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Evaluation of DNA polymorphisms amplified by arbitrary primers (RAPD) as genetically associated elements to differentiate virulent and non-virulent *Paracoccidioides brasiliensis* isolates

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Abstract

Randomly amplified polymorphic DNA (RAPD) analysis of 35 *Paracoccidioides brasiliensis* isolates was carried out to evaluate the correlation of RAPD profiles with the virulence degree or the type of the clinical manifestations of human paracoccidioidomycosis. The dendrogram presented two main groups sharing 64% genetic similarity. Group A included two isolates from patients with chronic paracoccidioidomycosis; group B comprised the following isolates showing 65% similarity: two non-virulent, six attenuated, five virulent, eight from patients with chronic paracoccidioidomycosis and two from patients with acute paracoccidioidomycosis. The virulent Pb18 isolate and six attenuated or non-virulent samples derived from it were genetically indistinguishable (100% of similarity). Thus, in our study, RAPD patterns could not discriminate among 35 *P. brasiliensis* isolates according to their differences either in the degree of virulence or in the type of the clinical manifestation of this fungal infection.

Keywords: Randomly amplified polymorphic DNA; DNA polymorphism; Virulent isolates; Arbitrary primers; *Paracoccidioides brasiliensis*

1. Introduction

Paracoccidioides brasiliensis, the aetiological agent of paracoccidioidomycosis (PCM), is a dimorphic fungus which develops a mycelial form at room temperature and a multiple-budding yeast form in the infected host or in culture at 37°C. PCM evolution varies among human patients who may then present different clinical manifestations ranging from localised, benign forms to disseminated, severe disease [1]. Different manifestations of this infection may be a result of host-related factors (age, sex, immune system condition) as well as characteristics of the fungus, mainly its virulence [2]. Four degrees of *P. brasiliensis* virulence were observed in susceptible mice after intraperitoneal infection: highly virulent, virulent, intermediate and slightly virulent [3–5]. Some *P. brasiliensis* isolates lose their virulence after in vitro subculturing for long periods of time [5,6]. So far, the degree of virulence has been inferred from the severity of the disease in mice or the differences in the clinical manifestations of human PCM. The lack of the *P. brasiliensis* isolate with a well known capacity of infecting hosts has been the main obstacle to the identification of one (or more) virulence factor as well since it interferes in some way with the interpretation of the experiments studying the resistance mechanisms of hosts to this fungus.

The progress of molecular biology techniques made it possible to investigate the diversity of pathogen strains based on genomic characteristics [7–10]. Randomly amplified polymorphic *DNA* analysis (RAPD) [11] has been successfully used to detect genomic variations among isolates of fungi causing human diseases such as *Candida* sp. [12,13], *Cryptococcus neoformans* [14,15], *Aspergillus fumigatus* [16,17] and *P. brasiliensis* [18–21]. Some authors have used RAPD analysis to investigate possible relation-

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ships between *P. brasiliensis* clusters and geographical origin, virulence or type of pathology. Recently, Calcagno and co-workers [18] have demonstrated that genetic differences could be associated with geographical regions but not to different clinical manifestations of human PCM. Molinari-Madlum and co-workers [20] have shown that RAPD patterns correlated with the virulence degree of *P. brasiliensis* isolates. In the present work we evaluate RAPD profiles as genetically associated elements to discriminate *P. brasiliensis* isolates with identification of their virulence degree, obtained from results of experimental infection of mice, or the clinical form of human PCM.

2. Materials and methods

2.1. P. brasiliensis isolates

The following isolates were obtained from the fungal culture collection of the Departamento de Imunologia, Instituto de Ciências Biomédicas, São Paulo, Brazil: (a) Pb18/00 = highly virulent; (b) Pb18AV, Pb18/93, Pb18/94, Pb18/95, Pb18/96, Pb18/98 and Pb18/99 = isolates derived from Pb18, which have lost their virulence after subculturing for long period; (c) Pb265 = non-virulent; (d) Pb339 = originally obtained from A. Restrepo (Laboratory of Mycology, Hospital Pablo Tobón Uribe, Medellin, Colombia), intermediate virulence (weaker than Pb18/00); (e) Pb145 = virulent; Pb2052 = virulent (Goiás, Brazil); (f) Pb192 = intermediate virulence [3,22–24]; (g) Pb113, PbGr60.855, PbGr60.835, PbGr-raton, PbC-9, Pb99, Pb262, PbVitor, Pb63-Ba, Pbtatu = isolates without identification regarding to virulence or associated clinical form.

