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Characterization of *Paracoccidioides brasiliensis* Isolates by Random Amplified Polymorphic DNA Analysis

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We initially used 25 different random primers in order to test their ability to generate random amplified polymorphic DNA fragments from the dimorphic human pathogenic fungus *Paracoccidioides brasiliensis*. From the tested primers we chose five to distinguish between seven isolates of this microorganism. The DNA amplification patterns allowed clear differentiation of the seven isolates into two distinct groups with only 35% genomic identity. One of these groups contained two subgroups with 81% genetic similarity. The random amplified polymorphic DNA analysis method proved to be a good tool for analyzing and comparing different genomes of *P. brasiliensis* isolates.

Paracoccidioidomycosis is a human systemic mycosis caused by the thermal dimorphic fungus *Paracoccidioides brasiliensis*. The disseminated, mucocutaneous, or pulmonary form of the disease may be the result of host-related factors as well as characteristics of the infecting agent (1, 13, 15). Since it is assumed that *P. brasiliensis* isolates could differ in their abilities to cause disease in humans, strain determination is very important. An efficient means of characterizing individual isolates is needed, since until now the denomination of strains in *P. brasiliensis* has been related to the virulence and biochemical characteristics of the fungal isolates.

Several investigators have reported the use of arbitrary primers for the identification of strains in different populations (4, 17, 18). Randomly chosen primer sequences, whose distributions within the genome would be different, are able to distinguish among different isolates. This technique has been successfully used to discriminate among isolates of several organisms such as *Aspergillus fumigatus* (2), *Rhizobium leguminosarum* (7), *Hirsutella longicolla* (16), and *Histoplasma capsulatum* (19) and has been applied to the study of genomic variations among other fungal species (6). In the present study, we examined the use of randomly selected primers to distinguish among isolates of *P. brasiliensis.*

Seven *P. brasiliensis* isolates were used in order to determine strain classification; they were strains Pb 01, 2052, 1684, S, and G, isolated by the Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil, and strains 662 and 7455 isolated by Instituto Nacional de Hygiene y Medicina Tropical Leopoldo Isquieta Péres, Guayaquil, Ecuador. For DNA isolation, yeast cultures of each isolate were grown at 37°C in semisolid Fava-Neto's medium (5) containing 1% (wt/vol) peptone, 0.5% (wt/vol) yeast extract, 0.3% (wt/vol) proteose peptone, 0.5% (wt/vol) beef extract, 0.5% (wt/vol) NaCl, 4% (wt/vol) glucose, and 1.4% (wt/vol) agar (pH 7.2). DNA was prepared as described by Borges et al. (3). Briefly, frozen cells were broken in liquid nitrogen by mechanical maceration and then the addition of Tris-spermidine buffer (40 mM Tris-HCl [pH 8.0], 4 mM spermidine, 10 mM EDTA, 0.1 M NaCl, 10 mM β-mercaptoethanol, and 0.1% sodium dodecyl sulfate). Two phenol extractions and one chloroform extraction were performed. The DNA was precipitated with 2.5 volumes of ethanol in the presence of 0.3 M NaCl, centrifuged, and resuspended in Tris EDTA buffer (20 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). Five primers named primers OPG-03, OPG-16, OPG-18, and OPG-19 (Operon Biotechnology) and primer 63 (synthesized by Laboratório de Biologia Molecular, IB, Universidade de Brasília) were used in the experiments. The five selected primers showed greater numbers and better definitions of amplified DNA fragments and also higher-intensity bands. The sequences of these primers are GAGCCCT CCA (OPG-03), AGCGTCCTCC (OPG-16), GGCTCATGTG (OPG-18), GTCAGGGCAA (OPG-19), and CAGCACCCAC (primer 63). The DNA amplification reactions were performed in a 25-μl system and consisted of 1 μl of *P. brasiliensis* DNA (5 ng/μl), 2.5 μl of 10× *Taq* buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.4], 20 mM $MgCl_2$), 2 μl of each deoxynucleoside triphosphate at 2.5 mM, 1 μl of primer (10 ng/μl), and 18 μl of water. *Taq* DNA polymerase (2.5 U; Cenbiot-RS; lot 93/1704; Biotechnology Center, Rio Grande do Sul, Porto Alegre, Brazil) was used for each reaction; the reaction mixture was then overlaid with 30 μl of mineral oil. Amplification was performed in an MJ Research Mini-Cycler, and the amplification program was 98°C for 2 min for the initial denaturation, 50°C for 2 min, and 35°C for 1 min. This was followed by enzyme addition and then 35 cycles of 72°C (2.5 min), 92°C (1.5 min), and 35°C (1 min) and a final extension period of 72°C for 5 min. The randomly amplified fragments were analyzed by electrophoresis on a 0.8% agarose gel and were visualized by ethidium bromide staining. The DNA fragment profiles produced by the primers were analyzed by calculating the simple matching coefficient of similarity by using the NTSYS-pc140 computer program as described by Rohlf (12). Data were scored for the presence or absence of amplification products.

