Extracellular enzymatic activity and serotype of *Cryptococcus neoformans* strains isolated from AIDS patients in Brazil

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Summary

One hundred and fifty-one *Cryptococcus neoformans* strains isolated from AIDS patients in Brazil and maintained in the Adolfo Lutz Institute (São Paulo, Brazil) were tested for phospholipase, protease and other extracellular enzymatic activities and their serotypes determined. Production of extracellular phospholipase and protease was tested by the agar plate methods. Determination of extracellular enzyme profile of the strains was performed by using the API-ZYM kit system, which can test 19 different enzymes. The serotypes were determined by cell agglutination using the Crypto-check method. Among the 151 strains, 147 were indentified as serotype A and four strains were serotype AD. Production of extracellular phospholipase and protease was extensive and observable at early stages of incubation. All of the tested strains were positive for the production of both enzymes. In the API-ZYM tests, more than 90 % of the 151 tested strains were positive for esterase C4 (No. 3), esterase lipase C8 (No. 4), leucine arylamidase (No. 6), phosphatase acid (No. 11), naphtol-AS-BI-phosphohydrolase (No. 12), α-glucosidase (No. 16) and ß-glucosidase (No. 17). Differences in enzymatic activities between the Brazilian strains and strains isolated in other countries were observed. The phospholipase, protease and other enzyme activities may play a role in host tissue invasion by *C. neoformans*.

Key words *Cryptococcus neoformans*, Enzymatic activity, Virulence factors, Serotype

Actividad enzimática extracelular y serotipo en cepas de *Cryptococcus neoformans* de pacientes con sida en Brasil

Resumen

Se estudiaron las actividades fosfolipasa, proteasa y otras actividades enzimáticas extracelulares de 151 cepas de *Cryptococcus neoformans* aisladas de pacientes con sida y mantenidas en el Instituto Adolfo Lutz (São Paulo, Brasil). La actividad enzimática extracelular se determinó por medio del test API-ZYM, que evalúa 19 enzimas extracelulares, y el serotipo mediante aglutinación celular (Crypto-check, Patron, Japón). De las 151 cepas de *C. neoformans* ensayadas por aglutinación 147 resultaron identificadas como serotipo A y solamente cuatro como serotipo AD. La producción de fosfolipasa y proteasa resultó muy abundante, sobre todo en los primeros días de incubación. Es importante añadir que todas las cepas presentaron actividades fosfolipasa y proteasa. Por medio del test API-ZYM más del 90% de las cepas de *C. neoformans* resultaron positivas para los siguientes enzimas: esterasa C4, esterasa-lipasa C8, leucina-arilamidasa, fosfatasa acida, naftol-AS-BI-fosfohidrolasa, α-glucosidasa y ß-glucosidasa. Se han observado diferencias entre las actividades enzimàticas de las cepas brasileñas y las aisladas en otras naciones. Las actividades fosfolipasa, proteasa y de otros enzimas extracelulares pueden facilitar la invasión de *C. neoformans* en el tejido del huésped.

Palabras clave *Cryptococcus neoformans*, Actividad enzimática, Factores de virulencia, Serotipo

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Cryptococcus neoformans is a basidiomycetous yeast-like fungus with world-wide distribution [12,21,29]. Its inhalation from an environmental source may cause pulmonary and neurological disease in humans and in animals. In healthy individuals, *C. neoformans* infections are rarely reported, but in immunocompromised patients the fungus causes life-threating infections [1,4,15,20,22]. In these patients, in particular in AIDS patients, the incidence of cryptococcosis (mainly cryptococcal meningitis) has markedly increased, as reported in the most recently literature [1,4,7,15,16,20,36]. However, it is evident in late 1990s that the Highly Active Antiretrovial Therapy (HAART) has resulted in a decrease in the incidence of opportunistic infections, including cryptococcosis, in HIV-infected patients [30,31]. In Brazil, despite the existence of the HAART and other advanced therapies, it has been observed stable morbidity and lethality rates associated with AIDS and cryptococcosis continue to be a prevalent disease [24]. It is important to know serotypes and extracellular enzyme production in relation to epidemiology and virulence of *C. neoformans* [2,8-11,17,19,25,28,33-35]. In the present work the virulence of 151 Brazilian AIDS strains, in addition to serotype determination, has been evaluated by monitoring their extracellular enzymatic activities.

