Original

Extracellular enzymatic activities in *Cryptococcus neoformans* strains isolated from AIDS patients in different countries

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Summary

Three hundred and ten *Cryptococcus neoformans* strains isolated from AIDS patients in five different countries (151 from Brazil, 23 from Italy, 28 from Spain, 104 from Thailand and four from Turkey) were tested by the API-ZYM kit to detect their extracellular enzymatic activity. The enzymes esterase (C4) (n°3), esterase lipase (C8) (n°4), leucine arylamidase (n°6) and acid phosphatase (n°11) were commonly positive in most of the strains (more than 95%). These enzymes could be considered a useful tool not only for *C. neoformans* identification, but in particular for their possible relationship to new *C. neoformans* virulence factors and also for epidemiological research. Interestingly, it is also the high positive percentage of α-glucosidase and ß-glucosidase detected in all isolates. The serotype A was the most predominant serotype in all countries, except for Italy where the serotype D was predominant. Further studies are needed to draw a clear correlation between the API-ZYM profile and serotype.

Key words *Cryptococcus neoformans,* AIDS, Enzymatic activities, Serotypes, Virulence

Actividad enzimática extracelular en *Cryptococcus neoformans* en diferentes países

Resumen

Trescientas diez cepas de *Cryptococcus neoformans* aisladas de pacientes con sida de cinco países (151 de Brasil, 23 de Italia, 28 de España, 104 de Tailandia y cuatro de Turquía) fueron analizadas con el test API-ZYM para detectar su actividad enzimática extracelular. Las enzimas esterasa (C4) (n°3), esterasa lipasa (C8) (n°4), leucina arilamidasa (n°6) y fosfatasa ácida (n°11) resultaron positivas en la mayoría de las cepas (más del 95%). Estas enzimas podrían considerarse como una herramienta útil, no sólo para la identificación de *C. neoformans*, sino también estudiar factores de virulencia y realizar estudios epidemiológicos. Es también interesante el alto porcentaje de cepas positivas a la α- y ß-glucosidasa presente en todos los países. El serotipo A fue el más frecuente en todos los países, excepto en Italia, donde el serotipo D fue predominante. Se necesitan más estudios para establecer una clara correlación entre el perfil API-ZYM y el serotipo de *C. neoformans*.

Palabras clave *Cryptococcus neoformans,* Actividad enzimática, Serotipos, Sida, Virulencia

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Cryptococcus neoformans is a basidiomycetous yeast with world wide distribution [5,14]. Its inhalation from environmental source may cause pulmonary and neurological disease in susceptible human and animals. *C. neoformans* infections are rarely reported in immunocompetent hosts, but in immunocompromised patients the fungus could cause life-threatening infections [5]. In these patients, the incidence of cryptococcosis (mainly cryptococcal meningitis) has markedly increased, as recently reported [5,14]. However, it was evident in late 1990s that the Highly Active Antiretroviral Therapy (HAART) resulted in a decrease in the incidence of opportunistic infections, including cryptococcosis, in HIV infected patients [12,15,16]. It is important to know serotypes and extracellular enzymes production in relation to epidemiology and virulence of *C. neoformans* [2,6-10,18].

In the present study, *C. neoformans* isolated from five different countries were examined in order to elucidate extracellular enzyme profiles and a possible correlation between the profiles and geographical distribution of strains.

Materials and methods

Culture for tests. Three hundred and ten *C. neoformans* strains isolated from AIDS patients in five different countries (151 from Brazil, 23 from Italy, 28 from Spain, 104 from Thailand and four from Turkey) were tested by the API-ZYM kit (BioMérieux SA, France) to detect their extracellular enzymatic activity and by the Crypto-check kit (Iatron Laboratories, Japan) for their serotype determination.

The cells were transferred onto fresh malt agar slants and incubated at 25 °C. After five days of the incubation at 25 °C a loopful (1 x 10^8 cell/ml) of each strain was inoculated into 200 ml of sterile 2% malt extract liquid medium and shaken on a gyratory shaker at 120 rpm for 24 h at 28 °C until the cells reached their early log-phase of growth.

