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Original Article

The Role of Chlamydospores of *Paracoccidioides brasiliensis*

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Abstract

The role of chlamydospores in the conversion process from a mycelial-to-yeast form using the slide culture method was studied. Three clinical isolates and two other isolates from armadillo, belonging to the fungal species *Paracoccidioides brasiliensis*, were cultured on Sabouraud dextrose agar (SDA), potato dextrose agar (PDA) and brain heart infusion dextrose agar (BHIDA). Initially, the mycelial forms of each isolate were grown at 25°C for 7, 14, 30 or 60 days on slide cultures and then the temperature was shifted to 35°C. Interestingly, the slide cultures of all the isolates at 25°C formed chlamydospores on either SDA or BHIDA, whereas, on PDA medium, aleurioconidia were formed. If the slide cultures on BHIDA were incubated at 35°C for 7 to 14 days, multiple budding forms could be observed. This phenomenon was not evident in the slide cultures of SDA or PDA. The results of this morphological study indicate that in *P. brasiliensis*, chlamydospores may play an important role in the conversion process from a mycelial-to-yeast form.

Key words: chlamydospore, nutrition, *Paracoccidioides brasiliensis*, thermo-dependent dimorphism

Introduction

Paracoccidioides brasiliensis, a thermo-dependent dimorphic fungus is the causative agent of paracoccidioidomycosis which is a kind of deep mycosis prevalent in South and Central America[1-3]. *P. brasiliensis* grows in its yeast form both *in vivo* and *in vitro* at 35°C to 37°C. The globose yeast cells have thick cell walls from which many daughter cells bud out. At room temperature it grows in the mycelial form as branching septate hyphae from which chlamydospores or aleurioconidia are produced. There have been many reports on the conversion process of this fungus from the mycelial to yeast form and the role of aleurioconidia in this process has been reported by several groups of researchers[1-6]. There are also a few reports including our own, however, stating that chlamydospores are intermediate structures that play the main role in the mycelial-to-yeast conversion process[7-12].

Even though it is widely known that temperature is the most important factor for the mycelia to yeast transition in *P. brasiliensis*[1-3], we speculate, that certain nutrients of animal tissue origin are also important determining factors in this transitional process.

The present study was carried out to clarify the role of chlamydospores of *P. brasiliensis* in the thermo-dependent conversion process using a slide culture method.

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Materials and Methods

P. brasiliensis isolates

Three clinical isolates of Pb-9 (IFM 41620), Pb-18 (IFM 41621) and AOKI (IFM 41632) along with two other strains of armadillo origin, Tatu (IFM 46463) and Tatu 1 (IFM 47183) were used in this experiment. The fungi were maintained at room temperature on potato dextrose agar (PDA, Difco, MI, USA) slants.

Media

Sabouraud dextrose (2%) agar (SDA) (polypeptone and dextrose from Wako, Japan and agar from Difco, USA), potato dextrose agar (PDA) and brain heart infusion (Difco, USA) dextrose (1%) agar (BHIDA) were used.

Culture of the fungi

The organisms, which were maintained on PDA at room temperature, converted to their yeast forms on BHIDA slants at 35°C and the yeast forms were maintained by twice a week sub-culture on the same medium at the same temperature. For slide culture, the yeast form cells were suspended in Sabouraud dextrose broth (SDB), potato dextrose broth (PDB) or brain heart infusion dextrose broth (BHIDB) and the cell concentrations were adjusted at 10^7 cells/m*l*. Then 1 m*l* from each suspension was mixed with 25 m*l* of SDA, PDA or BHIDA at 42°C in 20 mm ×90 mm plastic petri dishes. Approximately 5 × 5 × 5 mm^3 agar blocks containing 4 × 10^4 yeast cells were cut and put on sterilized slide glasses set upon U-shaped tubes and then were covered with cover glasses; about 5 m*l* of sterilized distilled water was added to each petri dish to prevent dehydration.

The slide cultures were incubated at 25°C for 7, 14, 30 or 60 days, after which the incubation temperature was raised to 35°C for 14 days and observed under a light microscope fixed and stained with lactophenol cotton blue. All these procedures were performed in triplicate. When the cytoplasma were stained with lactophenol cotton blue, their structures were recorded as morphological characteristics. The hyphae which were detected only as margins without the cytoplasma staining by lactophenol cotton blue were treated as ghost-like structures.