Materials and methods

Fungal strains. One hundred and fifty-one *C. neoformans* strains from the culture collection of Adolfo Lutz Institute (São Paulo, Brazil), isolated from AIDS patients, were transferred onto fresh malt agar slants and incubated at 25 °C. After five days, the strains were tested for production of phospholipase and protease and for their extracellular enzymatic activity.

Serotype determination. Fresh cells of each strain grown on malt agar at 30 °C for two days were tested by the cell agglutination test with a Crypto-check kit (Iatron, Japan).

Phospholipase production. Determination of phospholipase production was performed by using egg-yolk agar plates according to Polak [26]. In this method, egg-yolk digested by phospholipase produces precipitation around colonies. Ten microliters of a thick suspension of each strain was placed in the center of the egg-yolk agar plate (9-cm diameter Petri dishes). After incubation at 37 °C for four, six and eight days, the colony diameter (*a*) and the diameter of colony plus precipitation zone (*b*) were measured. The phospholipase activity was expressed as Pz value (*a*/*b*) as described by Price et al. [27]. The Pz value of three different samples of each strain was measured to obtain the average value. According to this definition, low Pz values mean high phospholipase production and, inversely, high Pz values indicate low production. A total of 151 strains were grouped into one of the following three classes: high Pz group between 1 and 0.700 (+); moderate Pz group between 0.699 and 0.400; low Pz group between 0.399 and 0.100 (+++).

Protease production. Determination of protease production was performed by using agar plates containing bovine serum albumin (BSA) according to Aoki et al. [2]. Ten microliters of a thick suspension of each strain were placed in the center of four BSA agar plates (9-cm diameter Petri dishes). After incubation at 37 °C for two, three, five and eight days, the dishes were stained with Coomassie Brilliant Blue G-250 (CBB). Protease secreted by fungal cells produces a CBB-unstained zone around colonies.

The colony diameter (*a*) and the diameter of colony plus unstained zone (*b*) were measured. As in the phospholipase activity, the ratio (Pz=*b*/*a*) of each sample was calculated. The Pz value of three different samples of each strain was measured to obtain the average value. A total of 151 strains were grouped into the following three classes: high Pz group between 1 and 0.700 (+); moderate Pz group between 0.699 and 0.400; low Pz group between 0.399 and 0.100 (+++).

Extracellular enzymatic profile. After five days of incubation on malt extract agar slants at 25 °C, a loopful of cells was inoculated into 200 ml of 2% malt extract broth and shaken on a giratory shaker at 120 rpm for 24 h at 28 °C until the culture reached early log phase of growth. The cells were washed three times by centrifugation in the same sterile malt broth and their enzymatic activity was then tested by using the semi-quantitative API-ZYM system according to the manufacturer's instructions. For this purpose, the washed cells of each *C. neoformans* strain were suspended in physiologic saline (0.9% sodium chloride) to reach an optical density (OD) of 0.1 at 550 nm. Sixty five microliters of each inoculum was dispensed into each well of the 20 API-ZYM strip microtubes and incubateed at 37 °C for 4 h. After this incubation period, a drop of ZYM A and ZYM B reagents was added. The color reaction was read after 5 min according to the API-ZYM reading color-scale, which ranges from 0 (negative reaction) to 5 nmols (maximum positive reaction), i.e. 1 corresponds to 5 nmols, 2 to 10 nmols, 3 to 20 nmols, 4 to 30 nmols and 5 to 40 nmols or more of each API-ZYM substrate metabolized by the strains.

Results

Serotype. Among the 151 *C. neoformans* strains tested, 147 were serotype A (var. *grubii*) and four strains were found to be *C. neoformans* serotype AD (Table 1). There were no *C. neoformans* serotype D strains and no var. *gattii* strains (serotypes B and C).

