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Paracoccidioides brasiliensis ISOLATES OBTAINED FROM PATIENTS WITH ACUTE AND CHRONIC DISEASE EXHIBIT MORPHOLOGICAL DIFFERENCES AFTER ANIMAL PASSAGE

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SUMMARY

The basis for virulence in *Paracoccidioides brasiliensis* is not completely understood. There is a consensus that the sequencial *in vitro* subcultivation of *P. brasiliensis* leads to loss of its pathogenicity, which can be reverted by reisolation from animal passage. Attention to morphological and biochemical properties that are regained or demonstrated after animal passage may provide new insights into factors related to the pathogenicity and virulence of *P. brasiliensis*. We evaluated morphological characters: the percentage of budding cells, number of buds by cell and the diameter of 100 mother cells of yeast-like cells of 30 *P. brasiliensis* isolates, before and after animal passage. The isolates were obtained from patients with different clinical forms of paracoccidioidomycosis (PCM): acute form (group A, n=15) and chronic form (group C, n=15). The measurement of the yeast cell sizes was carried out with the aid of an Olympus CBB microscope coupled with a micrometer disc. We measured the major transverse and longitudinal axes of 100 viable cells of each preparation. The percentage of budding cells as also the number of buds by cell was not influenced by animal passage, regardless of the source of the strain (acute or chronic groups). The size values of *P. brasiliensis* isolates from groups A and C, measured before the animal passage exhibited the same behavior. After animal passage, there was a statistically significant difference between the cell sizes of *P. brasiliensis* isolates recovered from testicles inoculated with strains from groups A and C. The maximum diameter of mother cells from group A isolates exhibited a size of 42.1µm in contrast with 32.9µm exhibited by mother cells from group C ($p<0.05$). The diameter of 1500 mother cells from group A isolates exhibited a medium size of 16.0µm (SD ± 4.0), a value significantly higher than the 14.1µm (SD = ± 3.3) exhibited by 1500 mother cells from group C isolates ($p<0.05$). Our results reinforce the polymorphism exhibited by *P. brasiliensis* in biological material and the need for further investigations to elucidate the role of morphological parameters of the fungus in the natural history of the disease.

KEYWORDS: *Paracoccidioides brasiliensis;* Morphology; Paracoccidioidomycosis.

INTRODUCTION

Paracoccidioidomycosis (PCM) is the most frequent endemic systemic mycosis in Latin America. It usually affects rural male workers aged 30 to 50 years[20]. Healthy subjects living in endemic areas may be infected by inhalation of infecting propagules of *P. brasiliensis* and develop one of two clinical forms: (i) acute or subacute form, with severe involvement of the mononuclear phagocyte system; or (ii) the chronic form, with insidious evolution and involvement of one or more organs[6,10].

There is mounting evidence that different clinical forms of the disease may be related to host factors (e.g. age, cell-mediated immunity) as well as characteristics of the infecting agent. There are clear indications that *P. brasiliensis* strains exhibit great biological diversity in terms of morphology, growth rate[13], biochemistry[11], antigenicity and virulence[9,21]. Differences of size and budding cells among *P. brasiliensis* strains have also been reported[1,3,4,5,13,15]. However, the relationship between these morphological variations and clinical forms of the disease has not yet been studied.

We evaluated the morphometrical variability of *P. brasiliensis* strains isolated from the two clinical forms of the disease in samples of cultures obtained before and after hamster testicular infection.

MATERIAL AND METHODS

Microorganisms: We studied 30 clinical strains of *P. brasiliensis* previously obtained from different biological material of two groups of patients: 15 were isolated from patients with the acute form of the disease (group A) and the others from cases with the chronic form (group C). Cultures had been maintained in the mycelial phase on Sabouraud-dextrose agar at 25 ºC for variable period of times.

