Note

Characterization of *Paracoccidioides brasiliensis* atypical isolates by random amplified polymorphic DNA analysis

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Summary

Two atypical *Paracoccidioides brasiliensis* strains (yeast form at room temperature) have been isolated from chronically infected patients living in Brazil. Different random primers were used to characterize these isolates and compare them to typical strains. The RAPD patterns allowed the differentiation of all the selected isolates. Their genetic distance ranged from 5% to 80% of non-shared bands depending on the strains and the primer used. The RAPD data were used to build a Wagner phenogram, which showed two major branches with more than 56% of genetic distance separating them. No significant difference was observed between the atypical isolates and the others suggesting that specific genes are involved in the dimorphism phenomenon.

Key words *Paracoccidioides brasiliensis*, Atypical isolates, RAPD analysis

Caracterización de aislamientos atípicos de *Paracoccidioides brasiliensis* por análisis del ADN polimórfico amplificado aleatoriamente

Resumen

Se aislaron dos cepas atípicas de *Paracoccidioides brasiliensis* (con forma de levadura a temperatura ambiente) en dos pacientes con infección crónica en Brasil. Se emplearon diferentes cebadores aleatorios para caracterizar estos aislamientos y compararlos con cepas típicas. Los patrones de RAPD permitieron diferenciar todos los aislamientos seleccionados con una distancia genética de entre 5 y 80% de bandas no compartidas dependiendo de las cepas y el cebador empleados. Los datos de RAPD fueron utilizados para construir un fenograma de Wagner que mostraba dos ramas principales con una distancia genética entre ellas superior al 56%. No se observaron diferencias significativas entre los aislamientos atípicos y los demás, sugiriendo que genes específicos están implicados en el fenómeno del dimorfismo.

Palabras clave *Paracoccidioides brasiliensis*, Aislamientos atípicos, Análisis RAPD

Paracoccidioides brasiliensis is the etiological agent which causes paracoccidioidomycosis, a systemic infection occurring exclusively in Latin America, where it is one of the most prevalent forms of deep mycosis [1-3].

There is a consensus about the existence of different *P. brasiliensis* strains. Variations in growth rate, morphology, ultra-structure [4] and biochemical characteristics [5] have been detected in several fungal isolates. In addition, variations in the biochemical composition of different fungal strains have been related to genetic determinants [6], to time of *in vitro* storage [3,5,7] and to type of culture medium in which the isolates develop [8,9]. By definition, *P. brasiliensis* is a dimorphic fungus that develops a mycelium form at room temperature and a yeast form at 37 °C [1-3]. However atypical strains that maintain the yeast-like phase in cultures at room temperature, have been obtained by chemical treatment [6], or nutritional restriction [10,11].

Recently we described a case of naturally occurring atypical *P. brasiliensis* (JT-2), recovered from a chronically infected female [12]. In this work we presented another atypical strain, named 1430, isolated by Dr. Jorge Lopes from a 55-year-old farm worker, suffering from chronic infection, in Santa Maria, Rio Grande do Sul, Brazil. These two strains were the first naturally occurring cases of *P. brasiliensis*, which did not present the conventional yeast –mycelial dimorphism. In this research we use the RAPD analysis to compare these two atypical isolates with four other strains exhibiting the classic dimorphism which included; one reference sample

