Virulence attenuation and phenotypic variation of *Paracoccidioides brasiliensis* isolates obtained from armadillos and patients

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Paracoccidioides brasiliensis *is the etiological agent of paracoccidioidomycosis, the most important systemic mycosis in Latin America. The virulence profiles of five isolates of* P. brasiliensis *were studied in two different moments and correlated with some colonial phenotypic aspects. We observed a significant decrease in the virulence and an intense phenotypic variation in the mycelial colony. The recognition of all ranges of phenotypic and virulence variation of* P. brasiliensis*, as well as its physiological and genetic basis, will be important for a better comprehension of its pathogenic and epidemiological features.*

Key words: *Paracoccidioides brasiliensis* - virulence - hamster - *Dasypus novemcinctus*

Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America (Wanke & Londero 1994).

It has been routinely observed the lost or attenuation of the virulence in the isolates that have been maintained for long periods in the laboratory condition (Brummer et al. 1990). Although it has been assumed that the virulence could be recovered after animal passage, the genetic and/or physiologic basis for such phenomenon is completely unclear.

We have been studying the virulence and genetic profiles of *P. brasiliensis* obtained from armadillos, demonstrating that these animals can harbour a wide range of eco-pathogenotypes, probably the same ones that cause PCM in humans (Sano et al. 1999a,b, Hebeler-Barbosa et al. 2003a,b). In the present paper, we document the attenuation of virulence in isolates of *P. brasiliensis* that seems to be directly associated with the time of isolation, and the existence of an intense phenotypic plasticity inter and intra isolates that could be important for fungus biology and its hosts interaction.

Three clinical (Bt60, Bt84, and Bt85) and two armadillo (T1F1 and T10B1) *P. brasiliensis* isolates were evaluated concerning its mycological aspects in the yeast phase cultured in tube slants with glucose-peptone-yeast extract agar (GPYA medium) at 35°C and in the mycelial phase by its growth rate, giant colonies, and the presence of colony sectors in potato dextrose agar (PDA) plates at 25°C, and virulence profiles by counting colony-forming units (CFU) using the hamster intratesticular model, as described by Hebeler-Barbosa et al. (2003a). The virulence of the armadillos isolates were also compared with the results obtained, in the same experimental condition, when these ones were isolated six and five years ago, respectively (Hebeler-Barbosa et al. 2003a) by the Unpaired t test or the non-parametric Mann-Whitney test.

Analysis of variance for log (CFU/g) was used to investigate differences between isolates and moments in each organ. A p-value < 0.05 was considered indicative of statistical significance. The possible correlation between the mycelial growth rate and virulence, and the age of the isolates (months of maintenance in laboratory condition) and virulence were also analyzed by the coefficient of determination (r squared).

All the colonies showed a linear growing pattern, during all the period of observation. The medial diameters of the colonies ranged from 21 to 38.6 mm, after 60 days of cultivation in PDA at 25ºC (Table). It was possible to detect two distinct growing patterns among *P. brasiliensis* isolates: high (Bt60, Bt84, T1F1, and T10B1) and low (Bt 85), that were statically different ($p < 0.05$).

The giant colonies presented typical cotton-like surface that varied from white to beige colour; several of them showed the wrinkled aspect and presence of fissures (Table and Figure). The isolate Bt85, that showed the lowest growing rate, presented an intense wrinkled aspect (Fig. g, h). A variant colony (Bt84a) (Fig. e, f) that differed markedly from its original colony (Bt84) (Fig. c, d), by showing a cream-like and glabrous aspect, was detected. Presence of sectors was observed in Bt60 (Fig. a) and T10B1 (Fig. k) and in T1F1 it was observed the presence of rings of growing in the colony border (Fig. i). In the reverse, it was observed variation in the colour tint (tonality), from the cream yellow to dark brown.

All the five isolates obtained from patients and armadillos showed ability to infect the hamster testis. There were no significant differences in the testis CFU counts between the periods of 30 and 60 days of inoculation. The