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***In vitro* evaluation of fungal strains morphometrical characters:** Before beginning the experiment, samples of cultures were sequentially plated 3 times on PYGA (peptone yeast extract glucose agar)[18], and incubated at 35 ºC up to complete transformation from mycelial to yeast form of the fungus. Afterwards, every strain was cultured on PYGA and incubated at 35 ºC for four days. In order to evaluate the yeast morphology and viability of the strains, a inoculum suspension with turbidity adjusted to 50-60% ± 1% transmission (T), by spectrophotometry (530nm wavelength) was prepared on PBS (pH 7.2). Aliquots of 100µL of the mentioned suspension were stained with equal volume of Janus Green B 0.02% vital dye[2] and placed on a glass slide. Using a Neubauer chamber, the elements not stained by vital dye were counted as viable cells. Under microscopic observation we recorded the following parameters: the percentage of budding cells, the number of buds exhibited by each cell and the diameter of the mother cells. The measurement of the yeast cell sizes was carried out with the aid of an Olympus CBB microscope coupled with a micrometer disc. We measured the major transverse and longitudinal axes of 100 viable cells of each preparation[19].

***In vivo* evaluation of fungal strains morphometrical characters:** Before beginning the experiment, an inoculum suspension of each strain containing 2.0 x 10^6 viable cells/mL of PBS was prepared with the aid of a hemocytometer. Thirty sixty-day old male hamsters with weights between 90 and 100g were challenged by inoculation of a 100µL of standard inoculum into the right testicle[8,12]. The animals were kept in individual cages and were given food and water *ad libitum.* Animals were observed daily for the presence of orchitis. In the presence of orchitis, hamsters were sacrificed and the right testicle was removed. Samples of fluids obtained by punction of each animal testicle were placed on a glass slide for further evaluation. All morphological parameters were studied according to the methodology previously described for *in vitro* evaluation of *P. brasiliensis* strains.

Statistical analysis: The morphometrical characters of fungal cells from *P. brasiliensis* strains were contrasted in two steps: (i) comparison of morphological parameters obtained with 30 isolates before and after animal infection, regardless of the origin of the strain; (ii) comparison of the morphological parameters exhibited by *P. brasiliensis* strains from the two groups (A and C) recorded after animal infection. These variables were analyzed by Student t-test, performed with the aid of the Statistica for Windows Microsoft Corporation version 6.0.

RESULTS

We were able to convert to the yeast phase all 30 *P. brasiliensis* isolates that had been maintained in the mycelial phase for variable periods of times. However, 2 isolates (A15 and C27) exhibited persistence of pseudo-hyphae and hyphae forms even after more than 10 sequential cultures on PYGA at 35 ºC. The high viability of all yeast cells, confirmed by using Janus Green B vital dye, ranged from 80 to 99% (mean=90%).

All animals were successfully challenged intratesticularly and the enlargement of the inoculated testis was observed after periods of 5 to 10 weeks (mean 60 days). During the 30 experiments performed in the present series, four (13%) animals exhibited progressive lost of vitality and weight, and death occurred before the documentation of orchitis. The animal passage was successfully repeated with those isolates and all challenged hamsters developed orchitis.

Table 1
Percentage of budding cells and number of buds by cell exhibited by 30 isolates of ***P. brasiliensis*** evaluated before and after animal passage

Isolates	Before animal passage		After animal passage	
	% of budding cell	Mean of buds/cell	% of budding cell	Mean of buds/cell
A1	88	4.07	15	0.24
A2	64	1.31	39	0.51
A3	74	4.25	58	1.72
A4	93	3.95	20	0.24
A5	90	4.89	41	0.61
A6	60	1.51	29	0.35
A7	85	2.64	17	0.21
A8	71	1.66	43	1.06
A9	87	3.66	20	0.32
A10	51	1.17	49	0.80
A11	86	1.64	19	0.32
A12	70	1.68	23	0.63
A13	51	1.09	00	0.00
A14	63	1.41	34	0.51
A15	37	0.70	58	1.00
M ± SD		2.37 ± 1.4		0.57 ± 0.4
C16	60	1.30	00	0.00
C17	79	2.45	22	0.31
C18	71	1.27	35	0.39
C19	71	1.21	22	0.49
C20	59	1.12	30	0.67
C21	82	1.63	37	1.62
C22	54	0.96	27	0.34
C23	84	2.41	12	0.14
C24	72	1.39	74	1.53
C25	68	1.40	26	0.40
C26	60	1.24	27	0.37
C27	73	3.49	30	0.63
C28	71	1.32	22	0.49
C29	71	1.38	22	0.49
C30	71	1.47	22	0.49
M (± SD)		1.60 ± 0.7		0.56 ± 0.4

