Original

Enzymatic profile of *Cryptococcus neoformans* strains by using the API-ZYM system

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Summary

Determination of the enzymatic profile of 41 *Cryptococcus neoformans* strains, 20 isolated from AIDS patients and 21 from bird droppings, was performed by using the API-ZYM commercial kit system (Bio-Mérieux, France), which tests 19 different kinds of enzymes.

All the strains showed positive enzymatic activity to the esterase (C4) (n°3). On the contrary, alkaline phosphatase (n°2), cystine arylamidase (n°8), trypsin (n°9), chymotripsin (n°10), α-galactosidase (n°13), β-glucuronidase (n°15), α-mannosidase (n°19), α-fucosidase (n°20) were negative in all the strains. The other 10 enzymes (n°4,5,6,7,11,12,14,16,17,18) were distributed among the strains in different positive percentages.

From the results of each enzymatic profile obtained, the 20 AIDS strains were grouped into 15 types, while the 21 bird dropping strains were grouped into 14 types. Interestingly, only one enzyme profile type occurred in the strains isolated from the AIDS patients and from the bird droppings. These results suggest that the API ZYM system is useful in discriminating between the AIDS strains and the bird dropping strains.

Key words

Cryptococcosis, *Cryptococcus neoformans*, epidemiology, enzymatic activity, AIDS

Actividad enzimática de diferentes cepas de *Cryptococcus neoformans* obtenida con el sistema API-ZYM

Resumen

La actividad enzimática de 41 cepas de *Cryptococcus neoformans*, 20 aisladas en pacientes con Sida y 21 aisladas en heces de varias aves, ha sido investigada por medio del sistema API-ZYM (Biomérieux, Francia).

Todas las cepas examinadas mostraron una actividad enzimática esterasa (C4). No fue observada actividad enzimática de ocho enzimas: fosfatasa alcalina (n°2), cistina arilamidasa (n°8), tripsina (n°9), quimiotripsina (n°10), α-galactosidasa (n°13), β-glucosidasa (n°15) α-manosidasa (n°19) y α-fucosidasa (n°2). Se observó un porcentaje de actividad enzimática diferente para los otros 10 enzimas del sistema API-ZYM en las 41 cepas de *C. neoformans* examinadas.

Según los resultados obtenidos las 20 cepas de *C. neoformans* aisladas en pacientes con Sida fueron incluídas en 15 diferentes biotipos mientras que las 21 cepas aisladas entre las aves fueron incluídas en 14 biotipos diferentes.

Un solo perfil de la actividad enzimática fue común entre las cepas aisladas de pacientes con Sida y en cepas aisladas de heces de aves. Los resultados obtenidos sugieren que el sistema API-ZYM podria ser útil en la identificación y separación de biotipos en cepas aisladas en pacientes inmunocomprometidos y en cepas aisladas en el medio ambiente.

Palabras clave

Criptococosis, *Cryptococcus neoformans*, Epidemiología, Actividad enzimática, Sida

Cryptococcus neoformans, after *Candida albicans* is a major cause of opportunistic and disseminated mycotic infections in immunocompromised and in particular in AIDS patients [1-11]. For this reason criptococcosis according to Ajello [12] should not only be considered as "sleeping disease" but according to Drouhet [13] it should be regarded as " the mycosis of the future".

Coordinated researches on *C. neoformans* epidemiology, genetic, biochemistry, enzymatic activity and serology are very useful in order to better evaluate its aetiology and also to understand its epidemiology.

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Commercial methods for a faster and easier identification of medically important yeasts, based on their enzymatic activity, have already been proposed. These methods proved to be very useful for *C. albicans* and some for *C. neoformans* identification [14,15].

In the present study, the API-ZYM commercial kit system (bio-Mérieux, France) was tested on 41 *C. neoformans* strains, 20 isolated from AIDS patients and 21 from bird droppings in order to identify not only a characteristic enzymatic profile of this yeast, but also to observe the different enzymatic activity between the strains isolated from AIDS and from bird droppings.