Figure 1 shows representative results from our random amplified polymorphic DNA (RAPD) analysis. The results of random amplification of total DNA with primers 63, OPG-16, OPG-18, OPG-19, and OPG-03 are shown in Fig. 1A, B, C, D, and E, respectively. On the basis of those data, the similarity

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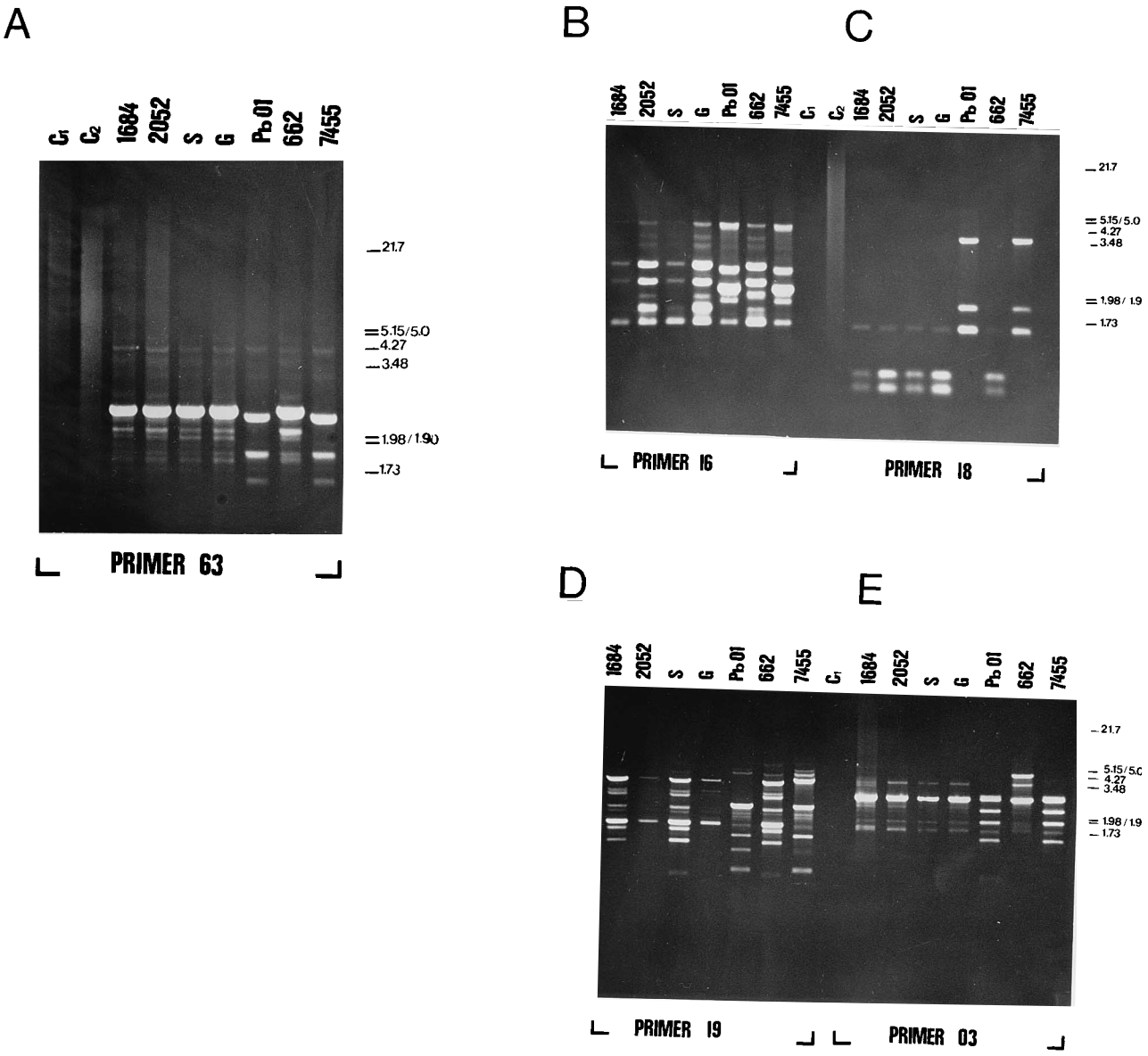