M: Mean of buds/cell of the 100 cells from each isolate; SD: standard deviation; A: Acute group; C: Chronic group

Yeast cells of *P. brasiliensis* were always recovered from the fluids of infected testicles. The variability of the morphological aspects of the yeast cells is shown in Figure 1. About 80% of the isolates presented spherical cells, seconded by oval and elongated ones. Of note, it was also possible to observe small pseudo-hyphae and hyphae forms among samples obtained from 2 isolates (A15 and C27). In contrast to the high incidence (>70%) of budding yeast cells observed with isolates maintained in cultures, samples of *P. brasiliensis* obtained after animal passage exhibited only 29% of the yeast cells as budding forms (Table 1). The mean of budding cells observed with all isolates was 2 buds/cell before animal passage and 0.6 buds/cell after animal passage. The percentage of budding cells as also the number of buds by cell was not

influenced by animal passage, regardless of the source of the strain (acute or chronic groups).

The estimated diameter size and the micromorphometrical aspects of *P. brasiliensis* in yeast-like form are depicted in figure 2. Table 2 shows the sizes of the *P. brasiliensis* isolates from groups A and C measured before and after animal passage. The size values of *P. brasiliensis* isolates from groups A and C, measured before the animal passage exhibited the same behavior. The diameter of all 30 isolates before animal passage, recorded by the analyses of 100 mother cells of each strain, exhibited a minimum size of 2.6µm, a maximum of 35.5µm and a medium of 12.3µm (SD = ± 4.7). The same parameters measured after animal passage were: minimum = 5.3µm, maximum 42.1µm, medium = 12.2µm (SD ± 4.4) (p=0.31).

Table 2
Morphometrical characteristics of 30 *P. brasiliensis* isolates recorded before and after animal passage

	Before animal passage				After animal passage			
Isolates	MAX	MIN	M	± SD	MAX	MIN	M	± SD
A1	23.7	5.3	13.0	3.9	28.9	5.3	11.7	4.0
A2	34.2	7.9	19.3	5.9	42.1	10.5	20.2	5.2
A3	26.3	5.3	13.1	4.2	21.0	5.3	10.7	3.6
A4	18.4	5.3	9.7	3.3	15.8	5.3	9.0	2.4
A5	26.3	5.3	12.5	3.9	23.7	5.3	13.1	4.5
A6	21.0	5.3	10.1	3.4	23.7	5.3	11.7	3.5
A7	28.9	7.9	16.4	4.7	23.7	5.3	12.6	3.8
A8	26.3	5.3	11.2	3.6	18.4	5.3	9.8	2.5
A9	26.3	5.3	10.6	4.0	22.4·	5.3	11.9	3.7
A10	26.3	7.9	11.9	3.2	31.6	5.3	14.5	4.4
A11	26.3	6.6	13.2	3.9	26.3	5.3	12.5	3.9
A12	31.6	5.3	13.5	4.4	26.3	5.3	12.5	3.9
A13	18.4	5.3	10.4	2.8	31.6	5.3	14.5	4.4
A14	21.0	5.3	10.6	3.8	23.7	5.3	12.1	4.2
A15	15.8	5.3	7.7	2.2	34.2	6.6	13.0	6.1
C16	21.0	5.3	11.6	3.2	18.4	7.9	13.0	3.3
C17	23.7	9.2	14.6	3.2	26.7	5.3	10.5	3.4
C18	26.3	5.3	14.6	4.9	26.3	5.3	11.5	3.8
C19	17.1	5.3	10.1	3.3	23.7	5.3	12.6	3.4
C20	35.5	7.9	18.6	6.6	32.9	5.3	17.8	5.3
C21	15.8	5.3	9.0	2.7	21.0	5.3	10.3	2.7
C22	26.3	5.3	14.6	4.2	21.0	6.6	11.2	3.3
C23	26.7	5.3	15.8	4.9	21.0	5.3	11.7	3.2
C24	18.4	5.3	9.2	2.4	18.4	5.3	9.4	2.7
C25	21.0	2.6	10.6	3.6	18.4	5.3	9.3	2.1
C26	21.0	5.3	9.8	2.3	21.0	6.6	11.8	3.2
C27	31.6	7.9	12.2	4.2	18.4	5.3	9.0	2.9
C28	26.3	7.9	12.5	4.4	23.7	5.3	12.7	3.4
C29	21.0	3.9	10.8	3.4	23.7	5.3	12.7	3.4
C30	21.0	5.3	11.8	3.1	23.7	5.3	12.6	3.4