Table 1. Serotypes of 151 *Cryptococcus neoformans* strains isolated from AIDS patients.

Serotype	No. of strains (%)
A	147 (97.4)
AD	4 (2.6)

Phospholipase activity. Production of phospholipase was very well observed after four days of incubation at 37 °C and the Pz ranged between 0.930 and 0.280 on day 8 of incubation. The majority of the strains showed high (103/151) (+) or moderate (47/151) (++) Pz after four days. Only one strain (1/151) showed a very low Pz (+++). The average Pz of the strains was 0.716 after four days and didn't change from day 8 of the experiment (Table 2).

After six days of incubation, the majority of the strains (97 out of 151) showed moderate Pz (++), while 53 showed high Pz (+) and only one very low Pz (+++) (Table 2). The average Pz was 0.650 at day 6. Similar results were obtained after eight days of incubation. The 151 strains were grouped in 30 with high Pz (+), 121 with moderate Pz (++) and none with low Pz (+++). The Pz was gradually decreased from 0.716 on day 4 to 0.608 on day 8 of incubation (Table 2).

Protease activity. Protease activity, in contrast to phospholipase activity, was higher and was observed earlier, being detectable after two days of incubation at 37 °C (data not reported). After three days, 67 strains showed moderate Pz (++) and 84 very low Pz (+++). No strain showed high Pz (+) (Table 3).

After five days of incubation, most strains (114/151) showed very low Pz (+++), 36 moderate Pz (++) and only one strain had a high Pz (+). Similar results were obtained after eight days of incubation. No strain was grouped in the high Pz (+) with low protease activity. Twenty-nine and 122 strains were grouped in the moderate Pz (++) and the low Pz (+++), respectively. The average Pz was decreased gradually from 0.39 after three days to 0.33 after eight days of incubation and did not change between days 5 and 8 (Table 3).

Extracellular enzyme profile by API-ZYM. Enzymatic profiles of the 151 strains are shown in table 4. The enzymes were roughly grouped into four classes according to the positive percentage among the strains.

In the first class, where the positive percentage was more than 90%, the following enzymes were included: esterase C4 (No. 3), esterase lipase C8 (No. 4), leucine arylamidase (No. 6), phosphatase acid (No. 11), naphtol-AS-BI-phosphohydrolase (No. 12), α-glucosidase (No. 16), and ß-glucosidase (No. 17). In particular, phosphatase acid (No. 11) was found in all the strains tested. In the second class, with a positive percentage around 50%, only ß-glucuronidase (No. 15) was included. In the third class, in which the positive percentage was less than 50%, phosphatase alcaline (No. 2) was found. In the fourth class, no positive activity resulted for the enzymes numbered 5, 7-10, 13, 14, 18, 19 and 20, as seen in table 4.

Table 2. Phospholipase production in 151 *Cryptococcus neoformans* strains grouped into three Pz classes.

Pz class	Phospholipase production	No. of strains		
		Day 4	Day 6	Day 8
1.000-0.700	+	103	53	30
0.699-0.400	++	47	97	121
0.399-0.000	+++	1	1	0
Average Pz		0.716	0.650	0.608

Table 3. Protease production in 151 *Cryptococcus neoformans* strains grouped into three Pz classes.

Pz class	Protease production	No. of strains		
		Day 3	Day 5	Day 8
1.000-0.700	+	0	1	0
0.699-0.400	++	67	36	29
0.399-0.000	+++	84	114	122
Average Pz		0.39	0.35	0.33

Table 4. API-ZYM profile of 151 *Cryptococcus neoformans* strains.

