

IN VITRO ACTION OF SOME DISINFECTANTS ON *Paracoccidioides brasiliensis* YEAST FORMS

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SUMMARY

The fungicidal action of sodium hypochlorite (0.3, 1, 2.5, 5 and 10%); formaldehyde (2, 5, and 10%); and ethyl alcohol (70%) on yeast forms of *Paracoccidioides brasiliensis* Pb 18 and a newly-isolated Goiana strain was described. Contact between the fungus and the disinfectants was maintained for 1, 2, 24, 48 and 72 hours at room temperature. Viability was evaluated by the fluorescein diacetate-ethidium bromide treatment, culture in solid and liquid media (36°C and 26°C); yeast to mycelial germination at room temperature; and radiometric study of metabolic activity. All concentrations of disinfectants were found to be effective in inactivating Pb 18 and Goiana strains, except for the 1-hour contact with 2% formaldehyde, in which fluorescein diacetate-ethidium bromide treatment was found to reveal 40 and 27% of viable cells, respectively. The yeast to mycelial germination method was considered to reveal faster and similar results as compared to culture in solid and liquid media.

KEY WORDS: *Paracoccidioides brasiliensis*; Disinfectants; Fluorescein diacetate-ethidium bromide; Yeast to mycelial germination; Radiometric method; Sodium hypochlorite.

INTRODUCTION

The "in vitro" action of some disinfectants on *P. brasiliensis* has been described since 1938. The activity of lugol, thimerosal and iodine containing compounds and acetone, toluene, ether

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on yeast forms of this fungus was documented respectively by ALMEIDA¹, ALMEIDA et al² and LACAZ et al⁸. In 1946, SILVA & LACAZ¹¹ also reported the action of ether on filamentous forms of the same fungus.

Formaldehyde was commonly used in laboratories at least during 18 hours in order to inactivate *P. brasiliensis*, sometimes causing side effects, mainly to the eyes and upper respiratory pathways. Furthermore, since 1984, sodium hypochlorite has been successfully used for HIV virus inactivation¹².

In two study, we determined the fungicidal effect of sodium hypochlorite on *P. brasiliensis*, established its time of action and effective concentrations and compared its effect with that of the other disinfectants.

MATERIALS AND METHODS

Sodium hypochlorite (Polyfarma), previously titrated at the São Paulo "Adolfo Lutz" Institute (8.5% of active CI) at concentrations of 0.3, 1, 2.5, 5 and 10%; formaldehyde (Merck at 37%, aqueous solution) at concentrations of 2, 5 and 10%; and 70% ethyl alcohol, prepared from absolute ethanol (Merck, P. A.) were tested. An identical volume of soy broth (Tryptic Soy Broth) was used as control medium.

Yeast form suspensions of strain Pb 18 and a virulent strain toward mice, kindly supplied by Mara S. Carvalhaes (Federal University of Goiânia) cultured at 36°C for 7 days, in Fava Netto medium⁶ were used. Two ml suspensions, containing 5×10^6 fungi/ml, suspended in phosphate-buffered saline, with a viability above 85% by fluorescein diacetate-ethidium bromide treatment⁴, were centrifuged at 550g for 10'. The supernatant was discarded and the fungi were resuspended in 2 ml of different disinfectant concentrations.

The cell suspensions were incubated for 1, 2, 24, 48 and 72 hours, at room temperature, in duplicates. Controls were incubated with soy broth. Afterwards the suspensions were centrifuged and washed 3 times in phosphate-buffered saline (PBS). The cells were then distributed in triplicate for radioisotopic study and in dupli-

cate for: 1) cultures in solid and liquid media; 2) yeast to mycelial germination; 3) staining with fluorescein diacetate-ethidium bromide. Non fixed samples, obtained from pellets after centrifugation at 550 g for 10', were visualized by light microscopy, on slides. For electron microscopy observations, pellets obtained by 9500 g centrifugation for 30', were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hours, and immediately postfixed in 1% OsO₄ in the same buffer; treated by 0.5% aqueous uranyl acetate solution overnight; dehydrated in a graded ethanol series and embedded in araldite. Silver sections were then examined with an electron microscope.