Results

Table 1 represents the cumulative findings of the different phenotypes observed in cultures at 25°C on SDA, PDA and BHIDA on days 7, 14, 30 and 60 of the incubation period. All isolates showed mycelial growth.

Chlamydospores were formed on slide cultures of isolate Pb-9 at 30 to 60 days after inoculation on SDA (Fig. 1). Isolate Pb-18 formed chlamydospores on slide cultures 60 days after inoculation and Tatu also formed them at 14, 30 and 60 days after inoculation on SDA.

When PDA was used for slide cultures, aleurioconidia were formed only by isolate AOKI at 30 to 60 days and by isolate Tatu 1 at 14, 30 and 60 days after incubation at 25°C (Fig. 2). Except for isolate Tatu 1 at 25°C 14 days after the initial incubation, the aleurioconidia were attached on ghost-like hyphae.

In the case of BHIDA, chlamydospores appeared instead of aleurioconidia: isolates Pb-9 and Pb-18 at 60 days, AOKI at 14, 30 and 60 days and Tatu 1 at 30 and 60 days after

Table 1. Cellular phenotypes of *P. brasiliensis* forming at 25°C cultures in different media

Isolate	Culture period (days)	Growth medium		
		SDA	PDA	BHIDA
Pb-9	7	−	−	−
	14	−	−	−
	30	CL	−	−
	60	CL	−	CL
Pb-18	7	−	−	−
	14	−	−	−
	30	−	−	−
	60	CL	−	CL
AOKI	7	−	−	−
	14	−	−	CL
	30	−	G, AC	G, CL
	60	−	G, AC	G, CL
Tatu	7	−	−	−
	14	CL	−	−
	30	CL	−	−
	60	CL	−	−
Tatu 1	7	−	−	−
	14	−	AC	−
	30	−	G, AC	CL
	60	−	G, AC	G, CL

−, pure mycelial growth; CL, chlamydospore; AC, aleurioconidia; G, ghost-like structure.

Table 2. The chlamydospore formation of *P. brasiliensis* on SDA

Isolate	Days of pre-culture at 25°C	Culture period at 35°C (days)				
		3	5	7	10	14
Pb-9	7 (−)	CL	CL	CL	CL	CL
	14 (−)	CL	CL	CL	CL	CL
	30 (CL)	CL	CL	CL	CL	G, CL
	60 (CL)	CL	CL	CL	G, CL	G, CL
Pb-18	7 (−)	CL	CL	CL	CL	CL
	14 (−)	CL	CL	CL	CL	CL
	30 (−)	CL	CL	CL	CL	G, CL
	60 (CL)	CL	CL	CL	G, CL	G, CL
AOKI	7 (−)	CL	CL	CL	CL	CL
	14 (−)	CL	CL	CL	CL	CL
	30 (−)	CL	CL	CL	CL	G, CL
	60 (−)	−	CL	CL	G, CL	G, CL
Tatu	7 (−)	CL	CL	CL	CL	G, CL
	14 (CL)	CL	CL	CL	CL	G, CL
	30 (CL)	CL	CL	CL	G, CL	G, CL
	60 (CL)	CL	G, CL	G, CL	G, CL	G, CL
Tatu 1	7 (−)	CL	CL	CL	CL	G, CL
	14 (−)	CL	CL	CL	G, CL	G, CL
	30 (−)	CL	CL	CL	G, CL	G, CL
	60 (−)	CL	CL	G, CL	G, CL	G, CL

−, pure mycelial growth; CL, chlamydospore; G, ghost-like structure.