MAX. The largest size in µm found among 100 observed cells from each isolate; MIN: The smallest size in µm found among 100 observed cells from each isolate; M: Mean of 100 measurements of cells from each isolate; SD: standard deviation

After animal passage, there was a statistically significant difference between the cell sizes of *P. brasiliensis* isolates recovered from testicles inoculated with strains from groups A and C. The maximum diameter of mother cells from group A isolates exhibited a size of 42.1µm in contrast with 32.9µm exhibited by mother cells from group C ($p<0.05$). The diameter of 1500 mother cells from group A isolates exhibited a medium size of 16.0µm (SD ± 4.0), a value significantly higher than the 14.1µm (SD = ± 3.3) exhibited by 1500 mother cells from group C isolates ($p<0.05$).

DISCUSSION

The basis for virulence in *P. brasiliensis* is not completely understood[6,16]. There is a consensus that sequential *in vitro* passage of *P. brasiliensis* leads to loss of virulence. Furthermore, the animal passage of attenuated *P. brasiliensis* may restore some virulence factors[7,14]. Attention to morphological and biochemical properties that are regained or demonstrated after animal passage may provide new insights into factors related to the pathogenicity and virulence of *P. brasiliensis*.

In the present study we investigated the effect of animal passage on the morphometrical characteristics of 30 isolates of *P. brasiliensis*. The selection of hamsters for animal passage of *P. brasiliensis* strains was based on previous publications addressing the efficacy of the intratesticular route of infection as well as the success of recovering the fungus from inoculated testis[8,12]. In our series of experiments orchitis developed in all challenged hamsters after 5 to 10 weeks and the recovery rate of *P. brasiliensis* from infected testis was 100%.

After animal passage, the yeast-like cells of *P. brasiliensis* exhibited a limited number of budding yeasts compared to the number observed before animal passage. The adversity of the environment caused by host defense mechanisms, pH modification of the infected tissue, as well as the high concentration of organisms found in the animal testis may have led to decrease in the reproduction of the fungus at the moment we obtained the sample for analysis.

The polymorphism of *P. brasiliensis* in clinical specimens has been well documented and includes variable sizes of isolated or multibudding yeasts and sometimes elongated distorted cells or chains of yeasts[1,3,4,5,13,15,16,17]. In the present series we were able to demonstrate a high variability of forms exhibited by *P. brasiliensis* in fluids obtained from testis of infected animals. As depicted in figure 1, the fungus revealed not only yeast forms with and without budding cells but also rare small hyphae and amorphous structures. Of note, the occurrence of filamentous forms of *P. brasiliensis* in clinical specimens has rarely been reported[15,17].