The isolates of *P. brasiliensis* were obtained also from patients presenting different clinical forms of the disease: Bt44 and Bt92 (acute form); Bt1, Btx, Bt40, Bt45, Bt50, Bt56, Bt57, Bt91, Bt93, Bt100 (chronic form). All these isolates were recently obtained from Faculdade de Medicina, UNESP, Botucatu, SP, Brazil.

The differences in virulence of *P. brasiliensis* isolates were observed in the course of experimental intraperitoneal infection of isogenic susceptible mice (B10.A). About 8 weeks after inoculation with a virulent isolate, one can observe a high degree of viable yeast cells dissemination to the lungs, liver and spleen (colony forming units (CFU) g^{-1} tissue) and a high number of granulomas in the peritoneum and other organs (histopathological examinations). On the contrary, a non-virulent isolate causes only very few granulomas and no viable yeast cells can be seen in the organs.

After in vitro subcultivation for long periods, a virulent *P. brasiliensis* decreased its virulence. The generated variant was then non-lethal to susceptible mice. However, its virulence can be recovered by passage in susceptible mice. After such reisolation, it is comparable to the virulence of the original isolate.

2.2. Fungal growth conditions

Fungal isolates were kept in mycelial form using semi-solid PYG medium (peptone–yeast extract glucose) at 25°C. Isolates were transferred to Fava Netto's medium [25] and cultured at 36°C with subcultivation every 7 days to obtain the yeast phase. Fungal yeast cells were then used in DNA extraction procedure.

2.3. DNA extraction

P. brasiliensis DNA was extracted according to Carlson et al. [9] with some modifications: yeast cells were broken in liquid nitrogen and then suspended in the extracting buffer (2 M Tris–HCl, pH 9.5, 0.5 M EDTA, pH 8.0, 1.4 M NaCl, 2% CTAB, 1% sarcosyl, 20 μl β-mercaptoethanol). Samples were incubated for 30–60 min and centrifuged at $10\,000 \times g$ for 10 min. Supernatants were transferred to clean tubes, kept at room temperature for a while, and then one volume of chloroform:isoamyl alcohol (24:1) was added. Samples were homogenised and centrifuged at $10\,000 \times g$ for 10 min. Aqueous phase was transferred to a clean Eppendorf tube and 1 volume of isopropanol was added. Samples were homogenised, incubated at room temperature for 30 min and centrifuged at $10\,000 \times g$ for 10 min. Pellets were washed with cold ethanol and centrifuged at 4°C for 10 min. DNA samples were desiccated and resuspended in TE (Tris–EDTA) buffer.

Table 1
Amplification fragments generated in RAPD analysis of *P. brasiliensis* isolates

Primer	Fragment size (kb)	*P. brasiliensis* isolates
OPA-03	0.59 and 0.54	56, 93, 262
	0.70	113
	0.77	44
OPD-20	0.46	56, 262
	0.60	50, 57
	1.70	56, 93, 262, 99
	3.90	339, 56, Gr60855, Gr-raton
OPG-03	0.93	339, 56, Gr60835, Gr60855, Gr-raton
	2.40	339, 50, Gr60835, Gr60855, Gr-raton, 99
OPG-10	0.61	18/96, 192, 45, 57, C-9
	1.30	2052, 339, 56, Gr60835, tatu, 63-Ba
	1.80	56, 93, 262
	2.80	56, 91, 93, 100, 262, tatu, 63-Ba
OPN-10	1.07	56, 93
	1.44	339, 262, Gr60835, Gr60855, Gr-raton
OPK-11	0.67	56, 93, Vitor, tatu, 63-Ba
	0.81	56, 93, 145, 262, 113
OPO-06	0.66	99
	0.73	50
	1.20	339, 56, 93, 262
	1.60	Gr60835, Gr-raton
Pr-1	1.10	339, Gr60835, Gr60855, Gr-raton
	4.40	113
Pr-2	1.10	56, 93