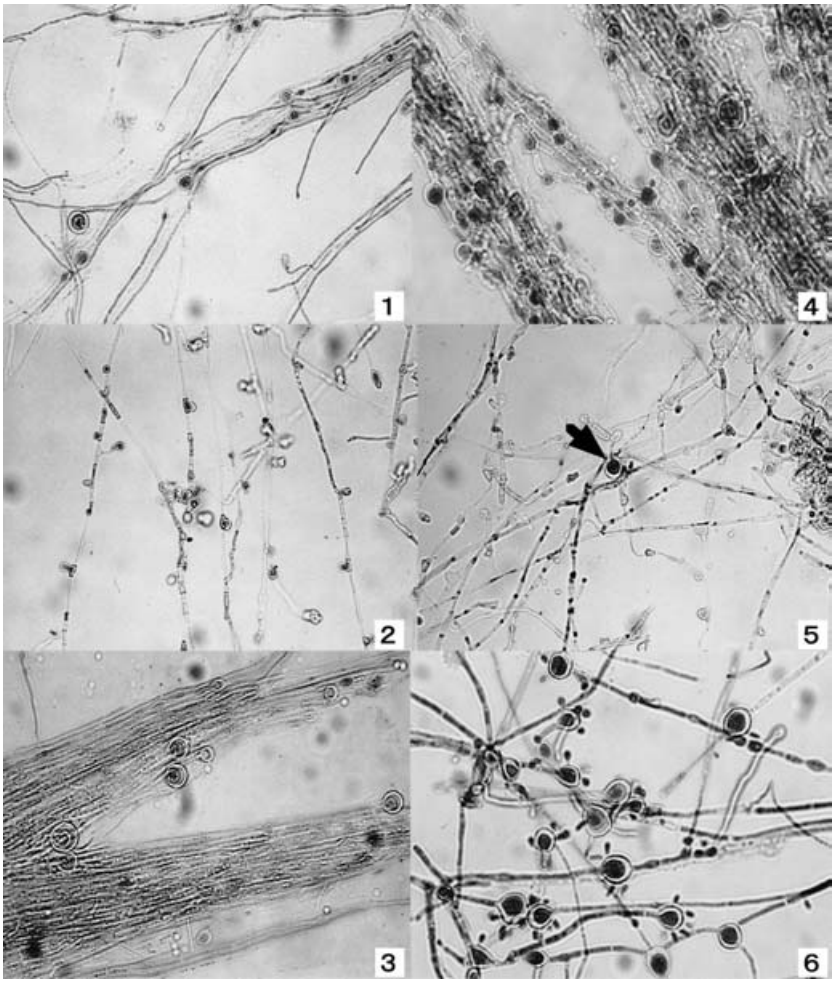

Fig. 1. Chlamydospores of the isolate Pb-9 cultured at 25°C on SDA for 60 days (×400, lactophenol cotton blue staining).
Fig. 2. Aleurioconidia attached to ghost-like hyphae of the isolate Tatu 1 cultured at 25°C on PDA for 30 days (×400, lactophenol cotton blue staining).
Fig. 3. Chlamydospores of the isolate AOKI cultured at 25°C on BHIDA for 14 days (×400, lactophenol cotton blue staining).
Fig. 4. Many chlamydospores of the isolate Tatu 1 cultured for 14 days at 35°C on SDA after being pre-cultured at 25°C for 14 days (×400, lactophenol cotton blue staining).
Fig. 5. The isolate Tatu 1 cultured for 10 days at 35°C after being pre-cultured at 25°C for 30 days on PDA formed aleurioconidia that became ghost-like cells and were no longer stained with lactophenol cotton blue; a chlamydospore can also be seen at the center (arrow, ×400, lactophenol cotton blue staining).
Fig. 6. Multiple budding yeast cells of the isolate AOKI cultured for 3 days at 35°C after being pre-cultured at 25°C for 7 days on BHIDA are shown (×400, lactophenol cotton blue staining).

incubation (Fig. 3). Chlamydospores of isolates AOKI and Tatu 1 were located on ghost-like hyphae at 25°C for 30 or 60 days after initial incubation.

Table 2 shows the results of slide cultures on SDA, which were initially kept at 25°C and then grown at 35°C for 14 days. Regardless of the duration of culture, all isolates produced chlamydospores from day 3 onwards and many chlamydospores were observed on the slide cultures of isolate Tatu 1 (Fig. 4). Hyphae became ghost-like structures after the temperature was shifted from 25°C to 35°C for at least 5 days.

The results of slide culture on PDA at 35°C are shown in Table 3. Chlamydospores were produced from all the isolates except isolate Tatu 1 which was cultured initially at 25°C for 60 days. The aleuriodonidia of isolate Tatu 1 produced in the initial cultivation disappeared and became a ghost-like structure at the final observation. In general, when the culture condition was shifted from 25°C to 35°C, the aleurioconidia that formed at the 25°C culture appeared swollen and transformed into chlamydospores, or remained as ghost-like cells (Fig. 5). The hyphae also faded out regardless of the isolates in the final observations except for the multiple budding cells in isolate Pb-9.