Extracellular enzymatic profile. The cells were then centrifugally washed three times in the same sterile malt liquid medium 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 (0D) of 0.1 at 550 nm. Sixty five microliters of each inoculum was dispensed into each well of the API-ZYM strip microtubes and incubated at 37 °C for 4 h. After incubation, a drop of ZYM A and ZYM B reagents were added. Color intensities were read according to the API-ZYM reading color scale, which ranges from 0 (negative reaction) to 5 (maximum positive reaction); approximately scale 1 corresponds to 5 nmols, 2 to 10 nmols, 3 to 20 nmols, 4 to 30 nmols, 5 to 40 nmols or more of each API-ZYM substrate metabolized by the strains. Each strain was tested three times in triplicate to confirm the results obtained.

Serotype determination. Fresh cells of each *C. neoformans* strain grown on malt agar at 30 °C for two days were tested by the Crypto-check kit (Iatron Laboratories, Japan) cell agglutination test according to the manufacture's instructions.

Statistical analysis. The statistical analysis was according to the comparison between two proportions and to the Kruskal-Wallis tests.

Results

Extracellular enzyme activities. The results of the API-ZYM tests are shown in table 1. All of the strains (95-100%) showed esterase (C4) (n°3), esterase lipase (C8) (n°4), leucine arylamidase (n°6) and phosphatase acid (n°11) activities. Similarly, the positive percentages of naphthol-AS-BI-phosphohydrolase (n°12) and ß-glucosidase (n°17) were very high (87.5-100%) in the strains from Brazil, Thailand, Spain and Turkey, but were lower

Table 1. *Cryptococcus neoformans* enzymatic activities in different countries.

		API-ZYM (positive activity in percentage)					
No	Enzyme	Australia*	Italy	Brazil	Thailand	Spain	Turkey
1	Control						
2	Phosphatase alkaline	100	0	18	0	0	0
3	Esterase (C4)	100	100	98	100	100	100
4	Esterase lipase (C8)	100	95	98	100	100	100
5	Lipase (C14)	100	10	0	0	0	0
6	Leucine arylamidase	100	95	95	100	100	100
7	Valine arylamidase	100	5	0	15,5	0	0
8	Cystine arylamidase	83	0	0	0	0	0
9	Trypsin	8	0	0	0	0	0
10	Chymotrypsin	0	0	0	0	0	0
11	Phosphatase acid	100	80	100	97,9	100	100
12	Phosphoamidase	50	75	93	100	100	100
13	α-galactosidase	0	0	0	2,1	0	0
14	ß-galactosidase	0	10	0	4,2	0	0
15	ß-glucuronidase	83	0	50	2,1	32	25
16	α-glucosidase	100	25	91	89,6	68	75
17	ß-glucosidase	100	60	98	87,5	100	100
18	N-acetyl-ß-glucosaminidase	83	18	0	0	16	0
19	α-mannosidase	41	0	0	0	8	25
20	α-fucosidase	0	0	0	0	0	0

*Data reported in [7].

in the Italian strains (75 and 60% respectively; table 2). The difference was statistically significant among the Brazilian, Thai and Spanish *C. neoformans* strains (P ranging between 0.000 and 0.018), but not with the Turkish strains (P ranging between 0.324 and 0.642 (Table 2). Moderate or high activity of the enzyme α-glucosidase (n°16) was observed in the Brazilian, Thai, Spanish and Turkish *C. neoformans* strains. A low positive percentage of this enzyme resulted only from the Italian strains (25%). The statistical analysis was negative only in the Turkish strains (P = 0.164) and positive for *C. neoformans* strains from the other countries (P ranging between 0.000 and 0.006; table 2). The other enzymes showed low positive percentages in the strains from all the countries.

According to the results published by Chen et al. [8] the esterase (C4) (n°3), esterase lipase (C8) (n°4), leucine arylamidase (n°6) and phosphatase acid (n°11) enzymes showed high activity. This high activity was also observed in the Brazilian, Italian, Spanish, Thai and Turkish *C. neoformans* strains tested in this work. It is not known if the results obtained in this work are reproducible when the same *C. neoformans* strain is tested more than once. Further researches would be performed in this field.