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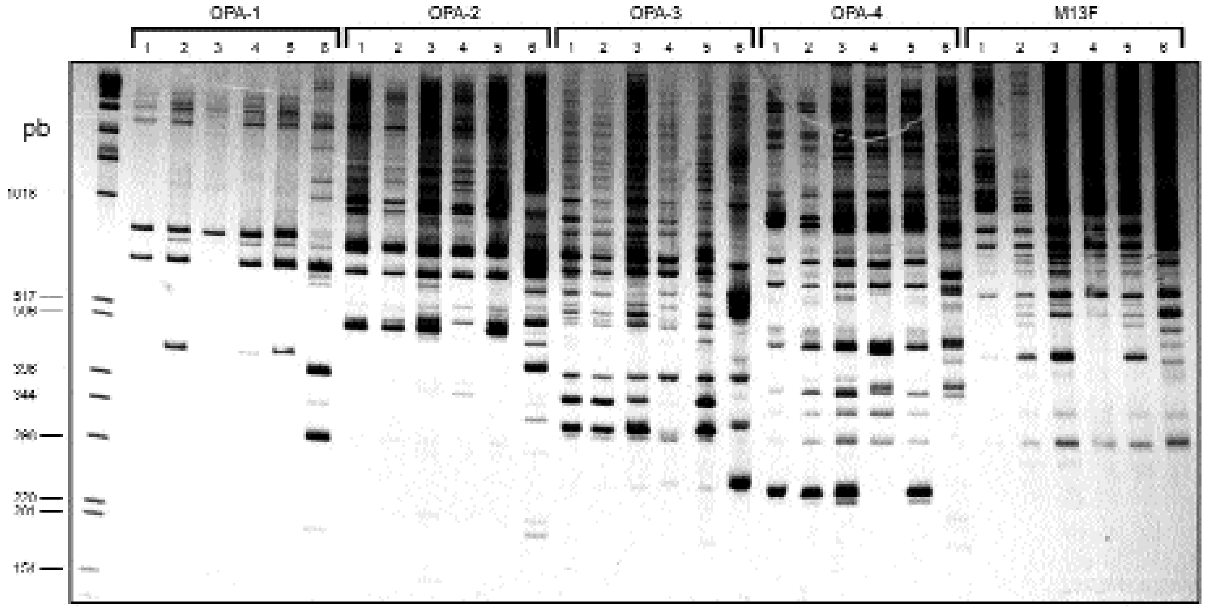

Figure 1. RAPD profiles showing polymorphism among the six analyzed *P. brasiliensis* isolates. The primers used were OPA-1, OPA-2, OPA-3, OPA-4 and M13F. 1- strain JT-2 (atypical isolate); 2- strain 1430 (atypical isolate); 3- strain Armadillo; 4- strain Penguin; 5- strain JT-1 (ATCC 90659); 6- strain RAJ-2.

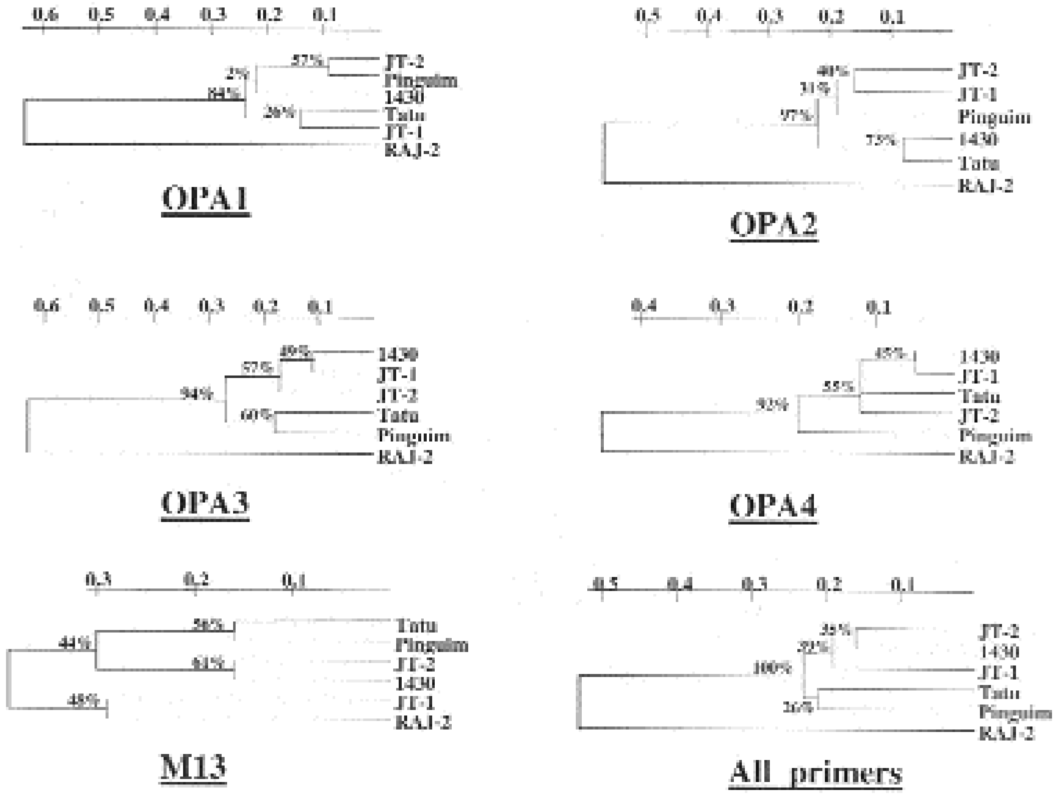

Figure 2. Phenograms of *P. brasiliensis* isolates based on UPGMA method derived from RAPD assays generated by using each primer separately and combined.