In *C. neoformans*, as for other medically important yeasts, fungi and bacteria [15-23], it was possible to obtain a typical enzymatic profile. This profile, together with other biomolecular and biochemical characters typical of each *C. neoformans* strain would be very useful not only for epidemiological purposes but also to better identify different *C. neoformans* strains. The results mentioned below suggest differences in the enzymatic profile between the AIDS and the bird dropping strains.

MATERIALS AND METHODS

Twenty *C. neoformans* strains isolated from AIDS patients, stored in the culture collection of the Infectious Disease Institute of Turin's University and 21 isolated from bird droppings and provided by the Institute of Microbiology at Messina University, were used in the present study. These strains were transferred onto fresh malt agar slants and incubated at 25°C. After five days a loopful ($1x10^8$ cell/ml), was inoculated into 200 ml of sterile 2% malt extract liquid medium and shaken on a gyrotory shaker at 120 rpm for 24 h at 28°C until the cells reached their early log-phase of growth.

The cells were centrifugally washed three times in the same sterile malt extract liquid medium and then tested for their enzymatic activity by using the semi-quantitative API-ZYM system according to manufacturer's instruction. For this purpose each *C. neoformans* strain was inoculated into 5 ml ($1x10^6$ cell/ml) of physiologic saline solution (0.9%) sodium chloride. The optical density (OD) at 550 nm of each suspension was about 0.1. Sixty-five µl of each inoculum was dispensed into each of the 20 API-ZYM strip microtubes and incubated at 37°C in thermostat for 4 hours in the apposite APY-ZYM chamber humidified with 5 ml of distilled water. After this incubation period, a drop of ZYM A and ZYM B reagents was added to each of the twenty microtubes.

The color reaction was read after 5 minutes, 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, 5 to 40 nmols or more of each APY-ZYM substrate metabolized by the strains. The positive enzymatic activity of each strain was observed and the strains with the same or with different enzymatic pattern were grouped (Table 4).

The chi-square test was performed to evaluate differences in the occurrence of positive enzymes between the AIDS and the bird dropping strains (Table 3). Similarly, differences in the average amount (nmols) of each substrate metabolized by the API-ZYM enzymes was statistically checked by the t-student test (Table 5).

Each strain was tested three times in triplicate on different days with different solutions to confirm the results obtained.

RESULTS

Enzyme profiles of the AIDS and bird dropping strains. The presence and the activity of 19 enzymes in the 41 *C. neoformans* strains examined with the API-ZYM system are shown in Table 1. Eleven of the 19 enzymes (n°3-7,11-14 and 16-18) were found to be positive either both or the just AIDS and the bird dropping strains, although their incidence varied from strain to strain.

All the AIDS and the bird dropping *C. neoformans* strains tested showed a positive enzymatic activity to the n°3 enzyme esterase (C4). The majority of the strains tested resulted positive for esterase-lipase (C8) (n°4), cystine arylamidase (n°6), acid phosphatase (n°11), naphtol-AS-BI-phosphohydrolase (n°12) and β-glucosidase (n°17). The occurence of lipase (C14) (n°5), valine arylamidase (n°7), β-galactosidase (n°14) and N-acetyl-β-glucosoaminidase (n°18) was low in the strains. All the strains tested were negative for alkaline phosphatase (n°2), cystine arylamidase (n°8), trypsin (n°9), chymotrypsin (n°10), α-galactosidase (n°13), β-glucuronidase (n°15), α-mannosidase (n°19) and α-fucosidase (n°20).

The intensity of the positive enzymes tested by the API-ZYM color reaction scale ranged from 1 to 5 (Table 1). Although most of the enzyme activities ranged from low to moderate (color scale 1 to 3), a high activity value (color scale 4 to 5) was distributed in esterase (C4) (n°3), leucine-arylamidase (n°6), acid phosphatase (n°11), α-glucosidase (n°16) and β-glucosidase (n°17) in eight AIDS strains and five bird dropping strains (Table 1)

Enzymes grouped by their occurence among the strains. The enzymes were grouped into five classes according to the positive percentage among the AIDS and the bird dropping *C. neoformans* strains (Table 2).