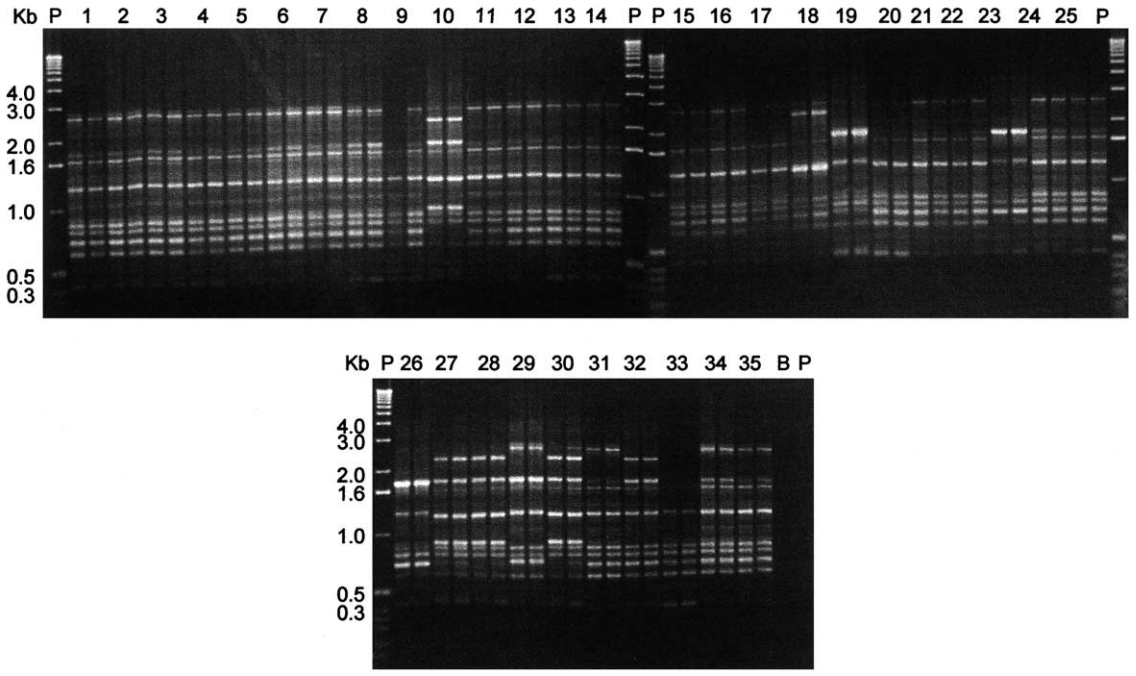

Fig. 1. RAPD patterns of the isolates of *P. brasiliensis* analysed with primer OPG-03. Lane P: 1-kb DNA standard. Lanes 1–14: Pb18/93, Pb18/94, Pb18/95, Pb18/96, Pb18/98, Pb18/99, Pb18/00, Pb2052, Pb265, Pb339, Pb192, Pb18AV, Bt1, Btx. Lanes 15–25: Bt40, Bt44, Bt45, Bt50, Bt56, Bt57, Bt91, Bt92, Bt93, Bt100, Bt145. Lanes 26–35: Pb262, PbGr60835, PbGr60855, Pb113, PbGr-raton, PbVitor, Pb99, PbC-9, Pbtatu, Pb63-Ba. Lane B: negative control.

2 μl (50 mg ml^{-1}) of RNase was added and samples were incubated at 37°C for 1 h. Then, chloroform:isoamyl alcohol (24:1) was added. After centrifugation at 10 000 × *g* for 5 min, ethanol containing 10% of sodium acetate was added. Ethanol was removed and pellets were washed with 70% ethanol. Desiccated DNA samples were resuspended in 50 μl of TE buffer.