Some isolates obtained from patients with acute form of PCM generated larger yeast cells after animal passage than those obtained from fluids of animals infected by isolates from patients with the chronic form of PCM. Apparently, yeast cells of *P. brasiliensis* maintained *in vitro* for long periods of time tend to become more homogeneous in size. Animal passage could be a stimulus for recovery of the original characteristics of the organism, including virulence factors. Consequently, the morphological differences we observed with *P. brasiliensis* isolates after animal passage suggest that the size of the yeast-like form of the

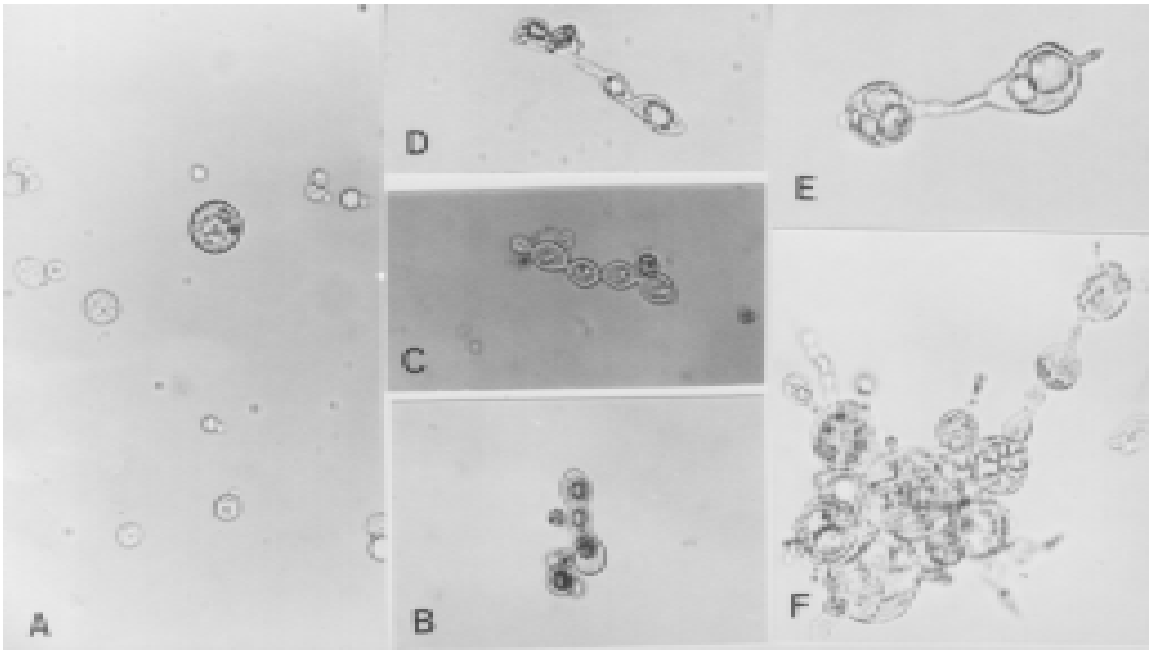

Fig. 1 - Morphological aspects of *P. brasiliensis* in yeast-like form after 4 days of incubation at 35 ºC in PYGA (400x): a) isodiametrical forms; b) Oval and elongated cells; c) chains of yeasts; d) small hyphae; e. f) amorphous structures.

