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

Valerio Vidotto[1], Soho Ito-Kuwa[2], Kenjrou Nakamura[2], Shigeji Aoki[2], Marcia Melhem[3], Kazutaka Fukushima[4] and Enrico Bollo[5]

[1]Dipartimento Discipline Medico-Chirurgiche, Sezione Malattie Infettive, Università di Torino, Torino, Italy; [2]Advanced Research Center, Nippon Dental University, Niigata, Japan; [3]Instituto Adolfo Lutz, Cerquira Cesar, Sao Paulo, Brazil; [4]Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chuo-Ku, Chiba, Japan; [5]Dipartimento Patologia Animale, Sezione Anatomia Patologica, Facoltà di Veterinaria, Università di Torino, Grugliasco, Italy

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

Dirección para correspondencia:
Dr. Valerio Vidotto
Dipartimento Discipline Medico-Chirurgiche
Sezione Malattie Infettive
Università di Torino
C.so Svizzera 164
10.140 Torino, Italy
Tel./Fax: +39 011 439 3860
E-mail: valerio.vidotto@libero.it

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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].