Viability methods

Cultures were maintained at 36 and 26°C in solid and liquid media; such as Sabouraud agar, solid; NEGRONI⁹ and BHI, liquid, for 30 days.

The radiometric detection of *P. brasiliensis* metabolic activity was carried out according to CAMARGO et al⁵, with slight alterations. In summary, the system used for detection of ¹⁴CO₂ consisted of 15 ml of aerobic 6 B medium in a 25-ml flask containing 2.0 uCi of ¹⁴C substrates (Johnston Laboratories), and 0.5 ml of fungal suspension containing 10⁸ *P. brasiliensis* yeast forms incubated with disinfectants. Controls consisted of fungi resuspended in media without disinfectants. Flasks containing autoclaved fungi were used to determine baseline uptake. Flasks were incubated at 35°C and fungal metabolism was measured weekly in Bactec 301 B apparatus, (Johnston Laboratories), with logarithmic scale up to 1000 index units = 25 nanocuries of activity ¹⁴C. Results were expressed in index units, with baseline readings with autoclaved fungi ranging from 2 to 6 index units. After 2 days incubation, ¹⁴CO₂ production was fairly constant, representing about 40 index units per day up to 20 days. Then, it fell off to reach baseline levels by 50 days. Readings above 20 units were regarded as positive for growth.

The yeast to mycelial germination in vitro was performed according to GOIHMAN-YAHR et al⁷, with the following alteration: soy broth was used for maintaining cultures in tubes at room temperature for 48 hours. Two readings

were performed, the first immediately after disinfectant treatment, the second after 48 h at room temperature incubation. A non treated control sample was also included. At least 200 *P. brasiliensis* cells were counted, for scoring percentages of pseudomycelium, yeast cells and budding forms. All tests were made in duplicates. The rise in hyphae counts after room temperature incubation was a clear indication of fungal viability.

RESULTS

Considering the viability of fungal suspensions treated by disinfectants, there was agree-

ment among methods used in all observations (table 1), except for fluorescein diacetate-ethidium bromide staining in suspensions treated with 2% formaldehyde for 1 hour. After this treatment, nearly 30% of forms were regarded as viable, while simultaneously no viability was obtained by culture, yeast to mycelial germination and radiometric methods.

The yeast to mycelial germination study revealed an absence of hyphae in all disinfectant-treated samples. Nevertheless, in controls, hyphae percentages ranging from 30.2% ± 14.0 to 41.3% ± 10.9 (media ± standard deviation) were observed after 48-hour incubation of fungi at room

TABLE 1
Viability of *Paracoccidioides brasiliensis* (Pb 18) after 1, 2, 24 and 48 hours of exposure to sodium hypochlorite, formaldehyde and ethyl alcohol.

Disinfectant	Concentration	Period of treatment																											
		1 hour				2 hours				24 hours				48 hours															
		C	MT	R	Br	C	MT	R	Br	C	MT	R	Br	C	MT	R	Br												
Sodium hypochlorite	10.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0												
	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0												
	2.5	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0												
	1.0	0	3.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0												
	0.3	0	1.7	0	0	0	0	0	0	0	1.1	0	0	0	1.8	0	0												
Formaldehyde	10.0	0	1.1	0	1.5	—	—	—	—	0	2.0	0	0	0	0.5	0	0												
	5.0	0	2.2	0	2.0	—	—	—	—	0	0.9	0	0	0	0.7	0	0												
	2.0	0	2.8	0	40.0	0	1.0	—	—	0	0.7	0	0	0	0.4	0	0												
Ethyl Alcohol	70.0	0	0.7	0	0	—	—	—	—	0	0	0	0	0	0.3	0	0												
Control	—	34.8				82.5				41.3				30.2				81.3				41.3				88.7			
		± 8.5				± 5.0				± 8.0				± 14.0				± 9.5				± 11.0				± 4.2			

C: Culture in liquid and solid media (0 = no growth)

MT: Mycelial transformation in hyphae %. In control (soy broth): mean (4 experiments in duplicate) of hyphae % ± standard deviation.

R: Radiometric method in index units in relation to control.

Br: Fluorescein diacetate-ethidium bromide treatment (viable forms % in at least 200 cells counted: mean ± standard deviation).