In the first class, where the positive percentage was more than 75% among the strains tested, esterase (C4) (n°3), esterase-lipase (C8) (n°4), leucyne arylamidase (n°6), acid phoshatase (n°11) and naphtol-AS-BI- phosphohydratase (n°12) enzymes were included. In particular the esterase (C4) (n°3) was found in 100% of the AIDS and the bird dropping strains. Leucyne arylamidase (n°6) was positive in a very high percentage (85%) only in the AIDS strains (Table 2-3).

In the second class with the positive percentage between 50-74%, consisted only of β-glucosidase (n°17) in the AIDS strains (Table 2-3).

The third class included with the positive percentage between 25- 49%, the α-glucosidase (n°16) enzyme in the AIDS strains and in the bird dropping strains. The leucyne arylamidase enzyme (n°6) with a 48% positive percentage activity and the β-glucosidase enzyme (n°17) with a 33% positive percentage activity in the bird dropping strains, were also included in the third class.

In the fourth class with the positive percentage less than 24%, 4 enzymes were included. Among them, lipase (C14) (n°5), valine arylamidase (n°7) and N-acetyl-β-glucosamidase (n°18) were commonly found both in the AIDS and in the bird dropping strains, while β-galactosidase (n°14) was positive only in the AIDS strains.

In the fifth class with no positive percentage in the 41 *C. neoformans* strains tested, the enzymes: alkaline phosphatase (n°2), cystine arylamidase (n°8), trypsin (n°9), chymotrypsin (n°10), α-galactosidase (n°13), β-glucoronidase (n°15), α-mannosidase (n°19) and α-fucosidase (n°20) were commonly negative in both the AIDS and the bird dropping strains. The β-galactosidase (n°14) was only negative in the bird dropping strains.

Table 1. Enzyme activity of the *Cryptococcus neoformans* AIDS and bird dropping strains according to the API ZYM system.

Substrates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AIDS strains																				
CN 5			2	2		1						1					1			
CN 6			2	2		1					1	2					1			
CN 7			3	1		4														
CN 8			1	1		1					3									
CN 9			1	3	2	1					3	5					3	1		
CN 10			3	2		4					5	1		2		4	2			
CN 13			4	2		5						2								
CN 14			2	1		3					3	1								
CN 16			1	1							5					1				
CN 17			2	2							1	1					1			
CN 18			1	1		1					1	1					5			
CN 19			2	3		1					1	1								
CN 20			3	2		2					2	2		2		3	2			
CN 21			1	1		1					1	1					2	1		
CN 22			2	1		2											1			
CN 25			3	2		1					2	2					2			
CN 19015			2	1		1					2	1					1			
AIDS I			2	2	1	1	1				2	1								
AIDS II			2	2		1					2					5	2			
CN 26 LQ			2								5	5				2				
Bird dropping strains																				
CN 21 M			2	1							1	1				1	3			
CN 30 M			3	1		5	1					1								
CN 40 M			3			2					2	4								
CN 55 M 1			2	2	1	1					1	1				1				
CN 74 M			2	2		1					1	1								
CN 79 M			1								5	4								
CN 1(10)			3	1								1								
CN 12(2)			3	2		1					1	2				1				
CN 12(8)			2	1								1								
CN 13(4)			2	1		1					1	2				1	1			
Can 1			2	1		1					1	1				1	1			
Can 2			2	2		1					1	1				1	1			
Can 4			1	1							1	1				1		1		
CN 2 U			2	1		1					2	2								
CN 3U B			1	1							2									
CN 3U D			2	1							1									
CN 3U E			2	2							2					2	1			
CN 3 U G			2								4	4				2	2			
CN 3 U I			3	2		1					5	4				2	2	1		
Cn 7rcg 1			2	1								1								
Cn 2 pac			2	1								2								

Table 2. The API-ZYM enzymes classed by the occurrence (positive percentage) in *Cryptococcus neoformans* strains.