Table 4 shows the results of slide culture of these isolates on BHIDA at 35°C; as is shown,

Table 3. The chlamydospore formation of *P. brasiliensis* on PDA

Isolate	Days of pre-culture at 25°C	Culture period at 35°C (days)				
		3	5	7	10	14
Pb-9	7 (−)	CL	CL	CL	MB	MB
	14 (−)	−	CL	CL	G, CL	G, CL
	30 (−)	−	CL	G, CL	G, CL	G, CL
	60 (−)	CL	G, CL	G, CL	G, CL	G, CL
Pb-18	7 (−)	CL	CL	CL	CL	G, CL
	14 (−)	−	−	CL	G, CL	G, CL
	30 (−)	−	−	−	G, CL	G, CL
	60 (−)	CL	CL	G, CL	G, CL	G, CL
AOKI	7 (−)	CL	CL	CL	CL	G, CL
	14 (−)	−	CL	G, CL	G, CL	G, CL
	30 (G, AC)	G, AC	G,AC,CL	G, CL	G, CL	G, CL
	60 (G, AC)	G, AC	G, AC	G, CL	G, CL	G, CL
Tatu	7 (−)	−	−	CL	CL	G, CL
	14 (−)	G, CL	G, CL	G, CL	G, CL	G, CL
	30 (−)	G, CL	G, CL	G, CL	G, CL	G, CL
	60 (−)	G, CL	G, CL	G, CL	G, CL	G, CL
Tatu 1	7 (−)	−	CL	CL	CL	G, CL
	14 (AC)	AC, CL	G, CL	G, CL	G, CL	G, CL
	30 (G, AC)	G, AC	G, AC	G,AC,CL	G,AC,CL	G, CL
	60 (G, AC)	G, AC	G, AC	G, AC	G, AC	G

−, pure mycelial growth; CL, chlamydospore; AC, aleurioconidia; MB, multiple budding yeast cells. G, ghost-like structure.

Table 4. The chlamydospore formation of *P. brasiliensis* on BHIDA

Isolate	Days of pre-culture at 25°C	Culture period at 35°C (days)				
		3	5	7	10	14
Pb-9	7 (−)	MBC	MBC	MBC	MBC	MBC
	14 (−)	MBC	MBC	MBC	MBC	MBC
	30 (−)	CL	CL	CL	CL	CL
	60 (CL)	CL	CL	CL	G, CL	G, CL
Pb-18	7 (−)	MBC	MBC	MBC	MBC	MBC
	14 (−)	CL	CL	CL	CL	CL
	30 (−)	−	CL	CL	CL	CL
	60 (CL)	CL	G, CL	G, CL	G, CL	G, CL
AOKI	7 (−)	MBC	MBC	MBC	MBC	MBC
	14 (CL)	CL	CL	CL	CL	CL
	30 (G, CL)	G, CL	G, CL	G, CL	G, CL	G, CL
	60 (G, CL)	G, CL	G, CL	G, CL	G, CL	G, CL
Tatu	7 (−)	CL	CL	MBC	MBC	MBC
	14 (−)	−	−	−	G, CL	G, CL
	30 (−)	G	G, CL	G, CL	G, CL	G, CL
	60 (−)	G	G	G	G	G, CL
Tatu 1	7 (−)	CL	CL	CL	MBC	MBC
	14 (−)	CL	CL	G, CL	G, CL	G, CL
	30 (CL)	G, CL	G, CL	G, CL	G, CL	G, CL
	60 (G, CL)	G, CL	G, CL	G, CL	G, CL	G, CL

−, pure mycelial growth; CL, chlamydospore; MBC, multiple budding yeast cells from chlamydosopores; G, ghost-like structure.

abundant multiple budding yeast cells appeared from all the isolates (Fig. 6). However, this took place only if the slide cultures were incubated at 35°C for 3 to 14 days after an initial incubation period of 7 to 14 days at 25°C. Chlamydospores remained as outstanding structures attached to hyphae which transformed into ghost-like structures or remained intact after the temperature shift from 25°C to 35°C. The ghost-like structures were predominant in the isolate Tatu, but a few chlamydospores could be found at the final observation.