—: not done.

temperature. No differences were observed between treated and untreated materials, as regards percentages of fungal forms other than hyphae (pseudomycelium, yeast cells and budding forms). In the highest sodium hypochlorite concentrations (5 and 10%) a significant decline in cell number was observed, probably by disinfectant action, promoting alterations, as shown in figure 2c, which made cell sedimentation difficult, even after centrifugation for electron microscope study.

Figure 1 illustrates morphological alterations visualized by light microscopy, after different periods of exposure to disinfectants.

The electron microscope study of *P. brasiliensis* (Pb18) control suspensions revealed numerous low-electronic-density ellipsoidal or round vacuoles, as well as granular material, which, in high magnification, is mainly represented by ribosomes and round profiles, possibly of nuclei (Fig. 2a). Fig. 2b shows a *P. brasiliensis* cell treated with 5% formaldehyde aqueous solution for 48 hours. Under such conditions, the preservation of submicroscopic structures was unsatisfactory probably due to employment of a commercial formaldehyde solution. The protoplast presents several low-electronic-density and variable-size vesicular profiles. The preservation of granular material was not satisfactory. As shown in Fig. 2c *P. brasiliensis* (Pb18) sample treated with 2.5% sodium hypochlorite for 48 hours, exhibited a ghost like appearance due to the extraction of protoplast and partially of its cell wall material. No cytoplasm or nuclear remains are noticed. Shadows are visible, without any remains of cytoplasm or nucleus.

DISCUSSION

In this study, there was an obvious similarity among the results obtained by different viability methods for *P. brasiliensis* yeast form suspensions. In 1982, RESTREPO et al¹⁰ also established a good correlation between diacetate fluorescein-ethidium bromide and yeast to mycelial germination methods, both requiring less time than colony-forming-units. It must be stressed that the diacetate-fluorescein ethidium bromide test is the fastest method for such assessment. However, in one instance, it did not prove to

be comparable with the other methods employed. Viability of 40% and 27% was observed for strains 18 and Goiana, respectively, through 2% formaldehyde treatment for 1 hour, in addition to an absence of growth by other methods. We might surmise that although alterations caused by such contact did not make fungi entirely non-viable, such alterations prevented them from growing at room temperature or at 36°C (negative results for culture, yeast to mycelial germination and radiometric method).

Yeast to mycelial germination can be demonstrated in this study to yield rapid results (approximately 48 hours). Its applicability in investigating *P. brasiliensis* viability of patients under treatment remains to be determined.

The data observed in this study lead us to infer that sodium hypochlorite is a substance capable of ensuring rapid and safe disinfection, in concentrations of 0.3 to 1%.

RESUMO

Ação "in vitro" de alguns desinfetantes sobre formas leveduriformes de *Paracoccidioides brasiliensis*.

Descreveu-se a ação fungicida do hipoclorito de sódio (0,3; 1; 2,5; 5 e 10%); do formaldeído (em solução aquosa a 2,5 e 10%); e álcool etílico a 70,0% sobre formas leveduriformes de 2 cepas de *Paracoccidioides brasiliensis*: Pb 18 e cepa Goiana, recentemente isolada. A incubação do fungo e desinfetantes foi realizada à temperatura ambiente por períodos de 1, 2, 24, 48 e 72 horas. A viabilidade foi avaliada pelo tratamento com diacetato de fluoresceína-brometo de etídeo; pela cultura em meios sólidos e líquidos a 36°C e 26°C; transformação de levedura em micélio à temperatura ambiente; e estudo radiométrico da atividade metabólica. Todas as concentrações de todos os desinfetantes estudados foram capazes de inativar ambas as cepas, exceto na incubação com formaldeído a 2% por 1 hora, em que o tratamento por diacetato de fluoresceína-brometo de etídeo revelou 40% e 27% de células viáveis, respectivamente, para a cepa Pb 18 e Goiana. A transformação de levedura em micélio foi considerada um método rápido, com resultados semelhantes ao cultivo em meios sólidos e líquidos.