2.4. RAPD analysis

Every RAPD reaction mixture (final volume 20 μl) contained 10 ng of genomic DNA; 2 μl of primer (0.4 μM); 1.6 μl of mix dNTP (200 μM); 0.8 μl de Mg^{+2} (2 mM); 1 U of Taq DNA polymerase (Gibco, BRL); 2 μl of 10× conc PCR buffer (Gibco, BRL; 200 mM Tris–HCl, pH 8.4, 500 mM KCl). Amplifications were carried out in a thermal controller (M.J. Research PTC-100, NV, USA) at 94°C for 3 min and 30 s, 40 cycles of 45 s at 94°C, 45 s at 36°C and 1 min and 45 s at 72°C, followed by 3 min and 30 s at 72°C. Randomly amplified products were analysed by electrophoresis on a 1.4% agarose gel. The primers (Operon Technologies, CA, USA) used were: **OPA-03**, 5′-AGTCAGCCAC3-′; **OPB-07**, 5′-GGTGACGCAG3-′; **OPD-20**, 5′-ACCCGGTCAC3-′; **OPG-03**, 5′-GAGCCCTCCA3-′; **OPG-10**, 5′-AGGGCCGTCT3-′; **OPG-18**, 5′-GGCTCATGTG3-′; **OPN-10**, 5′-ACAACTGGGG3-′; **OPK-11**, 5′-AATGCCCCAG3-′; **OPO-06**, 5′-CCACGGGAAG3-′; **OPP-03**, 5′-CTGATACGCC3-′; **OPC-12**, 5′-TGTCATCCCC3-′; **OPD-10**, 5′-GGTCTACACC3-′; **OPE-16**, 5′-GGTGACTGTG3-′; **OPK-4**, 5′-CCGCCCAAAC3-′; **OPK-8**, 5′-GAACACTGGG3-′; **Pr-1**, 5′-ATTGGGCGAT3-′; **Pr-2**, 5′-AACACACGAG3-′.

2.5. Analysis of RAPD data

Statistical analysis of data from RAPD was carried out using the NTSYS-pc, 1.7 (Numerical Taxonomy and Multivariate Analysis System, NY, USA). The dendrogram was determined by calculating Jaccard index [26].

3. Results

RAPD analysis of 25 *P. brasiliensis* isolates was performed to evaluate DNA fragment profiles as genetically associated virulence elements. These isolates were different from each other in their degree of virulence or the type of the human disease they caused. Ten isolates with unknown virulence/disease characteristics were additionally included in the experiments. Seventeen primers were initially tested and 10 were selected to construct the dendrogram (OPA-03, OPK-11, OPD-20, OPG-03, OPG-10, OPG-18, OPN-10, OPO-06, Pr-1, Pr-2) based on the generation of the greatest number of polymorphic DNA fragments and the best amplification patterns. Among these 10 primers, OPO-06, Pr-1, OPD-20, OPA-03 and OPG-03 were the most discriminatory; the rest were included only to check if the dendrogram remained unchanged. Representative RAPD patterns for all 35 tested isolates obtained with primer OPG-03 are shown in Fig. 1. The primer OPG-