Discussion

Various factors and phenotypes in fungi have been presumed to bestow to them their virulence potential, the absence of which may render it avirulent. With regard to the different phenotypes of a certain fungus, it is important to determine which of the particular phenotypic state/states is/are related to the development of pathogenicity in humans and animals so that precautionary or preventive measures can be designed and adopted. As in the case of *Filobasidiella neoformans* var. *neoformans*, basidiospore has been identified as an infectious phenotype[13]; views have varied greatly regarding the nature of the infectious propagule of *P. brasiliensis*. This experiment involving extensive morphological investigations is a simple and straightforward attempt to shed light on the type of the pathogenic form of *P. brasiliensis*.

Romano was the first to define the dimorphism of fungi in 1966 by dividing it into three categories: 1) temperature-dependent dimorphism, 2) temperature- and nutrition-dependent dimorphism and 3) nutrition-dependent dimorphism[14]. It has been known for some time that *P. brasiliensis* is a temperature-dependent dimorphic fungus. Restrepo and her colleagues had insisted that the mycelial form of *P. brasiliensis* converted to its yeast form through its aleurioconidia which could thus be considered to be this organism's infectious or pathogenic form for animals[15-17]. However, we have had doubts on this hypothesis, because our findings have shown that it was the chlamydospores that were triggered to their yeast forms by multiple budding under appropriate nutritional and thermal conditions, whereas aleurioconidia did not produce multiply budded cells under any of these conditions[8-9]. Our observations have yet to be confirmed by *in vivo* assessment and comparisons.

The purpose of this report is to clarify that nutritional factors and temperature conditions definitely affect the conversion of *P. brasiliensis*

and that both these factors are closely related.

In 1987, Miyaji *et al*. pointed out by using an agar implantation method that the conversion from a mycelial-to-yeast form occurred through chlamydospores[9] and this view was also previously hinted at by Salazar *et al*.[18].

In this experiment the authors have used three different culture media (PDA, SDA and BHIDA) to answer the question of nutritional influence. When SDA or BHIDA was used and the slide cultures were incubated at 25°C, the hyphae were producing chlamydospores. When BHIDA was used a particularly dominant formation of chlamydospores was observed; obviously the nutrients of animal origin and 1% dextrose present in BHIDA influenced this type of development. Villar *et al*. also demonstrated that a variant of *P. brasiliensis* grew in its yeast form even at room temperature in the presence of fetal calf serum[19], while, when PDA was used only aleurioconidia were produced. This result indicated that aleurioconidia grew in a medium containing nutrients of plant origin and a longer culture period produced more of it. This also implied that aleurioconidium could be the predominant and resistant form under unfavorable environmental conditions, as when the growth medium was incorporated with nutrients of animal origin and the incubation temperature was raised at the range of 35°C～37°C, a high frequency of chlamydospore formation was observed. Tanaka of this laboratory in 2001 found that some fresh isolates from armadillo grew as cerebriform colonies on PDA slants at room temperature which predominantly consisted of chlamydospores[12]. This finding also strongly supports the view that chlamydospores may be the natural form of *P. brasiliensis*.

Finally, the current authors would like to refer to the process of infectivity of this fungus in humans and animals. The fungus, *P. brasiliensis* may be found in both enriched and poor soil. It may take a mycelial form with a predominance of chlamydospores in a nutrient-rich soil, whereas, in a soil poor in nutrient content, mycelia with aleuriospores would be found. Deforestation and agricultural intervention may have changed the composition of a soil from a poor to a rich one and thus enhanced the formation of chlamydospores; this could be the reason the tribal laborers at a coffee plantation in Brazil reportedly suffered from paracoccidioidomycosis[20]. During winter the fungi may remain in a dormant stage, while with the advent of spring and the rise of the climatic temperature, the fungi start extending hyphae and producing chlamydospores. It is at this time that the farmers also usually begin their cultivation; as a result, they run the risk of inhaling short hyphal fragments containing chlamydospores or may accidentally contaminate a pre-existing wound and are thereby inflicted with paracoccidioidomycosis[21].

The findings of this study conclude that the mycelial forms of *P. brasiliensis* convert to its yeast forms *via* chlamydospores, and that this conversion process is dependent on both thermal and nutritional factors.

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