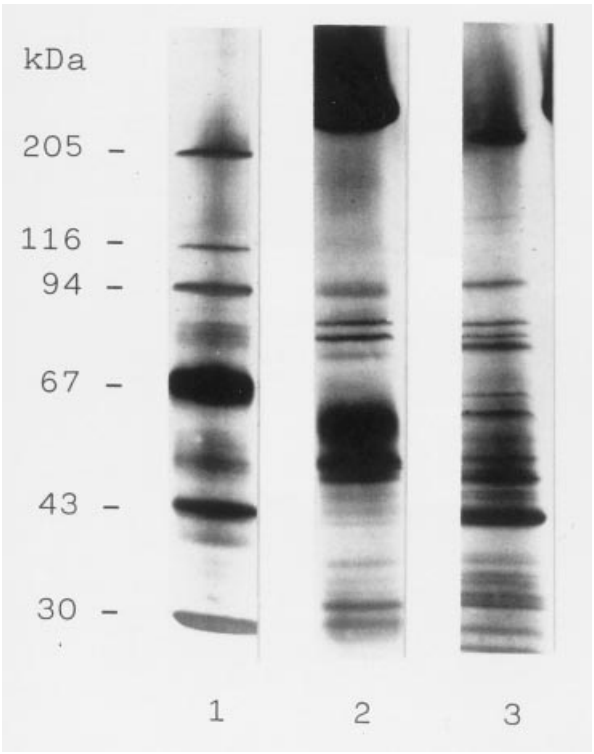

Fig. 7 SDS–PAGE. Lane 1: molecular weight markers; lane 2: exo-antigen of *P. brasiliensis* isolated from soil; lane 3: exo-antigen of the BAT strain isolated from a patient.

not used at coffee plantations during the winter (June to September) and the soil is extremely dry and dusty. Furthermore, the ecological conditions of the region where the new strain was isolated are similar to those described previously by several authors when describing the areas of natural occurrence of the fungus [3,5,26–28]. We named the new isolate the Ibiá strain.

The pathogenicity of the new isolate to rats and guinea pigs, its antigenic identity with other human strains of *Paracoccidioides* and its protein profile compared with that of the BAT strain [29] definitely confirm the isolation of *P. brasiliensis.* Low gp43 secretion was detected by electrophoresis. Although most *P. brasiliensis* strains produce large amounts of this protein, a recent study has shown that other strains may present little or negligible amounts of this molecule [30,31]. Moreover, it is unknown if isolates in nature behave like those isolated from patients. A detailed evaluation of the Ibiá strain may contribute to a better understanding of the biology of *P. brasiliensis*, especially with respect to its saprophytic characteristics in nature.

Despite the large number of samples obtained and of experiments conducted over a period of 1 year, isolation was achieved only once, confirming the rarity of this event. This probably depends on changing, unknown ecological conditions. The climate changes are a remarkable factor which have already been considered by several authors, and in the present study we have emphasized that the fungus was isolated from soil during the dry cold season of the year.

The isolation of *P. brasiliensis* from a single soil sample may also be interpreted as the result of airborne distribution of a propagule originating from another habitat. However, paracoccidioidomycosis is endemic in the region where the isolation was made, suggesting the continuous presence of the fungus at this site. The observation of a granuloma containing *P. brasiliensis* in the lung of an armadillo on a coffee plantation in Ibiá, Brazil [32], represents additional important evidence of the existence of an ecological niche for the fungus in this area.

The present study, which was conducted to develop an epidemiological model relating coffee culture to the transmission of *P. brasiliensis*, has led to the isolation of a new strain of this fungus, emphasizing the importance of agricultural activities as a predisposing factor to paracoccidioidomycosis. Perhaps it is not only coffee culture that provides the conditions for the fungus to maintain its saprophytic nature and to favour the infection of field hands working in environments where the formation of aerosols is frequent. The possibility of a heterothermal mammal and even the presence of arthropods closing the epidemiological link may account for the transitory finding of the fungus in the crops.

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