Revista Latinoamericana de Microbiología (1999) 41:139-143
Asociación Latinoamericana de Microbiología

Random Amplified Polymorphic DNA (RAPD) Analysis of *Paracoccidioides brasiliensis* Isolates.

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ABSTRACT. Five *Paracoccidioides brasiliensis* isolates of humans origin were analyzed using three arbitrary primers (3301, 3304 and 3307 of 10, 9 and 10 oligonucleotídes respectively) in random amplified polymorphic DNA (RAPD) analysis.The analysis of the complex RAPD profiles obtained were carried out using the Dice similarity coefficient that distinguished the isolate Pb 02 from the others (Pb 18, Pb 192, Pb 265 and Pb SN). The results revealed limited intraspecific genomic variations in these *P. brasiliensis* isolates and indicate that RAPD can be useful for analysis of *P.brasiliensis* genome for characterization or differentiation within this genus.
Key Words: RAPD, *Paracoccidioides brasiliensis*, Genetic analysis, PCR, Fungi.

RESUMEN. Cinco aislados de origen humano de *Paracoccidioides brasiliensis* fueron analizados usando tres oligonucleótidos arbitrarios (3301, 3304 y 3307 de 10, 9 y 10 oligonucleótidos respectivamente) mediante DNA polimórfico amplificado al azar (RAPD). El análisis de los perfiles del RAPD complejo obtenidos fueron llevados a cabo usando el coeficiente de similaridad de Dice que distingue el aislado Pb 02 de los otros (Pb 18, Pb 192, Pb 265 y Pb SN). Los resultados revelaron variaciones intraespecíficas limitadas en esos aislados de *P. Brasiliensis* e indicaron que el RAPD can be útil para el anélisis del genoma de *P. brasiliensis* para caracterización o diferenciación dentro de este género.
Palabras Clave: RAPD, *Paracoccidioides brasiliensis*, Análisis Genético, PCR, Hongos.

INTRODUCTION

Paracoccidioides brasiliensis, the etiological agent of paracoccidioidomycosis is a thermally dimorphic fungus that causes the most important and wide spread systemic mycosis in South America. The yeast form found in infected tissues develops at 37°C, whereas at 23°C conversion to a mycelial form occurs.[21] Among the wide range of clinical forms, from asymptomatic infection to severe and even lethal disease, the clinical manifestations are those of a chronic granulomatous disease with involvement of the lung, reticulo-endothelial system, mucocutaneous area and other organs. These pathologies can be mainly related to intrinsic characteristics of the patients, but also can be attributed to the fungus related factors.[9] Isolates of *P. brasiliensis* exhibit biochemical differences and variations in virulence, pathogenicity and immunogenicity.[12,17,23] Four basic patterns of virulence of *P. brasiliensis* isolates were described based on intraperitoneal infection of the susceptible inbred mice B10.A: slightly virulent (Pb 265 and IVIC Pb 267), intermediate (Pb 192,IVIC Pb 9 and Pb SN), virulent (Pb 2052) and highly virulent (Pb 18),[23] however, the genetic relationships between these isolates have not been investigated hitherto at the DNA level.

Molecular biology techniques have been used to try to solve taxonomic problems as well as to differentiate strains within pathogenic fungal species, providing powerful tools for the direct analysis of the genomes of many pathogens.[26] Restriction fragment length polymorphism (RFLP) of genomic DNA can be used to distinguish *Aspergillus fumigatus* isolates[5] and this technique has been used to study the epidemiology of some human fungal pathogens, like *Candida* sp.[16], *Histoplasma capsulatum* [13] and *Trichophyton rubrum.*[4] Randomly amplified polymorphic DNA (RAPD) profiles have been used to distinguish isolates of different species and strains within a given genus. Other studies have shown better results with RAPD than those obtained with RFLP in isolates of *H. capsulatum.*[14]

The RAPD relies on random amplification of genomic DNA in polymerase chain reactions using arbitrary primers. Some isolates of *A. fumigatus* have been distinguished on this basis.[1] Clinical samples of the pathogen *H. capsulatum* were divided in different classes using RFLP and RAPD analysis of genomic DNA, which can distinguish isolates into a single RFLP class.[14] RAPD has also been used to characterize genomic relationships among medi-

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cally important *Candida* species.[15] In this study, we have used RAPD profiles obtained with three different primers to compare different strains of *P. brasiliensis*.