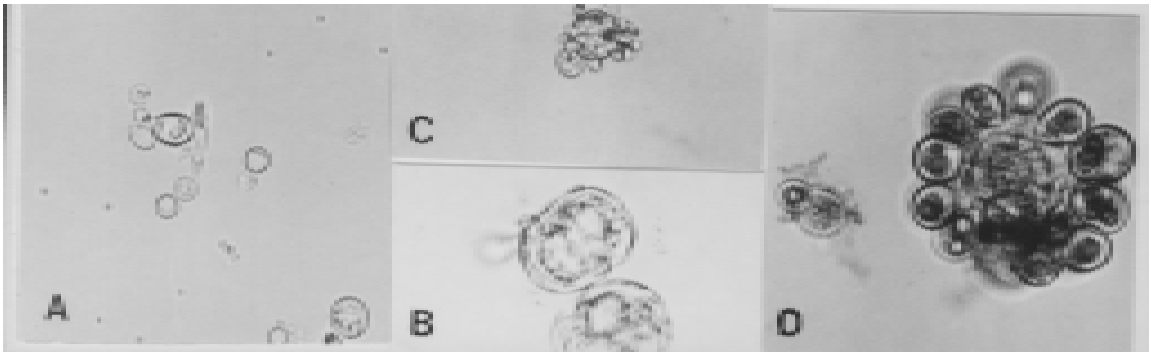

Fig. 2 - Diversity of sizes exhibited by *P. brasiliensis* C25 isolate in yeast-like form evaluated before and after animal passage (400x): a) Small cells ≤ 4µm, before animal passage; b) Large cells (≅ 25 µm) after animal passage; c) Medium cells from 13 to 18 µm with several buds; d) Multibudding cells stained by (Janus Green).

fungus may play a role in the virulence of the organism as well as in the severity of the disease.

According to LACAZ *et al*.[15], yeast like cells of *P. brasiliensis* isolated from fluids from enlarged lymph nodes of patients with PCM have large cells (up to 25µm) in contrast to the small cells (up to 5µm) seen in oral lesions. Our results reinforce the polymorphism exhibited by *P. brasiliensis* in clinical specimens and the need for further investigation to elucidate the role of morphological parameters in the natural history of this disease.

RESUMO

Isolados de *Paracoccidioides brasiliensis* obtidos de pacientes com doença crônica e aguda exibem diferenças morfológicas após passagem animal

Os mecanismos de virulência em *Paracoccidioides brasiliensis* não estão totalmente esclarecidos. Há um consenso que subcultivos sucessivos de *P. brasiliensis* acarretam a perda de sua patogenicidade que pode ser revertida pelo reisolamento do agente após passagem animal. As

propriedades morfológicas e bioquímicas que são recuperadas ou demonstradas após passagem animal podem fornecer novas informações quanto a fatores relacionados à patogenicidade e virulência de *P. brasiliensis.* Nós avaliamos características morfológicas: porcentagem de células brotantes, número de brotamentos por célula e determinação do diâmetro de 100 células-mãe em 30 isolados de *P. brasiliensis*, antes e após passagem animal. Os isolados foram obtidos de pacientes com diferentes formas clínicas de paracoccidioidomicose (PCM): forma aguda (grupo A n=15) e forma crônica (grupo C n=15). A mensuração do tamanho das células foi feita em microscópio óptico Olympus CBB acoplado com régua micrométrica. Nós medimos o maior eixo transversal e longitudinal de 100 células viáveis de cada preparação. A porcentagem de células brotantes bem como o número de brotamentos por célula não foram influenciados pela passagem animal independente da origem da amostra (grupo agudo ou crônico). Os valores dos tamanhos dos isolados de *P. brasiliensis* dos grupos A e C medidos antes e após passagem animal exibiram o mesmo comportamento. Após passagem animal, houve diferenças estatisticamente significativas entre o tamanho das células de *P. brasiliensis* isolados a partir de testículos inoculados com amostras dos grupos A e C. O diâmetro máximo das células mãe dos isolados do grupo A exibiram tamanho de 42.1µm, em contraste com 32.9µm exibidos pelas células mãe do grupo C ($p<0.05$). O diâmetro de 1500 células do grupo A exibiram tamanho médio de 16.0µm (SD ± 4.0), valor significativamente maior que 14.1µm (SD ± 3.3) exibidos pelas 1500 células do grupo C ($p<0.05$). Nossos resultados confirmam o polimorfismo exibido por *P. brasiliensis* em amostras biológicas e reforça a necessidade de mais investigações com o objetivo de elucidar o papel que parâmetros morfológicos do fungo possam assumir na história natural da doença.

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