The intensity of the positive enzymes tested by the API-ZYM color scale ranges from 1 to 5. According to the results obtained the highest [4-5] color scale activity was observed for the phosphatase acid (n°11) enzyme, from the Brazilian, Spanish and Turkish *C. neoformans* strains. Good color scale (3.07) activity was also observed from the Brazilian strains for the ß-glucosidase activity (Tables 3 and 4). Other enzymes showed moderate, low or no color reaction scale activity in all the isolates (Table 3).

Serotype determination. The most frequent *C. neoformans* serotype detected by the Iatron Crypto-check kit was the serotype A in all the countries, except from the Italian strains in which D was the most prevalent serotype (Table 5). According to the results obtained by the API-ZYM kit, the high number and percentage between the Brazilian and Thai *C. neoformans* strains, few enzymes [i.e. esterase (C4) (n°3), esterase lipase (C8) (n°4), leucine arylamidase (n°6) and phosphatase acid (n°11)] showed correlation with *C. neoformans* serotype A, the same correlation was observed among the other countries (data not shown). One strain of each serotype C and B were only observed from the Italian isolates. In all the five countries low number of the *C. neoformans* strains showed the AD serotype (Table 5).

Discussion

In fungal infections, disruption of host cell membranes and subsequent penetration are the first essential step to establish an infection. Extracellular enzymes are considered to have pivotal roles in such fungal invasive process. It is known that extracellular enzymes, protease [2,4,7] and phospholipase [6,10,18], can destroy host tissue and help fungal invasion. Furthermore, it is established that there is a relationship of these enzymes with virulence and pathogenicity not only in *Candida albicans* but also in *C. neoformans* and other medically important yeasts [7-8,14,17].

Detection of other extracellular enzymes in *C. neoformans* by the API-ZYM method could be very important to identify new *C. neoformans* virulence factors and its relationship to its pathogenicity. This could be the case for the esterase (C4) (n°3), esterase lipase (C8) (n°4), leucine arylamidase (n°6) and phosphatase acid (n°11) enzymes, since the activity was very high in the majority of the strains from different countries (95-100%). Among these enzymes detected by the API-ZYM kit, the phosphatase acid

Table 2. Comparison between two proportions test among different countries.

		Brazil	Italy	Spain	Thailand	Turkey
	Enzyme No	p	p	p	p	p
Brazil	12	–	0.018	0.018	0.000	0.642
	17	–	0.000	0.000	0.006	0.324
	16	–	0.000	0.006	0.000	0.164

Enzyme n. 12: Naphthol-AS-BI-phosphohydrolase
Enzyme n. 17: ß-glucosidase
Enzyme n. 16: α-glucosidase

Table 3. Kruskal–Wallis test among different countries.

		Brazil	Italy	Spain	Thailand	Turkey
	Enzyme No	p	p	p	p	p
Brazil	11	–	0.000	0.985	0.000	0.828
	17	–	0.000	0.615	0.000	0.354
Italy	11	0.000	–	0.000	0.740	0.000
	17	0.000	–	0.000	0.299	0.037
Spain	11	0.985	0.000	–	0.000	0.831
	17	0.615	0.000	–	0.000	0.521
Thailand	11	0.000	0.740	0.000	–	0.036
	17	0.000	0.299	0.000	–	0.367
Turkey	11	0.828	0.000	0.831	0.036	–
	17	0.354	0.037	0.521	0.367	–

Enzyme n. 11: Phosphatase acid
Enzyme n. 17: ß-glucosidase

Table 4. *Cryptococcus neoformans* enzymatic activity.