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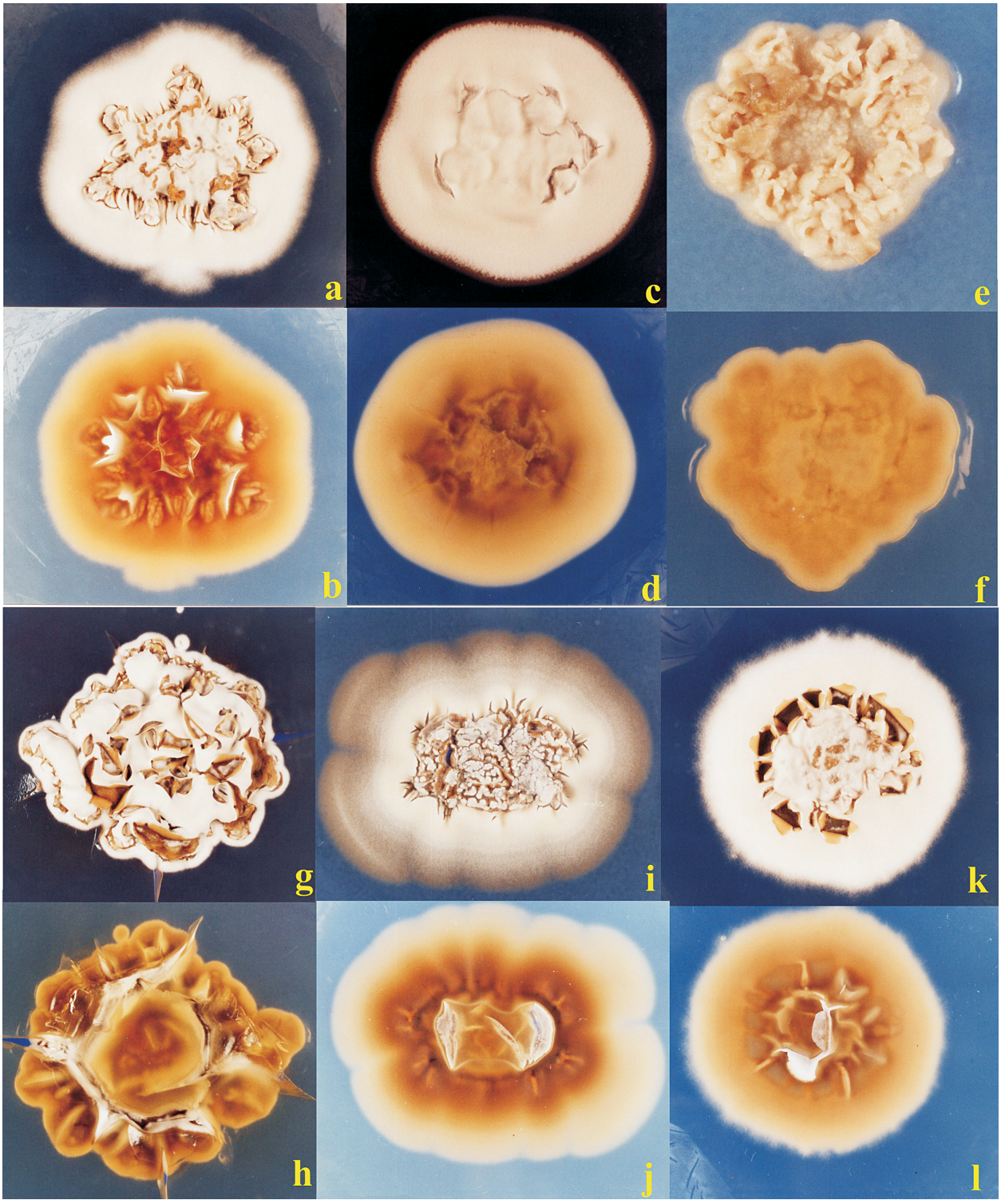
two armadillo's isolates showed CFU counts significantly higher than the two clinical isolates (Bt60 and Bt85), in the testis and also a higher ability to disseminate to spleen and liver. The armadillo isolate T10B1 presented the highest CFU counts in all the organs.

The virulence of T1F1 and T10B1 decrease markedly after periods of six and five years of in vitro subcultivation. The CFU counts of T1F1 in the testis (30 days after inoculation) and in the spleen and liver (30 and 60 days after inoculation) was significantly lower at present evaluation



Verse and reverse of giant colonies of isolate Bt60 (a, b); Bt84 (c, d), Bt 84a variant (e, f); Bt85 (g, h); T1F1 (i, j); and T10B1 (k, l).

in comparison with that observed six years ago in the same experimental condition. The CFU counts of T10B1 in the spleen and liver (30 days after inoculation) were significantly lower at present than that observed five years ago. In the period of 60 days after inoculation, while T10B1 produce highs CFU counts at present, all the hamster were killed five years ago.

It was not observed correlation between virulence and growth rates, since the coefficient of determination (r squared = 0,1775) obtained was not significant.

