Simultaneous infection of human host with genetically distinct isolates of *Paracoccidioides brasiliensis*

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This study is the first report on genetic differences between isolates of Paracoccidioides brasiliensis *from a single patient. We describe a simultaneous infection with genetically distinct isolates of* P. brasiliensis *in a patient with chronic paracoccidioidomycosis. The clinical isolates were obtained from lesions in different anatomical sites and were characterised by random amplified polymorphic DNA (RAPD) analysis. The RAPD technique can be helpful for distinguishing between clinical isolates. Different random primers were used to characterise these clinical isolates. The RAPD patterns allowed for differentiation between isolates and the construction of a phenetic tree, which showed more than 28% genetic variability in this fungal species, opening new possibilities for clinical studies of* P. brasiliensis. *Based on these results and preliminary clinical findings, we suggest that different genotypes of* P. brasiliensis *might infect the same patient, inducing the active form of the disease.*

Key words: *Paracoccidioides brasiliensis* - simultaneous infection - RAPD

Paracoccidioidomycosis is considered the most prevalent systemic mycosis in South America. The etiologic agent of this disease is the dimorphic fungus *Paracoccidioides brasiliensis and* areas of endemicity can be found in Brazil, Colombia, Venezuela and Argentina (San-Blás 1993, McEwen et al. 1995). In Brazil, the state of Mato Grosso (MT) has a high incidence with cases reported daily, forming a distinct geographic region for the acquisition of the disease (Hahn et al. 2003).

Although previous papers have attempted to link random amplified polymorphic DNA (RAPD) patterns to other parameters (virulence of the isolate, geographic origin, in vitro susceptibility to antifungal drugs, clinical forms of the disease, atypical isolates, comparison between clinical isolates and armadillo isolates), little is known about the extent of genetic variation within *P. brasiliensis* (Soares et al. 1995, Sano et al. 1999b, Hahn et al. 2003).

One study (Sano et al. 1999b) used RAPD-PCR to compare isolates obtained from humans and armadillos. Although one armadillo harbored several genotypes, no studies have revealed genetic heterogeneity between clinical isolates from separate lesions in the same patient.

The present paper aims to use RAPD to compare distinct isolates of *P. brasiliensis* obtained from lesions in the upper right arm and right upper quadrant of the face. The patient is a 46-year-old white male agricultural machinery operator with a case of chronic paracoccidioidomycosis having pulmonary involvement.

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PATIENT, MATERIALS AND METHODS

Direct mycological examination was undertaken by applying 20% potassium hydroxide to the patient's sputum and to biopsied tissue of the patient's arm and facial lesions. Cultures of biopsy tissue on Fava-Netto agar medium supplemented with chloramphenicol revealed fungal growth 14 days after inoculation and incubation at 35ºC. The colonies presented macroscopic and microscopic characteristics of the yeast-like form of *P. brasiliensis*. Histological examinations of the biopsy fragments proved positive for the yeast form of multifilament fungal cells, typical of *P. brasiliensis*.

Isolates from lesions in the right upper quadrant of the face and upper right arm were named Pb550F and Pb550B, respectively. The two isolates were sampled after growth for 5-6 days in solid Fava-Netto medium at 35ºC. Genomic DNA was obtained from each individual yeast culture by the method in Sambrook et al. (1989). Briefly, a loop of the yeast colony was transferred to an Eppendorf tube containing 0.5 mL TES buffer (50 mM EDTA, pH 8.0, 10 mM Tris-HCl, pH 8.0 and 20% sucrose), 5 µL of β-2 mercaptoethanol and 5 mg of novozym, incubated for 2 h at 30ºC and centrifuged at 5,000 rpm for 5 min. A 0.5 mL aliquot of TES buffer + 0.5 mL of 10% SDS was added to the precipitate and the mixture incubated at 65ºC for 30 min; then, 200 µL of 3 M sodium acetate, pH 5.0 and RNAse (10 µg/mL) were added and the mixture was incubated at 37ºC for 30 min, centrifuged at 5,000 rpm for 10 min and the supernatant transferred to a new, sterile 1.5 mL tube. For DNA extraction, an equal volume of phenol/chloroform (1:1) was added and the mixture was shaken for 15 min at 4ºC and centrifuged at 5,000 rpm for 10 min. This procedure was repeated twice. A 1/10 volume of 3 M sodium acetate, pH 5.0 and two volumes of 100% etha-

nol were added to the supernatant at 0ºC. The DNA was precipitated overnight at 0ºC, centrifuged at 10,000 rpm for 20 min and washed with 70% cold ethanol. The pellet was dried at RT and resuspended in 100 µL of sterile Milli-Q water. DNA concentration was determined by spectrophotometry at 260 nm and its purity determined by the $A_{260/280}$ ratio.