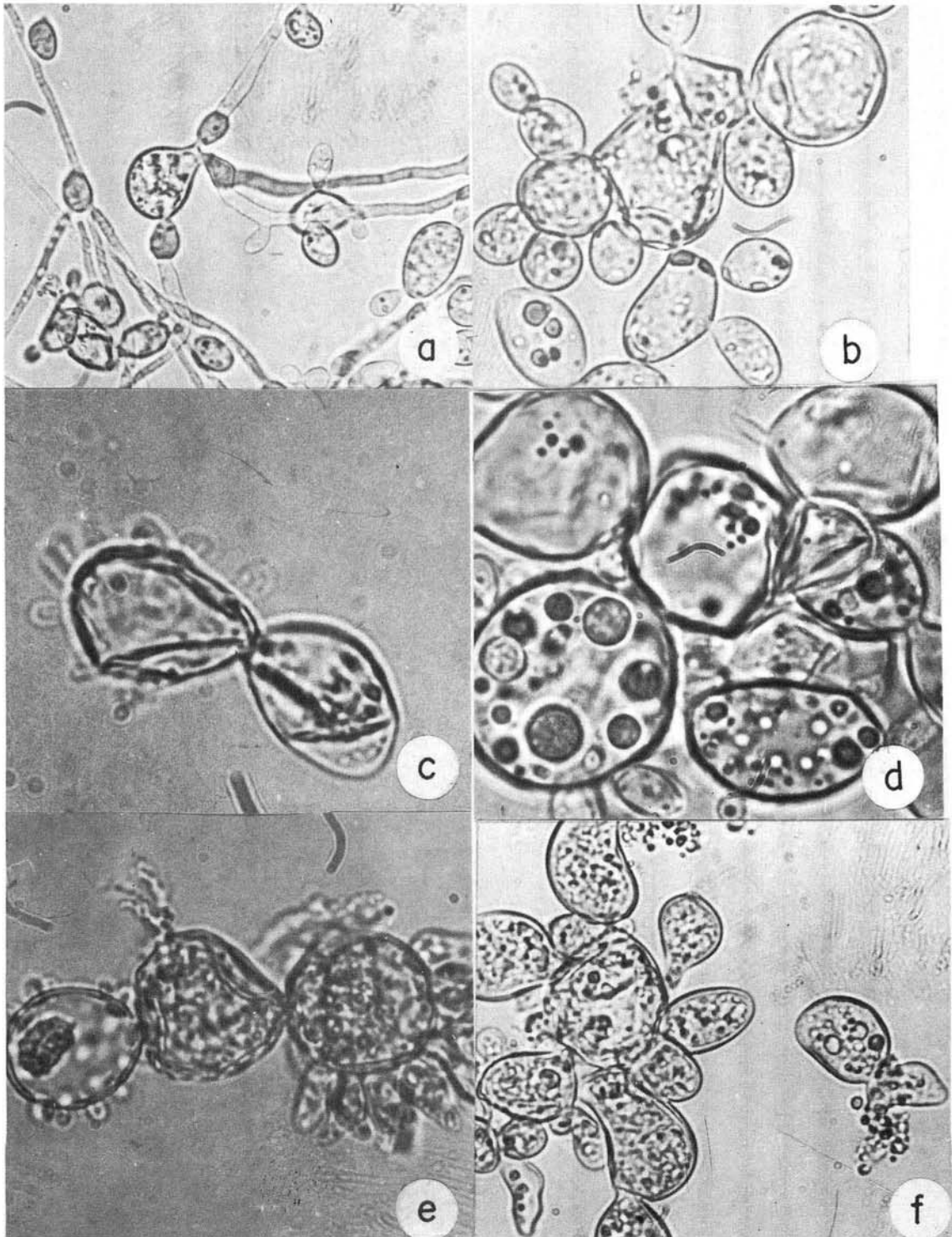


Fig. 1 *P. brasiliensis* yeast forms after disinfectants action: a) Untreated control cells incubated for 24 hours with phosphate buffered saline, showing yeast to micelial germination after 48 hours at room temperature; b) 1% sodium hypochlorite for 24 hours (X 250), c) 0,3% sodium hypochlorite for 24 hours (X 350); d) 0,3% sodium hypochlorite for 48 hours (X 500); e) 70% ethyl alcohol for 48 hours (X 200); f) 2% formaldehyde for 48 hours (X 160).