(ATCC 90659), one environmental isolate (Penguin) [13], one animal isolate (Armadillo) [14] and one clinical isolate from Cuiabá- MT, Brazil (RAJ-2).

All analyzed strains were maintained on solid Fava-Neto's medium at 35°C [15] and microorganisms in yeast-like form were collected in the exponential growth phase. DNA was prepared as described by Borges *et al.* [16] after an enzymatic digestion with glucanase (Glucanex- Novo Nordisk, USA). We initially tested 10 different arbitrary primers and chose the OPA-1 (CAGGCCCTTC), OPA-2 (TGCCGAGCTG), OPA-3 (AGTCAGCCAG), OPA-4 (AATCGGGCTG) and M13F (TGACCGGCAGAAAAATG) which produced more polymorphic and reproductive profiles. RAPD analyses were carried out as described by Williams *et al.* [17] in a Perkin-Elmer thermocycler. The reaction was achieved in a final volume of 10 µl of PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 3.5 mM $MgCl_2$) containing 1 picomol of primer; 0.5mM of each dNTPs; 0.3 U of *Taq DNA polymerase* (Gibco BRL, USA) and 1 ng of total DNA. Randomly amplified products were analyzed by electrophoresis in 8% polyacrilamide gel for 6 h at 125V and 30mA followed by silver staining as described by Santos *et al.* [18].

For RAPD data analysis the relative mobility positions of all bands present in each analyzed *P. brasiliensis* strain were calculated and transformed into a data matrix. We used the Nei & Li algorithm [19] contained in the TREECON computer package program [20] to calculate the genetic distances between the strains. The phenograms were then constructed by UPGMA (Unweighted Pair Group with Arithmetic Mean) method and the robustness of the tree topology was assessed resampling 1000 times by bootstrap [21-23]. We considered a particular branch as strongly supported if it appeared in more than 80% of the bootstrapped trees.

Figure 1 shows the *P. brasiliensis* RAPD patterns obtained with the five chosen primers On average we detected 8.9 ± 0.3 (average ± SD) bands varying from 154 to 1018 bp depending on the primer and the strain analyzed. No strain exhibited an identical pattern considering each primer separately or in combination. The percentage of shared bands between any two strains studied was on average 66 % ± 17 (average ± SD) ranging from 20% (observed between strains Armadillo and RAJ-2 with the primer OPA-1) to 92% (observed between strains 1430 and JT-1 with the primer OPA-4).

The RAPD data were used in a phylogenetic approach for the *Paracoccidioides* group. Figures 2 shows that, in general, the phenograms constructed from the data obtained with each primer separately or in combination are very similar to each other. With the exception observed for M13 profiling, all *P. brasiliensis* strains analyzed but one (RAJ-2) clustered systematically in one of the two major branches. The branch we named group I, encompasses the isolates JT-2, 1430 (atypical isolates) JT-1 (ATCC-90659), Armadillo and Penguin. The strains belonging to this branch were genetically more related presenting on average 74% of shared bands. To our surprise the isolate RAJ-2, a typical clinical isolate, from Cuiabá-MT, Brazil, was set apart from the others in a branch we named group II and presented a maximum of 44% of shared bands with group I. The separation of group I and group II was supported by 100% of bootstrap analysis in the combined data tree.

Our findings demonstrated that RAPD technique using five arbitrary primers could differentiate all *P. brasiliensis* strains but were not able to distinguish the atypical strains JT-2 and 1430 from the others. These results suggest that mutational events limited to a unique or very few genes are involved in the regulation of the *P. brasiliensis* dimorphism phenomenon. In this context several studies have demonstrated that nutritional factors, among others, may also be involved in the regulation of dimorphism in *P. brasiliensis* [10, 24-26]. In our case, despite numerous attempts, it has not yet been possible to adapt the atypical isolates JT-2 and 1430 to a synthetic medium [27], which suggests the existence of some nutritional deficiency. Additional studies, such as differential display, should determine which genes are involved in the dimorphism phenomenon of the *P. brasiliensis*.

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