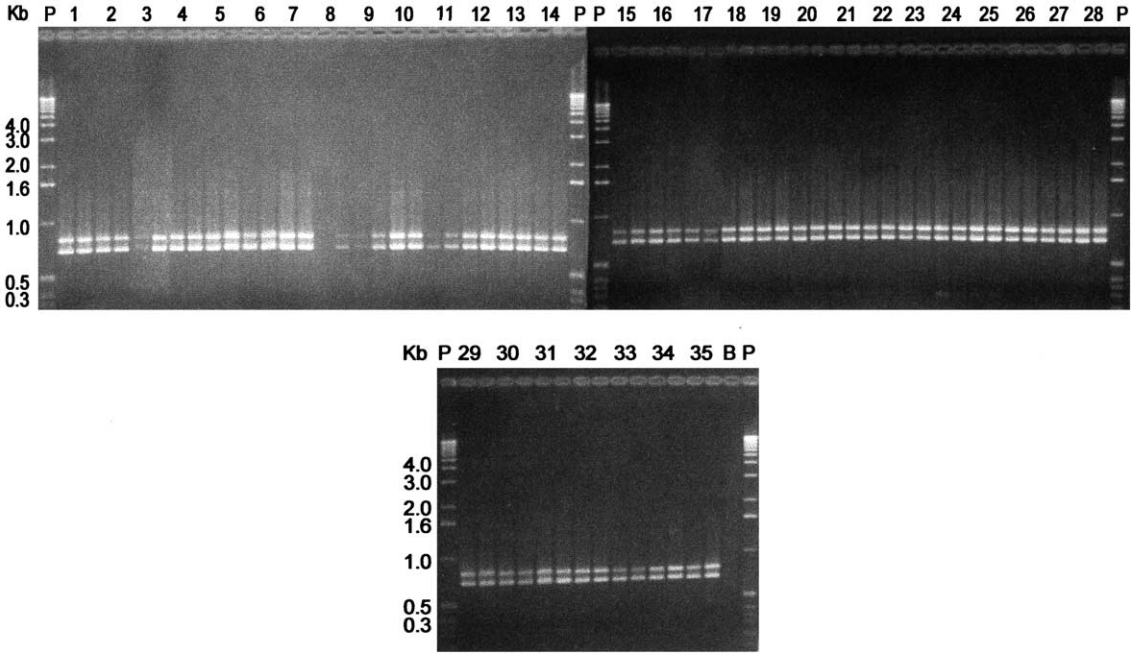

Fig. 2. RAPD patterns of the isolates of *P. brasiliensis* analysed with primer OPG-18. Lane P: 1-kb DNA standard. Lanes 1–14: Pb18/93, Pb18/94, Pb18/95, Pb18/96, Pb18/98, Pb18/99, Pb18/00, Pb2052, Pb265, Pb339, Pb192, Pb18AV, Bt1, Btx. Lanes 15–28: Bt40, Bt44, Bt45, Bt50, Bt56, Bt57, Bt91, Bt92, Bt93, Bt100, Bt145, PbGr60835, PbGr60855, Pb113. Lanes 29–35: Pb262, Gr-aton, PbVitor, Pb99, PbC-9, Pbtatu, Pb63-Ba. Lane B: negative control.

18 generated only two monomorphic DNA fragments with molecular sizes of 0.6 and 0.8 kb (Fig. 2). Fig. 3 shows the RAPD patterns of an isolate of *C. neoformans* that was used as an outgroup control. The 10 utilised primers generated 76 polymorphic and 23 monomorphic DNA products that varied from 0.3 to 4.5 kb in length (Table 1). These results were used to construct a binary data matrix of presence and absence of shared bands in the agarose gel and the data obtained from these comparisons were used to calculate the Jaccard coefficient of similarities.

The dendrogram was constructed using 35 isolates of *P. brasiliensis* and the outgroup control (*C. neoformans*) which had a coefficient of similarity of 17% (Fig. 4). Two main groups were formed: group A comprised Bt56, Bt93

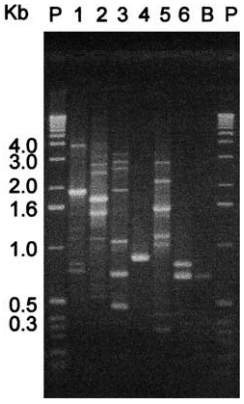

Fig. 3. RAPD patterns of the strain of *C. neoformans* (outgroup control) analysed with the following primers: OPN-10, OPG-03, OPO-06, OPD-20, OPA-03 (lanes 1–5). Lane 6: Pb18 tested with OPG-18. Lane P: 1-kb standard. Lane B: negative control.

(chronic form) and Pb262 (unknown virulence) which had similarity coefficients between 85 and 91%. Group B showed two subgroups (B1 and B2) plus two distinct groups, one represented by isolate Pb99 (unknown virulence) and the other by Bt50 (severe chronic form). Subgroup B1 included the isolates Pb339 (virulent), PbGr60835, PbGr60855 and PbGr-raton (unknown virulence) with coefficients of similarity between 89 and 91%. Subgroup B2 was formed by isolates Pb18/93, Pb18/94, Pb18/95, Pb18/96, Pb18/98, Pb18/99, Pb18AV (isolates originated from highly virulent Pb18 but have lost their virulence), Pb18/00, Pb2052 e Bt145 (highly virulent), Pb265 (non-virulent), Pb192 (intermediate virulence), Bt1, Btx, Bt40, Bt45, Bt57, Bt91 and Bt100 (chronic form), Bt44, Bt92 (acute form), Pbtatu, 63-Ba, PbVitor, PbC-9, Pb113 (unknown virulence). These samples in subgroup B2 had a similarity coefficient of about 80% (Fig. 4). The virulent isolate Pb18, recently isolated from infected mice, and samples derived from it which had lost their virulence presented a coefficient of similarity of 100%. This same coefficient was detected for strains that were isolated from patients with chronic and acute forms of human disease.