RAPD analysis was performed as in Williams et al. (1990) with minor modifications. Three primers were used: OPG 05 (5'-TG AGA CGG A-3'), OPG 14 (5'-GGA TGA GAC C-3') and OPG 16 (5'-AGC GTC CTC C-3') (OPERON-Biotechnology). For the DNA amplification reactions, a master mix containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each oligonucleotide, 2 U of Taq polymerase, 25 pmol of primer and 100-200 ng of genomic DNA (final volume, 25 µL) was used. This reaction mixture was overlaid with 20 µL of mineral oil to prevent evaporation. The amplification program was 94ºC for 2 min for the initial denaturation, followed by 40 cycles at 94ºC (1 min), 30ºC (2 min) and 72ºC (2 min) and a final extension period at 72ºC for 7 min. Amplification was performed with a Thermolyne (Amplitron II) thermocycler. The randomly amplified fragments were analyzed by 1.4% agarose gel electrophoresis and visualised with ethidium bromide staining.

PCR was performed as described by Gomes et al. (2000) to verify the presence of gp43. Agarose gel electrophoresis of PCR products was performed to visualise the amplification of a 0.7-Kb fragment in the gp43 gene from isolates Pb550F and Pb550B amplified with the 590 and 592 primer pair.

RESULTS

The *P. brasiliensis* strains obtained from the upper right arm and right upper quadrant of the face of a patient with chronic paracoccidioidomycosis revealed different RAPD patterns and the use of three primers enabled discrimination between the two isolates. Furthermore, the reproducible amplification products were sufficiently polymorphic to allow the genetic differentiation of the strains.

Representative RAPD patterns for both clinical isolates obtained with primers 3237, 3238 and 3239 (Fig. 1) were scanned and analyzed using the GelCompar software, version 4.0 (Applied Maths, Kortrijk, Belgium) and the data were used to construct a phenogram (Fig. 2). Genetic diversity varied between 15-28%, suggesting genetic variability between the clinical isolates (Fig. 2).

Genotypic characterisation of *P. brasiliensis* clinical isolates was obtained by visualisation of bands corresponding to a 0.7-kb fragment of the *gp43* gene of *P. brasiliensis* (Fig. 3). The existence of bands corresponding to the migration position of a 43-kDa glycoprotein, the exoantigens 550F and 550B, was verified for all samples.

DISCUSSION

Calcagno et al. (1998) used the RAPD technique to cluster 33 *P. brasiliensis* strains into five major groups related to geographical origin (Venezuela, Brazil, Peru, Colombia and Argentina), but not to pathological features of the disease. Molinari-Madlum et al. (1998)

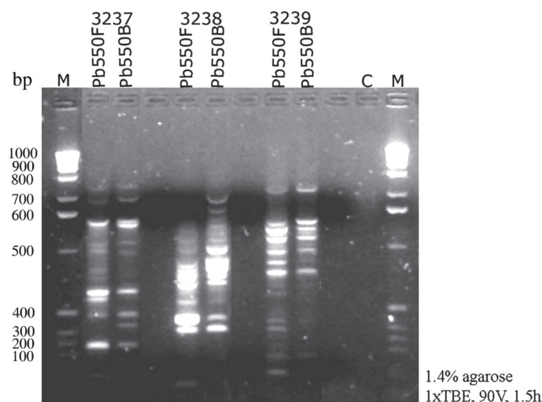

Fig. 1: random amplified polymorphic DNA profiles showing polymorphism between the two analyzed *Paracoccidioides brasiliensis* isolates (Pb550F and Pb550B). Primers user were 3237, 3238 and 3239. bp: base pairs; C: control (no DNA); M: 1 Kb DNA molecular weight marker.

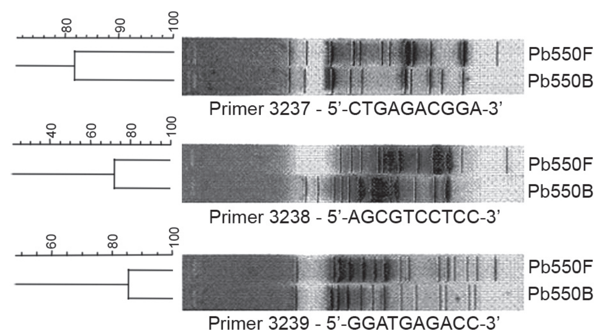

Fig. 2: phenogram of *Paracoccidioides brasiliensis* based on GelCompar software, version 4.0, from data obtained by random amplified polymorphic DNA analysis.