	API-ZYM Color Scale (0-5 nmol)				
Enzyme	Italia	Brazil	Thailand	Spain	Turkey
Control					
Phosphatase alkaline	0	1.77	0	0	0
Esterase (C4)	1.6	2.08	1.71	2.48	2.6
Esterase lipase (C8)	0.15	2.55	0.89	1.04	2
Lipase (C14)	1.6	0	0	0	0
Leucine arylamidase	0.05	2.79	0.98	1.84	2
Valine arylamidase	0	0	0.5	0	0
Cystine arylamidase	0	0	0	0	0
Trypsin	0	0	0	0	0
Chymotrypsin	1.95	0	0	0	0
Phosphatase acid	1.35	4.91	1.58	4.92	4.6
Phosphoamidase	0	4.13	1.17	2	1.8
α-galactosidase	0.2	0	1	0	0
ß-galactosidase	0	0	0.75	0	0
ß-glucuronidase	0.75	2.29	0.5	0.48	0.2
α-glucosidase	1.15	2.74	0.91	0.8	0.8
ß-glucosidase	0.1	3.07	0.51	2.76	1.8
N-acetyl-ß-glucosaminidase	0	0	0	0.16	0
α-mannosidase	0	0	0	0.08	0.2
α-fucosidase	0	0	0	0	0

Table 5. Serotype of *C. neoformans* strains isolated from five different countries.

Serotype	Italy (n=23)	Brazil (n=151)	Thailand (n=104)	Spain (n=28)	Turkey (n=4)
A	2 (8)	147 (97)	103 (99)	17 (61)	3 (75)
B	1 (4)	–	–	–	–
C	1 (4)	–	–	–	–
D	18 (80)	–	–	7 (25)	1 (25)
AD	1 (4)	4 (3)	1 (1)	4 (14)	–

Numbers in the parentheses indicate %.

seems to be more important for a possible *C. neoformans* virulence factor, not only because resulted positive in 100 % of the strains in all the five countries considered, but in particular due to its high color scale activity. According to the results obtained, although there are no experimental data in literature, it is also possible to consider these new enzymatic activities in *C. neoformans* as new virulence factors not only in *C. albicans* but also in other medically important yeast or fungi i.e *C. albicans*, *Candida dubliniensis* and *Aspergillus fumigatus*. Further research is needed in this field. In addition, the evaluation of other extracellular enzymes in *C. neoformans* detected by the API-ZYM method could provide differences in enzymatic activities in strains form different sources and countries [6,8], in particular for the α- and ß-glucosidase and naphthol-AS-BI-phosphohydrolase enzymes. The different enzymatic patterns and activity among strains from different countries could be considered a useful tool for *C. neoformans* epidemiological researches. According to the literature [10,13], the enzyme type could be a useful tool for a rapid, simple and inexpensive identification, of *C. neoformans* compared to the molecular technique.

Serotype A strains were predominant among the strains from Brazil, Thailand and Spain, being consistent with results reported previously for clinical isolates from these countries [3,12,15-16]. In contrast, the predominant serotype of Italian strains was D, followed by serotype A. This result is very close to that obtained with *C. neoformans* strains isolated from northern Italy [25]. According to the results in this study, the high number and percentage between the Brazilian and the Thai *C. neoformans* strains, few enzymes [i.e esterase C4 (n°3), esterase lipase C8 (n°4), leucine arylamidase (n°6), and phosphatase acid (n°11)] seemed to have such correlation with the *C. neoformans* serotype A. For this reason, it would be important to examine a possible correlation between the same *C. neoformans* serotype among different countries and the API-ZYM profile. In addition, the API-ZYM profile reported from USA [7] is distinct from those obtained in our study. Several enzymes (phosphatase alkaline, lipase C-14, valine arylamidase, cysteine arylamidase and N-acetyl-ß-glucosaminidase) which are negative or lowly positive in our study are highly positive in strains from USA. These differences may suggest serotype-dependent variations in the API-ZYM pattern. However, the API-ZYM test is a semi-quantitative method to detect the enzymatic activity of yeasts and fungi, for this reason a quantitative method must be applied. Thus, assays under more controlled conditions are required to reveal some correlations among strains of the same serotypes and geographical distribution.