4. Discussion

The degree of virulence of *P. brasiliensis* isolates has been determined as the consequence of the fungal infection in experimental susceptible mice or the type of clinical manifestation in the human disease. Lacking certain

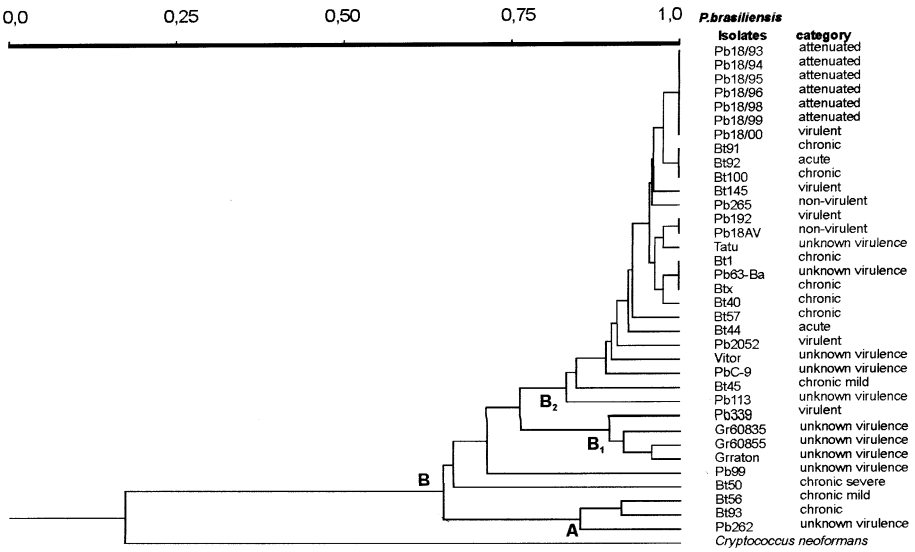

Fig. 4. Dendrogram of isolates of *P. brasiliensis* based on UPGMA cluster analysis of Jaccard coefficients. The *C. neoformans* was used as an outgroup.

P. brasiliensis isolates with a well-defined capacity of infecting hosts has been a barrier to finding a virulence factor of this fungus. Many authors have tried to correlate certain characteristics of *P. brasiliensis* isolates with virulence without success [23,24,27,28]. Recently, the RAPD technique has been used in the study of genomic diversity among *P. brasiliensis* isolates [18,20,21,29–31]. In the present work, 35 *P. brasiliensis* isolates were tested using this technique. Seventy-six polymorphic and 23 monomorphic DNA fragments were generated by the use of 10 primers. OPG-18 produced only two monomorphic fragments of 0.6 and 0.8 kb. Soares et al. [21] also observed two RAPD products of 3.6 and 1.8 kb generated by OPG-18. As these fragments were present in two isolates (Pb01; Pb7044) but not in the others (2052; G; 1684; S; 662), the authors suggested those as genetic markers to distinguish among the isolates of *P. brasiliensis*. In our studies using the same primer these fragments have not been revealed. Instead, we detected 0.6 and 0.8 kb fragments in all 35 *P. brasiliensis* isolates but not in the outgroup control *C. neoformans*. Although this may imply potential use of these fragments as *P. brasiliensis* genetic markers, it will be necessary to analyse a significant number of different fungal species to verify this suggestion.

The largest number of polymorphic fragments was obtained with the primer OPO-06. With OPN-10 a DNA fragment of 0.5 kb was generated only in the samples Bt56 (chronic), Bt93 (chronic), Pb2052 (virulent) and in the isolate Pb339 (virulent) which also revealed a DNA band of 0.6 kb. With OPG-03, Pb339 isolate revealed 0.9 and 2.4 kb fragments, while sample Bt56 (chronic) showed only 0.9 kb and sample Bt50 (chronic, severe) produced only 2.4 kb fragment. However, when all these differences were statistically analysed, no correlation with virulence or type of pathogenicity could be detected.