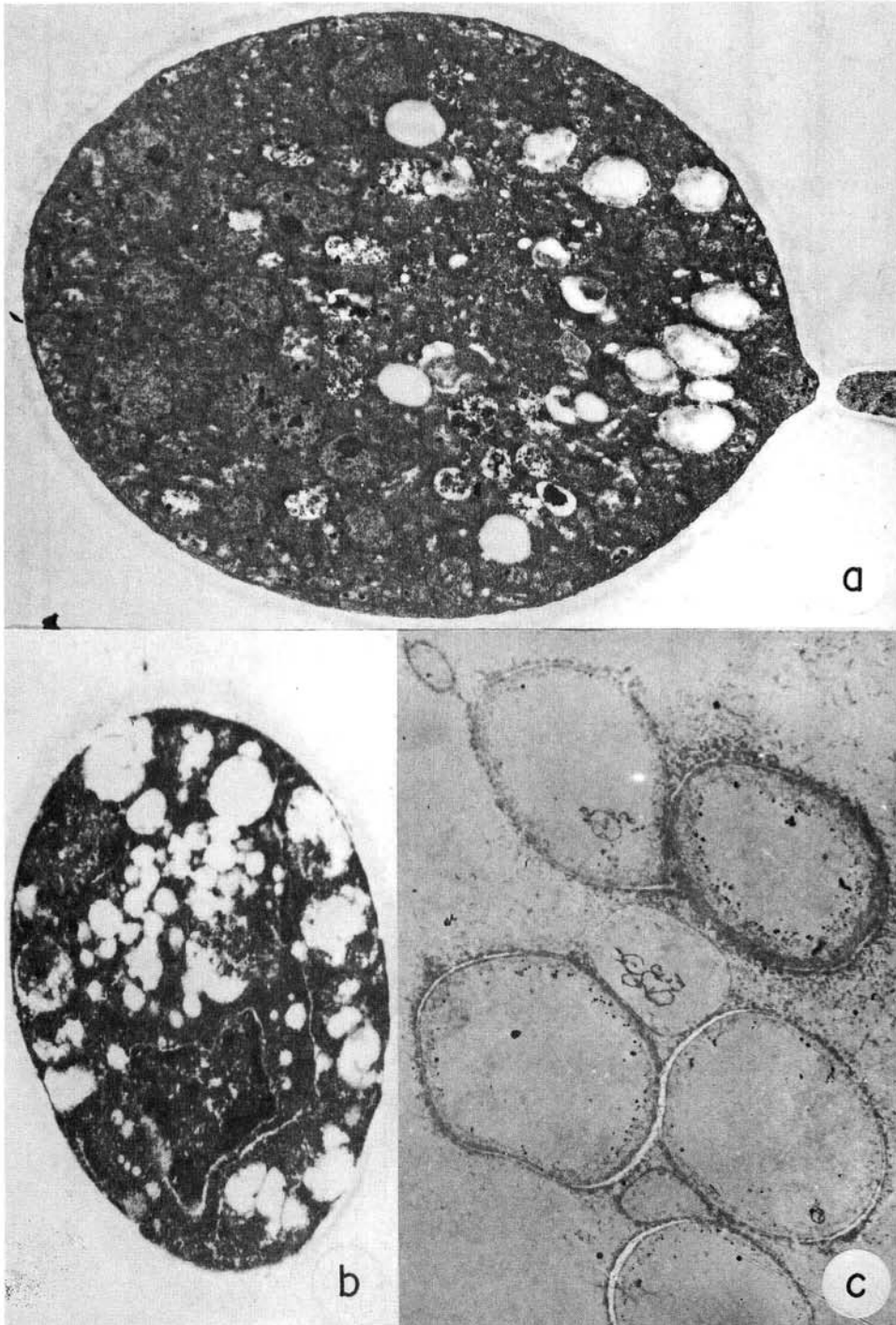


Fig. 2 — Electron microscopy of *Paracoccidioides brasiliensis* yeast forms after disinfectants action: a) untreated control (X 7.300); b) 5% formaldehyde for 48 hours (X 9.500); c) 2% sodium hypochloride for 48 hours (X 6.100).

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