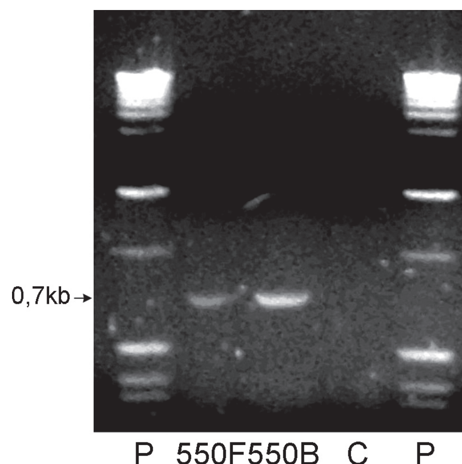

Fig. 3: agarose gel electrophoresis with the PCR products obtained from clinical isolates 550F and 550B, amplified by specific primers 590 and 592, which amplify a fragment of 0.7 kb from the gene of gp43 kDa from *Paracoccidioides brasiliensis*.

showed that RAPD patterns of *P. brasiliensis* isolates from Brazil and Ecuador were reasonably well correlated with the degree of virulence in mice. Motta et al. (2002), however, could not find any association between RAPD profiles of 35 strains of *P. brasiliensis* (with the majority isolated from Brazil), either in virulence in experimentally infected mice or with clinical forms of human disease.

Sano et al. (1999b) compared 63 isolates of *P. brasiliensis* obtained from thirty-nine banded armadillos (*Dasypus novemcinctus*) and 19 clinical isolates using the OPG-19 primer and RAPD analysis. One of the RAPD clusters was a *P. brasiliensis* genotype characteristic of animals and another was from individual armadillos possibly infected with *P. brasiliensis* of a different genotype. Sano et al. (1999a) reported on the pathogenicity of three *P. brasiliensis* isolates from a nine-banded armadillo (*D. novemcinctus*). This study identified a partial sequence of the GP43kDa gene for one of the isolates (D351), suggesting that the same armadillo might be susceptible to multiple *P. brasiliensis* isolates. Our study results are similar to those of Sano et al. (1999b). Genetic heterogeneity between clinical isolates from the same patient suggests that humans may be infected with *P. brasiliensis* cells of different genotypes.

As for human fungal infection, other authors have reported *Aspergillus fumigatus* and *Candida albicans* as exhibiting different genotypes (Verweij et al. 1996, Redding et al. 1997). Haynes et al. (1995) reported that multiple strains of *C. neoformans* may be responsible for a single episode of cryptococcosis and that recurrent infection may occur as a result of reinfection with a novel strain. There is, however, no information in the literature evaluating strains from distinct anatomical sites from the same patient with paracoccidioidomycosis.

Two strains from our patient exhibited genetic diversity of 18% (OPG 05), 28% (OPG 16) and 15% (OPG 14). The amplification of select DNA sequences in a future study will clarify the genetic diversity among our isolates. Hahn et al. (2003) showed a genetic association between isolated strains in MT (Brazil) from patients with good therapeutic responses to trimethoprim-sulfamethoxazole, in contrast to other strains isolated from the MT at the same time from patients who presented as relapses or failed treatment with trimethoprim-sulfamethoxazole.

These findings deserve attention when seen in light of the eco-epidemiological aspects of paracoccidioidomycosis. Matute et al. (2006) demonstrated the existence of three phylogenetic species (S1, PS2 and PS3) of *P. brasiliensis* and more recent studies indicate an additional distinct genetic group, named Pb01-like (Carrero et al. 2008, Theodoro et al. 2008, Teixeira et al. 2009). Despite being considered different phylogenetic species, all of these were capable of inducing disease in both humans and animals. Our study results provide preliminary evidence of simultaneous human infection with different *P. brasiliensis* isolates.

We suggest that genetic characteristics of *P. brasiliensis* isolates may influence and open new perspectives for clinical-epidemiological studies in paracoccidioidomycosis. Further characterisation of DNA differences between isolates may be a promising strategy for more clearly defining their biological importance.

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