MATERIAL AND METHODS

Five isolates of *P. brasiliensis* (Pb 02, Pb 18, Pb 192, Pb 265 and Pb SN) were obtained from the collection of the Faculty of Medicine of the University of São Paulo, Brazil. They were all isolated from human sources and maintained in semi-solid Fava Netto's medium at 35-37°C.[8] To obtain cells for DNA extraction, both mycelial and yeast forms were grown in McVeigh and Morton liquid synthetic medium[18] at room temperature for 30 days or at 37°C for 15 days, respectively. Cells harvested by filtration through sterile paper were washed three times with sterile saline solution (NaCl 0,85%) by centrifugation at 3.000 g, and the pellets frozen at -20°C. To remove the fungal cell wall, three different methods were tested: sonication,[19] liquid nitrogen[2] and zymolase.[10] Genomic DNA was then extracted according to Sambrook *et al.*[20], using phenol/chloroform followed by ethanol precipitation. RNA and proteins were removed by RNase and pronase treatments, respectively.

Three different arbitrary primers were used: 3301 (5'- TCGTAGGCAA - 3'); 3304 (5'- GCACTGTCA - 3') and 3307 (5'- AGTGCTACGT - 3').

Reactions were carried out as previously described by Dias-Neto *et al.*[6], using 0.8 units of Taq DNA polymerase (CENBIOT-RS, Brazil), 1 x Taq DNA polymerase buffer (1.5 mM $MgCl_2$); 200 μM of each dNTP; 50 mM KCl; 10 mM Tris-HCl, pH 8.5; 6.4 pmol of primer and either 0.1 and 1.0 ng of template DNA, in a total of 10 μl /reaction tube, that were overlaid with 20 μl of mineral oil. Amplification were carried out through the following temperature profile: 95°C for 5 min to denature; 30°C for 2 min for annealing; 72°C for 1 min to extension and 95°C for 30 seconds to denature followed by 33 cycles where the annealing was 40°C. A final cycle of 5 min at 72°C was added. Amplification products were run on a 4% polyacrylamide gel electrophoresis in TBE buffer (134 mM Tris-base pH 8.6; 80 mM boric acid and 30 mM EDTA Na_2). These gels were fixed with 10% ethanol/0.5% acetic acid for 20 min, stained with 0.2% silver nitrate for 30 min and revealed by reduction with 0.75 M NaOH; 0.1 M formaldehyde for 10 min.

A phenetic tree based on band sharing between all possible pairs in an analysis group was constructed using the Dice similarity coefficient S= 2a/2a + b + c, where a= the number of bands shared between organism 1 and 2, b= the number of bands present in 1 but not in 2 and c= the number of bands present in 2 but not in 1. Data derived from this formula represent the percentage of common bands between every two individuals.[7,24]

They were plotted to establish a matrix of similarity that was then used for unweighted pair group method analysis (UPGMA). The phenon line marked on the UPGMA phenogram is the average of the similarities among the pairs and indicates the point of reference for dividing the organism into separate groups.

RESULTS AND DISCUSSION

The most efficient methods that allow the best extraction of DNA reliable for amplification were zymolase and liquid nitrogen for mycelial and yeast forms respectively (Fig. 1). In spite of some DNA degradation presented by isolate Pb SN, there was no interference with the PCR amplification.

Fig. 2 shows the RAPD profiles of genomic DNA obtained from the mycelial form of *P. brasiliensis* isolates, where most of the segments amplified appear as monomorphic bands. In Figure 2A, lanes 4 and 5 (isolate Pb 02), we observe a strong and reproducible band below 603 bp obtained with primer 3307, which is not present in the other isolates (arrow). Figure 2B shows the amplification products of *P. brasiliensis* with primer 3304. We also observe that most of the bands were conserved among the isolates presenting few polymorphic segments. The most striking polymorphism was seen when we compare isolate Pb 02 with the others. A band between 1078 and 872 bp, was specific to this isolate (arrow). Figure 2C shows the amplification of *P. brasiliensis* isolates with primer 3301. Again, Pb 02 presents a unique band between 1353 and 1078 bp (arrow).

A total of 40 bands obtained with three primers that could be identified with confidence on the basis of their intensity and separation from others bands, were considered for analysis and phenogram construction (Figure 3). The obtained tree confirms the visual inspection of the RAPD profiles in that Pb 02 differed clearly from the others *P. brasiliensis* isolates.

PCR based on random amplification of genomic DNA (RAPD) has been used to identify biological samples,[27] and also for studies of known or unknown genomes of a large number of species, requiring a little amount of DNA and, even when the DNA is degraded, reproducible results can be obtained.

The isolates could be more readily distinguished by RAPD than RFLP analysis (data not shown). By RAPD we could observe that Pb 02 differed from the others with all primers used for DNA amplification. No differences in RAPD profiles were seen between mycelial and yeast form of *P. brasiliensis* (data not shown). The analysis of the RAPD profiles suggests that isolates Pb 18, Pb 192 and Pb SN and Pb 265 have similar patterns and that only isolate Pb 02 showed significant genomic differences as compared to the others isolates of *P. brasiliensis*, and these differences cannot be correlated with virulence.