The dendrogram constructed with the 35 *P. brasiliensis* isolates presented two groups (Fig. 4): group A included Bt56 (chronic, mild), Bt93 (chronic) e Pb262 (unknown virulence); group B was divided in subgroups B1 and B2, and two other distinct groups represented by samples Pb99 (unknown virulence) and Bt50 (chronic, severe). Subgroup B1 included samples Pb339 (virulent), PbGr60835, PbGr60855 and PbGr-raton (unknown virulence); subgroup B2 included isolates Pb18/93, Pb18/94, Pb18/95, Pb18/96, Pb18/98, Pb18/99 e Pb18AV (attenuated virulence), Pb18/00, Pb2052 e Bt145 (virulent), Pb265 (non-virulent), Pb192 (virulento), Bt1, Btx, Bt40, Bt57, Bt91 e Bt100 (chronic), Bt45 (chronic, mild), Bt44, Bt92 (acute), Pbtatu, Pb63-Ba, PbVitor, PbC-9, Pb113 (unknown virulence). Our results indicate that the highly virulent Pb18/00 (which was recently isolated from susceptible mice with a severe infection) and the six derivative forms of the virulent Pb18 (which had their virulence very attenuated or even lost) all exhibited a similarity coefficient of 100%. The same coefficient was found for the non-virulent Pb18AV and the virulent Pb192 isolates.

Brummer et al. [6] and Kashino et al. [5] observed that *P. brasiliensis* virulence could be attenuated or even lost after being subcultured for longer periods and that the recovery of virulence could be achieved by animal passages. In our experiments, although the isolates derivatives from Pb18 (virulent, attenuated and non-virulent) and also Pb192 (virulent) presented different capacities of infecting susceptible mice, they were all genetically identical showing 100% of similarities. In the same way, it was not possible to correlate RAPD patterns with the type of clinical forms of human disease. *P. brasiliensis* samples Bt91, Bt100, Bt1, Bt40 (chronic) as well as Bt92 and Bt44 (acute) displayed similarities of 100%. On the other hand, the results revealed 91% of genetic similarity between two

samples (Bt56 and Bt93), isolated from patients with chronic form and only 65% of similarity with sample Bt50, also isolated from a patient with chronic form.

In our study, we found similarity coefficients ranging from 65 to 100% among 35 *P. brasiliensis* isolates. The DNA sample from *C. neoformans*, used as an outgroup control, formed a distinct branch with 17% of genetic similarity. Our results are not in accordance with those from Molinari-Madlum and co-workers [20] who have distinguished among 15 *P. brasiliensis* isolates, two groups with 17% of genetic similarity and also demonstrated a correlation between RAPD patterns and the degree of virulence. Nevertheless, our results are similar to those of Calcagno and co-workers. [18] who studied RAPD patterns of 33 isolates of *P. brasiliensis* and found 63% to 100% of similarity among them. These authors found no correlation between RAPD patterns and the type of pathology but did find it with the geographical region where each fungal strain was isolated. Araujo et al. [32] has already described an isolate (Pb4940) obtained from a patient with neuroparacoccidioidomycosis in Goiás (Brazil), that had a unique RAPD pattern, forming a distinct and independent group which showed 30% of genetic similarity with other *P. brasiliensis* isolates obtained from other Brazilian geographic regions. In our study, two DNA samples formed two independent groups distinct from the other 33 *P. brasiliensis* isolates: Pb339, which was originally isolated in Colombia and the isolate Pb262 obtained in Uberlândia, Minas Gerais (Brazil). The former had 67% of similarity and the latter 65%. On the other hand, RAPD analysis of the highly virulent Pb18/00 was indistinguishable from the attenuated or non-virulent isolates derived from Pb18 after long periods of subculturing. It might be that genetic differences found among the 35 isolates derive from geographical differences in the origin of the samples. Further investigations using these isolates would be relevant.

Conclusively, DNA polymorphisms in our experiments revealed by amplification with arbitrary primers (RAPD) did not prove as a genetically associated element to differentiate virulent and non-virulent isolates of *P. brasiliensis*.

Acknowledgements

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