Enzyme number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AIDS strains			•	•	∇	•	∇				•	•		∇		◊	Σ	∇		
Bird dropping strains			•	•	∇	◊	∇				•	•				◊	◊	∇		

• higher than 75% (class 1); Σ between 50-74% (class 2); ◊ between 25-49% (class 3); ∇ less than 24% (class 4); 0% (class 5)

Table 3. Positive percentage activity of the AIDS and bird dropping (B.D.) *Cryptococcus neoformans* strains and Chi-square test.

Enzyme	Strains AIDS %	Strains B.D.%	P values
1	0	0	Control
2	0	0	-
3	100	100	-
4	95	86	0.207
5	10	5	0.635
6	85	48	0.028*
7	5	5	0.49
8	0	0	-
9	0	0	-
10	0	0	-
11	80	76	0.934
12	75	86	0.638
13	0	0	
14	10	0	0.447
15	0	0	-
16	25	48	0.239
17	60	33	0.162
18	10	10	0.635
19	0	0	-
20	0	0	-

*Significant

The statistical analysis of the chi-square test of each API-ZYM enzyme in the AIDS and the bird dropping *C. neoformans* strains revealed no significant differences except for leucine-arylamidase (n°6). Its chi-square test showed very significant result (P=0.028) (Table 3).

Patterns of the enzymatic profile. From the results obtained by using the API-ZYM system it was possible to identify the enzymatic profile of the 41 *C. neoformans* strains as shown in Table 4. For this reason the enzymatic profile could be considered a discriminant parameter between the AIDS and the bird dropping *C. neoformans* strains. As can be seen in this Table, the 20 strains isolated from the AIDS patients were grouped into 15 patterns according to their enzymatic profile. Among these patterns, one group with five strains and two groups with two different strains were observed. The other 12 groups contained only one strain. Similarly, the 21 *C. neoformans* strains isolated from the bird droppings were grouped into 14 patterns. There was one group with four strains, one group with three strains and two groups with two strains

Table 4. Enzymatic activity of the AIDS and bird dropping *Cryptococcus neoformans* strains according to the same or different enzymatic patterns.

Enzyme number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AIDS strains																				
CN6, CN18, CN25, CN19015			+	+		+					+	+					+			
CN10, CN20			+	+		+					+	+		+		+	+			
CN14, CN19			+	+		+					+	+								
CN5			+	+		+						+					+			
CN7			+	+		+														
CN8			+	+		+					+									
CN9			+	+	+	+					+	+					+	+		
CN13			+	+		+						+								
CN16			+	+							+					+				
CN17			+	+							+	+					+			
CN21			+	+		+					+	+					+	+		
CN22			+	+		+											+			
AIDS I			+	+	+	+	+	+			+	+								
AIDS II			+	+		+					+					+	+			
CN28LQ			+								+	+				+				
Bird dropping strains																				
CN(10), CN12(8), CN2pac, CN7rcg			+									+								
CN13(4), Can1, Can2			+	+		+					+	+				+	+			
CN2U, CN74M			+	+		+					+	+								
CN3UB, CN3UD			+	+							+									
CN21M			+	+							+	+				+	+			
CN30M			+	+		+	+					+								
CN40M			+			+					+	+								
CN55M(1)			+	+	+	+					+	+				+				
CN12(2)			+	+		+					+	+				+				
Can4			+	+							+	+				+				
Cn79M			+								+	+						+		
CN3UG			+								+	+				+	+			
CN3UI			+	+		+					+	+				+	+	+		
CN3UE			+	+							+					+	+			

Table 5. Average amount of metabolized substrate in the AIDS and the bird dropping (B.D.) *Cryptococcus neoformans* strains and t-test.