Soares *et al.*[25] using RAPD analysis of seven others

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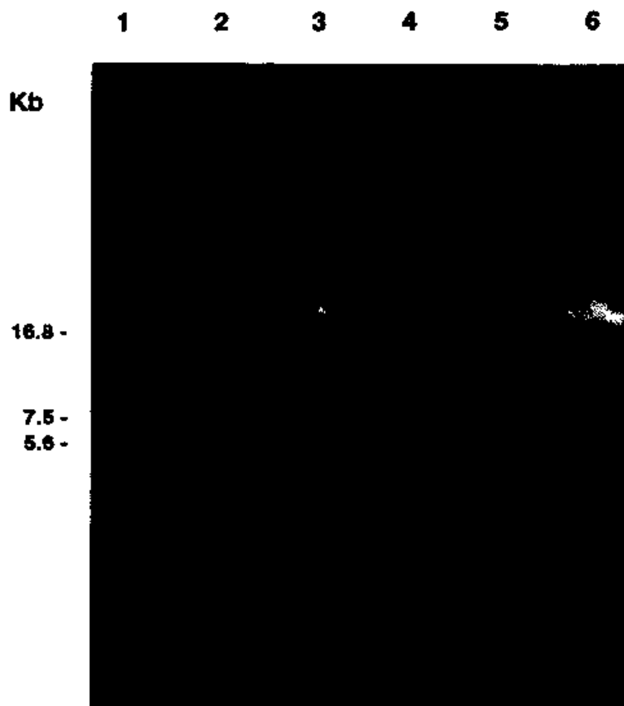

Fig. 1. Agarose gel electrophoresis (0.8%) of *P. brasiliensis* mycelial form DNA extracted after cell wall digestion with zymolase and staining with ethidium bromide. Lane 1: Bacteriophage lambda DNA (BamH1 digested), lanes 2 through 6 are *P. brasiliensis* isolates: Pb 02, Pb 192, Pb SN, Pb 18 and Pb 265.

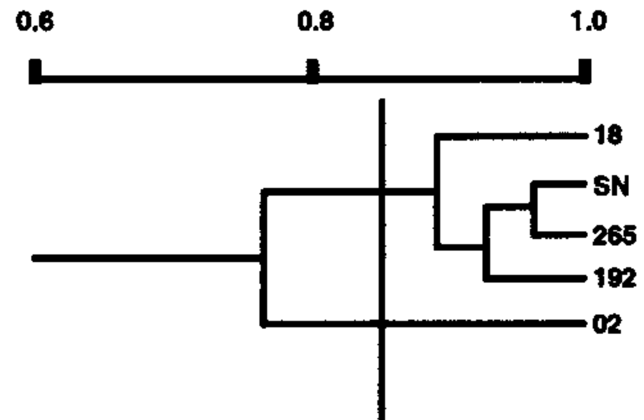

Fig. 3. UPGMA phenogram of *P. brasiliensis* isolates based on pairwise band sharing. A total of 40 bands of the RAPD profiles derived from primers 3307, 3304 and 3301 were used for Dice similarity coefficient analysis. The phenon line is represented by the vertical bar.

isolates of *P. brasiliensis*, (Pb 01, 2052, 1684, S, G, 662 and 7455) observed a high similarity among these samples and suggests the possible occurrence of genetically diverse subgroups in *P. brasiliensis*.

The RAPD analysis of *P. brasiliensis* strains from Argentina, Brasil, Colombia, Peru and Venezuela produced amplification products which were sufficiently polymorphic to allow differentiation of the strains.[3] Our studies also showed that others isolates (Pb 18, Pb 192, Pb 265, and Pb SN) has a high similarity.

The others techniques were proposed for identification of *P. brasiliensis* as PCR[11] and hybridization with the DNA probe specific.[22]

Our studies confirm that RAPD analysis of genomic DNA of *P. brasiliensis* is very useful, sensitive, and requires small amounts of DNA for successful amplification. Quantities of DNA under 0.1 ng or over 5 ng resulted in the absence of some of the amplified products observed between these amounts. Both concentrations used in these work (0.1 and 1ng) presented reproducible results as shown in Fig 2.

These results also revealed limited genomic variability among the *P. brasiliensis* isolates analyzed and indicate that RAPD is useful to detect genomic variabilities in *P. brasiliensis* isolates from different geographic regions and/or hosts, for characterization, differentiation and for epidemiological studies.

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2A

2B

2C

Fig. 2. RAPD profiles of genomic DNA of *P. brasiliensis* mycelial form obtained with primers 3307 (2A), 3304 (2B) and 3301 (2C) in a silver stained 4% polyacrylamide gel. Lane 1, molecular weight marker ϕX174 (*Hae* III digested); lanes 2 through 11 are repetitions of two concentrations of genomic DNA (1.0 and 0.1 ng) of each isolate: Pb 18, Pb 02, Pb SN, Pb 192 and Pb 265. Arrows shows unique bands.

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