FIG. 1. Comparison of arbitrary primer PCR profiles obtained with DNAs prepared from seven isolates of *P. brasiliensis*. The primers were 63 (A), OPG-16 (B), OPG-18 (C), OPG-19 (D), and OPG-03 (E). The amplification reactions are described in the text. The numbers indicate the molecular mass marker of bacteriophage λ DNA digested with *Eco*RI and *Hin*dIII.

coefficient analysis of the amplification profiles of DNAs from the seven *P. brasiliensis* strains were calculated and are presented in Table 1. It places the seven isolates into two groups with only 35% genomic identity: group 1, strains 2052, G, 1684, S, and 662; group 2, strains Pb 01 and 7455. It is noteworthy that group 1 can be divided into two subgroups, with the first one containing strains 2052 and G, which had identical RAPD genetic patterns, and the second containing strains 1684, 662, and S, for which the similarity coefficient was greater than 90%. Between these two subgroups the genetic similarity was about 81%. Isolates Pb 01 and 7455 were not related to the others; they were related to each other with a similarity coefficient of 94%.

Our data show that RAPD analysis provides a means of distinguishing *P. brasiliensis* isolates. It is noteworthy that

TABLE 1. Similarity matrix obtained from the amplification of polymorphic DNA fragments of *P. brasiliensis* isolates

Isolate	Similarity coefficients for isolates:						
	1684	2052	S	G	Pb 01	662	7455
1684	1.000						
2052	0.792	1.000					
S	0.962	0.830	1.000				
G	0.792	1.000	0.830	1.000			
Pb 01	0.283	0.340	0.245	0.340	1.000		
662	0.906	0.811	0.943	0.811	0.226	1.000	
7455	0.340	0.358	0.302	0.358	0.943	0.245	1.000

with all of the primers investigated isolates 7455 and Pb 01 behaved as a separate group. Until now we could not correlate the data for the two groups with other data, such as growth and differentiation curves or even the isolation origin. For example, the isolates in group 1 present very distinct kinetics of growth and differentiation in liquid medium, as determined in our laboratory. In addition, Pb 01 and 7455, which were placed in the same cluster by analysis with all the five primers investigated, were isolated from patients from geographically distinct areas: Guayaquil, Ecuador, and Goiânia, Brazil, respectively.

The OPG-18 primer showed a very singular RAPD pattern among the isolates. This primer amplified two different sets of DNA fragments among the seven isolates. The amplification profile suggests the occurrence of a DNA fragment insertion(s) in strains Pb 01 and 7455 or a deletion(s) in the other isolates. The DNA patterns observed suggest the possibility that fragments of DNA with 3.6 or 1.8 kb can be used as genetic markers to distinguish *P. brasiliensis* isolates (18). Little is known about the extent of genetic variation within *P. brasiliensis*; even the life cycle of the fungus is still unknown. The fidelity of group definition, which was observed with all of the primers used, suggests the possible occurrence of genetically diverse subgroups in *P. brasiliensis*. The DNA fragments obtained by OPG-18 primer amplification could be used to characterize the genetic variability among the isolates of *P. brasiliensis.*

Finally, an unsolved question about the absence of a correlation between virulence and other biochemical characteristics that could define an isolate of *P. brasiliensis* remains (8, 11, 14). In the typing of *H. capsulatum* by restriction fragment length polymorphism (RFLP) analysis with a nuclear gene, it was possible to separate into different classes avirulent and virulent isolates from North America (9). Strain identification and analysis of genomic diversity by RAPD with clinical isolates of *H. capsulatum* showed a much more diverse polymorphic DNA pattern than had been indicated by RFLP analysis (10). The possible correlation between RAPD patterns and virulence with different isolates of *P. brasiliensis* is under progress.

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