	AIDS		B.D.		
Substrates	nmol	sd	nmol	sd	P values
1					
2	0	0	0	0	-
3	11.75	1.15	11.66	5.08	0.853
4	8.5	4.89	5.71	3.27	0.037*
5	0.75	2.44	0.24	1.09	0.389
6	9.75	11.17	4.28	8.7	0.087
7	0.25	1.11	0.24	1.09	0.977
8	0	0	0	0	-
9	0	0	0	0	-
10	0	0	0	0	-
11	12.75	13.32	9.28	12.07	0.387
12	8.25	11.38	10	10.36	0.609
13	0	0	0	0	-
14	1	3.07	0	0	0.143
15	0	0	0	0	-
16	5.25	11.41	3.09	3.7	0.415
17	6.75	9.49	2.85	5.14	0.381
18	0.5	1.53	0.47	1.5	0.95
19	0	0	0	0	-
20	0	0	0	0	-

*Significant

each. The other 10 groups contained only one strain. In the results shown in Table 4, it should also be noted that were was only one enzymatic profile pattern, that included the two AIDS strains (CN10 and CN20) and the two bird dropping strains (CN2U and CN74M), common to the AIDS and the bird dropping strains.

Metabolized substrates (nmols) in the AIDS and in the bird dropping C. neoformans strains. On the basis of the data listed in Table 1, the average amount (nmols) of the metabolized substrates by API-ZYM enzymes, the only significant difference between the AIDS and the bird dropping strains, was observed for the esterase-lipase (C8) (n°4) enzyme by using t-Student test (Table 5).

DISCUSSION

Rapid enzymatic identification of *C. neoformans* by using the activity of several enzymes such as urease, phenoloxidase, nitratase, etc, have already been developed [13,14]. These methods evaluate only one enzymatic activity, but do not allow a wide range of enzymatic activity for rapid identification in *C. neoformans*. This goal has been obtained for the first time by using the API-ZYM kit system, which accordingly to the results obtained showed different enzymatic profiles from each of the 41 *C. neoformans* tested (Table 4). Although the number of the *C. neoformans* strains tested was not numerous, it is possible to hypothesize that *C. neoformans* has its own enzymatic profile, which is different from *C. albicans* and *Pityrosporum pachidermatis* or other medically important yeasts [15,23].

The high β-glucosidase enzymatic activity observed in the *C. neoformans* strains tested confirms, according to Casal and Linares, that *C. neoformans* is β-glucosidase positive, while in *C. albicans*, the activity of this enzyme is generally considered to be negative [21]. The recent phospholipase production observed in *C. neoformans* [11,24], the new and the high positive enzymatic percentage activity of more than 75% of the *C. neoformans* strains tested in enzymes n°3,4,6,11, according to Chen [25], reveals that there are several new extracellular enzymatic activities in *C. neoformans*, which may be involved in tissue invasion. Therefore, one can hypothesize, according to Papini *et al*. [21] as in *Microsporum canis*, that these new enzymes are essential not only for growth, metabolism of *C. neoformans*, but may also indicate a relationship between *C. neoformans* and its virulence.

According to the results obtained, enzymatic activity lower than 75% would not be considered to be a discriminant enzymatic parameter between the AIDS and the

bird dropping *C. neoformans* strains. On the contrary, the significant t student difference in the metabolized substrate (nmols) between the AIDS and the bird dropping *C. neoformans* strains for enzyme n°4 could be considered a discriminant parameter (Table 5). Enzyme n°6 with a positive percentage activity of more than 75% in the AIDS strains (Table 2), could be also considered as a typical discriminant enzyme between the AIDS and the bird dropping strains, although its chi-square test was very significant but not its t-test. Thus more detailed research in enzymes activities on *C. neoformans* by using a wide number of enzymatic activities such as the API-ZYM would be useful in order to help the identification and characterization of *C. neoformans* strains isolated from immunocompromised patients or from the environment. This technique permits rapid, low cost identification and does not require a great deal of laboratory experience. On the contrary while, molecular biology or genetic techniques such as DNA fingerprinting or karyotyping, seem to be promising tools for the identification not only for *C. albicans* [14] but also for different *C. neoformans* strains [26-34], these techniques are time-consuming ones and require considerable experience.

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