No.*	Enzyme name	No. of positive strains (%)	Average color scale
2	Phosphatase alcaline	18 (17.9)	1.77
3	Esterase (C4)	148 (98.0)	2.08
4	Esterase Lipase (C8)	148 (98.0)	2.55
5	Lipase (C14)	0 (0)	-
6	Leucine arylamidase	144 (95.4)	2.79
7	Valine arylamidase	0 (0)	-
8	Cystine arylamidase	0 (0)	-
9	Trypsine	0 (0)	-
10	Chymotripsine	0 (0)	-
11	Phosphatase acide	151 (100)	4.91
12	Naphtol-AS-BI-phosphohydrolase	141 (93.4)	4.13
13	α-galactosidase	0 (0)	-
14	ß-galactosidase	0 (0)	-
15	ß-glucuronidase	79 (52.3)	2.29
16	α-glucosidase	138 (91.4)	2.74
17	ß-glucosidase	149 (98.7)	3.70
18	N-acetyl-ß-glucosaminidase	0 (0)	-
19	α-mannosidase	0 (0)	-
20	α- fucosidase	0 (0)	

*No. 1 is for control and does not contain a substrate.

The intensity of the positive enzymes tested by the API-ZYM color reaction scale ranged from 1 to 5 nmols. The average values from the 151 strains tested ranged from 1.77 to 4.91 (Table 4). A high activity with the color scale 4-5 was distributed in phophatase acid (No. 11) and naphtol-AS-BI-phosphohydrolase (No. 12). Moderate activity was observed in ß-glucosidase.

Discussion

C. neoformans var. *grubii* serotype A strains are distributed throughout the world and this is the most frequent serotype isolated from AIDS patients even in tropical and subtropical regions where *C. neoformans* var. *gattii* (serotypes B and C) is also possibly endemic [5,6,13,14,20,21]. Ohkusu et al. [23] reported that 60 out of 61 strains isolated from AIDS patients in São Paulo were *C. neoformans* var. *grubii* serotype A and only one strain of *C. neoformans* var. *gattii* was serotype B. No *C. neoformans* var. *neoformans* serotype D was found. Among 23 strains isolated from non AIDS patients, 15 were *C. neoformans* var. *grubii* serotype A, the remaining eight were *C. neoformans* var. *gattii* all serotype B. In the present study, 147 of the 151 isolates belonged to serotype A and the remaining four were serotype AD. No strains of *C. neoformans* var. *gattii* (serotypes B or C) were found. These serotype distribution patterns in Brazilian (São Paulo State) strains are very similar to those obtained from AIDS patients in Thailand [18], but are in contrast to isolates from HIV-positive patients in Italy where serotype D is predominat (72.4%), followed by serotype A (23.8%) and AD (6.6%) [32]. Recently, Bertout et al. [3] showed that serotypes A, D and AD are genetically close by using the multilocus enzyme electrophoresis (MLEE) typing method and that *C. neoformans* strains readily switch from serotype A to D via the intermediate serotype A/D. It is of interest to reveal the ploidy and mating type of the four serotype AD strains found the present study. All of the 151 strains tested in this work produced extracellular phospholipase and protease, common virulence features within pathogenic yeasts [2,7-11,25,26,34]. As known in *Candida albicans*, these enzymes are considered to contribute to virulence of *C. neoformans* since the enzymes can destroy host tissue and help fungal invasion. In addition to protease [2,5,7,20] and phospholipase production [8,9,25,28,35] in *C. neoformans*, detection of other extracellular enzymes in *C. neoformans* by the API-ZYM method provides differences in enzymatic activities in strains sampled from different sources and different regions [10,19,33]. For example, enzymes no. 4 (lipase), 7 (valine arylamidase), 14 (ß-galactosidase) and 18 (N-acetyl-ß-glucosaminidase) were negative in Brazilian AIDS strains in the present study, while one or two strains among 19 Italian AIDS strains were positive. Moreover, the different enzymatic patterns and activities among strains from different origins may be useful tools for epidemiological studies and for evaluation of possible different virulence among the strains [10,11,19,33]. Nevertheless, more extensive analysis would be needed to support this statement. In fungal infection, disruption of host cell membranes and subsequent penetration are the first essential steps. The extracellular enzymes are considered to participate with important roles in such fungal invasive process [3,9,27,34]. This problem should be solved in further studies.

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