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References

1. Aoki S, Ito-Kuwa S, Nakamura K, Kato J, Ninomiya K, Vidotto V. Extracellular proteolytic activity of *Cryptococcus neoformans*. Mycopathologia 1994; 128: 143-150.
2. Baro T, Torres-Rodriguez JM, Morera Y, Alia C, Lopez O, Mendez R. Serotyping of *Cryptococcus neoformans* isolates from clinical and environmental sources in Spain. J Clin Microbiol 1999; 37: 1170-1172.
3. Buchanan KL, Murphy JW. What makes *Cryptococcus neoformans* a pathogen? Emerg Infect Dis 1998; 4: 71-83.
4. Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington DC, ASM Press, 1998.
5. Chen CA, Wright LC, Santangelo RT, Muller M, Moran VR, Kuchel PW, Sorrell TC. Identification of extracellular phospholipase B, lysophospholipase, and acyltransferase produced by *Cryptococcus neoformans*. Infect Immun 1997; 65: 405-411.
6. Chen LC, Blank ES, Casadevall A. Extracellular proteinase activity in *Cryptococcus neoformans*. Clin Diagn Lab Immunol 1996; 3: 570-574.
7. Chen LC, Pirofski LA, Casadevall A. Extracellular proteins of *Cryptococcus neoformans* and host antibody response. Infect Immun 1997; 65: 2599-2605.
8. Garcia-Martos P, Marin P, Hernandez-Molina JM, Garcia-Agudo L, Aoufi S, Mira J. Extracellular enzymatic activity in 11 *Cryptococcus* species. Mycopathologia 2000; 150: 1-4.
9. Kwong-Chung KJ, Sorrell TC, Dromer F, Fung E, Levitz SM. Cryptococcosis: clinical and biological aspects. Med Mycol 2000; 38 (Suppl 1): 205-213.
10. Mitchell TG, Perfect JR. Crytptococcosis in the era of AIDS – 100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 1995; 8: 515-546.
11. Nishikawa MM, Lazera MS, Barbosa GG, Trilles L, Balassiano BR, Macedo RCL, Bezerra CF, Perez MA, Cardarelli P, Wanke B. Serotyping of 467 *Cryptococcus neoformans* isolates from clinical and environmental sources in Brazil: Analysis of host and regional patterns. J Clin Microbiol 2003; 41: 73-77.
12. Ohkusu M, Tangonan N, Takeo K, Kishida E, Ohkubo M, Aoki S, Nakamura K, Fujii T, Siqueira IC, Maciel EA, Sakabe S, Almeida GM, Heins-Vaccari EM, Lacaz C da S. Serotype, mating type and ploidy of *Cryptococcus neoformans* strains isolated from patients in Brazil. Rev Inst Med Trop Sao Paulo 2002; 44: 299-302.
13. Pienthaweechai K, Ito-Kuwa S, Nakamura K, Aoki S, Vidotto V, Koga-Ito CY, Sinicco A. Phospholipase production in *Cryptococcus neoformans* isolated from AIDS patients in Thailand and in Italy. J Mycol Med 1998; 9: 45-46.
14. Santangelo RT, Nouri-Sorkhabi MH, Sorrell TC, Cagney MH, Chen SC, Kuchel PW, Wright LC. Biochemical and functional characterization of secreted phospholipase activities from *Cryptococcus neoformans* in their naturally occuring state. J Med Microbiol 1999; 48: 731-740.
15. Tintelnot K, Schar G, Polak A. Epidemiological data of cryptococcosis in Austria, Germany and Switzerland: part of the ECMM survey in Europe. Mycoses 2001; 44: 345-350.
16. Torres RA, Barr M. Impact of combination therapy for HIV infection on inpatient census. N Eng J Med 1997; 336: 1531-1532.
17. Tortorano AM, Viviani MA, Rigoni AL, Cogliati M, Roverselli A, Pagano A. Prevalence of serotype D in *Cryptococcus neoformans* isolates from HIV positive and HIV negative patients in Italy. Mycoses 1997; 40: 297-302.
18. Vidotto V, Sinicco A, Di Fraia D, Cardaropoli S, Aoki S, Ito-Kuwa S. Phospholipase activity in *Cryptococcus neoformans*. Mycopathologia 1996; 136: 119-123.