We document here a common fact currently observed by many labs that deals with different isolates of *P. brasiliensis*. The fungus is not a simple clone with a constant phenotype. Inter and intra isolates variations tend to occur in the fungus. This point, besides being a central theme in population and evolutionary biology, has also some practical aspects. The recognition of all range of phenotypic variation of a certain species of pathogenic fungus is fundamental for a precise diagnostic and the disease prognostic. The occurrence of plasticity in strains of pathogenic fungi has also been associated with an inherent ability to adapt to environmental changes, and during infection, could promote the emergence of variants that are able to evade host immune mechanisms (Franzot et al. 1998). *P. brasiliensis* shows an intense colonial plasticity, with variation inter and intra isolates. In certain fungi, the differences in growth rate could be associated with the ploidy level of the strains (Fincham et al. 1979). There is some indication that *P. brasiliensis* is a diploid fungus, and some eletrophoretic karyotiping studies have detected a significant variation in chromosome profiles (Montoya et al. 1997, Cano et al. 1998), also indicating that haploid and diploids strains can occur simultaneously between the isolates (Feitosa et al. 2003). Therefore, we have observed colonies with sectoring in isolates both from human and armadillos. The production of sectors in fungal colonies is a strong indication that the fungus is suffering mitotic chromosomal rearrangements with amplification of variability by parasexual processes (Pontecorvo & Roper 1956). In such process, abrupt changes in the fungus physiology can occur, mainly differences in their rate of growth and extent of spore (Finchman et al. 1979).

We have also observed one same strain displaying two distinct colonial aspect, such as observed in Bt84 isolate (complete glabrous or cottonous colony). In *Candida albicans*, it has been observed a phenomenon of phenotypic switching in the colony morphology (opaque or white) that could be related with fungal virulence (Soll 1992). In *Cryptococcus neoformans* a similar mechanism seems to be operating (Fries et al. 2002). It was demonstrated that a same strain of *C. neoformans* could undergo rapid genetic and phenotypic changes in vitro and in vivo that may contribute to survival in the host by providing a means to evade host defences (Franzot et al. 1998).

The virulence attenuation of *P. brasiliensis* isolates detected in the present study seems to be directly dependent on the time of in vitro subcultivation. The lost of virulence seems to be more evident in the organs of dissemination than in the inoculate site (testis, in the ham-

TABLE

Morphological colonial aspects of *Paracoccidioides brasiliensis* isolates in the mycelial phase cultured in potato dextrose agar, at 25ºC, for two months, and the yeast phase cultured in glucose peptone yeast extract agar, at 35ºC, for one week

Isolate	Host	Age of the isolate	Mycelial phase				Yeast phase aspects
			Front	Reverse	Sectors	Diameter (mm)	
T1F1	Armadillo	1996	Cotton like, white, grooves, and fissures, margin glabrous, and cream coloured, white, growth circles	Fissures, central area colouring nut-brown, clear margin, with cracks	No	34,2	Brain like, wrinkled and folded. Intense growing
T10B1	Armadillo	1997	Cotton like, white, irregular central area with folds and hollows, wrinkled and crakes	Growth biscuit colour, central area nut brown, with radials wrinkles and crakes	Yes	33,4	Brain like, wrinkled and folded. Intense growing
BT60	Human	1990	Cotton like, white, with dark margin, crakes and groves	Smooth, orange-shaped	Yes	38,6	Brain like, wrinkled and folded. Very intense growing
BT84	Human	1994	Cotton like, cream-colouring, with dark margin, few grooves	Smooth, cream-yellowish	No	34,3	Brain like, wrinkled and folded. Very intense growing
BT84a	Human	1994	Glabrous, cream colouring	Smooth superficial, cream colouring	No	ND	Brain like, wrinkled and folded. Intense growing
BT85	Human	1994	Cotton like, white colouring, wrinkles, in total area	Cracked central area, cream-yellowish colouring, and grooves	No	21,0	Brain like, wrinkled and folded. Very intense growing

BT84a was a variant obtained from the BT84 isolate; ND: not determined.

ster model). Although the genetic basis for such process is poorly understood, it makes sense to admit that the same rapid genetic changing the pathogenic fungus can undergo will cause completely different results if in vivo or in vitro condition. In vivo, the changing can be selected for more survival in the host and to evade immune defences; in vitro, it will contribute for variants with attenuated virulence. The virulence attenuation could also be associated with physiological and cytoplasmatic changing, since the nuclear genetic materials tend to be more conservative for variation. The involvement of cytoplasmatic genetic material in the fungal virulence, such as the presence of dsRNA mycovirus, has been observed in some phytopathogenic fungi, mainly inducing hypovirulence, and has never been explored in human pathogenic fungi (Choi & Nuss 1992). Such physiological and genetic variation also could account for some important differences in the antigen production already observed in *P. brasiliensis* (Franco et al. 1996).

In summary, we document here the virulence attenuation in *P. brasiliensis* according to the time of the fungus has been maintained in vitro condition, and also an intense phenotypic plasticity inter and intra isolates. This phenomenon seems to be the result of a same operating biological variation system in pathogenic fungi. More studies on the physiology and genetic basis of this system should be carried out, since it is directly associated with the ability of the fungus in causing diseases. In practical terms, it is highly recommended that the isolates collection should be preserved in frozen or lyophilized states and with individual records for its subcultures in vitro condition.

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