Extracellular activity in *Cryptococcus neoformans* strains isolated from AIDS patients and from environmental sources

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Summary

Nineteen *Cryptococcus neoformans* strains isolated from AIDS patients and 16 from bird droppings were tested for their extracellular activity. Typical enzymatic activity that was different from other medically important yeasts was found. The results obtained may indicate that there are new extracellular enzymatic activities that imply a relationship between *C. neoformans* and its virulence. A correlation among the different enzymatic activities was also investigated and according to the results obtained no relationship was observed among any of the recorded extracellular enzymatic activities. Research on *C. neoformans*'s extracellular enzymatic activity is useful not only to better understand its metabolism but in particular to establish a possible relationship between its virulence and pathogenicity.

Key words

Cryptococcus neoformans, Phospholipase, Protease, Phenoloxidase, Virulence

Actividad extracelular en cepas de *Cryptococcus neoformans* procedentes de pacientes con sida y del ambiente

Resumen

Se examinaron las actividades enzimáticas extracelulares de 19 cepas de *Cryptococcus neoformans* aisladas de pacientes con sida y 16 de heces de aves. Los resultados obtenidos demostraron una actividad enzimática diferente de otras levaduras de interés médico. Según los resultados obtenidos, es posible identificar una relación entre las nuevas actividades enzimáticas extracelulares de *C. neoformans* y su virulencia. No existe ninguna correlación entre las diferentes actividades enzimáticas extracelulares de *C. neoformans*. Las investigaciones sobre la diferentes actividades enzimáticas extracelulares de *C. neoformans* pueden ser muy útiles para profundizar en el conocimiento del metabolismo de esta levadura y en la relación entre su virulencia y su patogenicidad.

Palabras clave

Cryptococcus neoformans, Virulencia, Fosfolipasa, Proteasa, Fenoloxidasa

Extracellular enzymatic activity in *Candida albicans* has been well known for a long time with a great number of published reports. In *Cryptococcus neoformans*, limited research has been performed in this field until now, although the extracellular activity of this yeast was reported since 1986 by Brueske, Aoki *et al*., Chen *et al.* and Jacobson *et al.* [1-3].

Research on *C. neoformans*'s enzymatic activity is useful not only to better understand its metabolism, but in particular to estabilish a possible relationship between its virulence and pathogenicity. Studies on this activity, together with genetic, serological and biochemical investigations, would be very useful to characterize different *C. neoformans* strains and to elucidate the epidemiology of cryptococcosis. In this study several extracellular enzymatic activities were tested on different clinical and environmental *C. neoformans* strains, in order to observe a possible relationship between these enzymes and *C. neoformans*'s virulence.

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MATERIALS AND METHODS

Nineteen *C. neoformans* strains, from the Infectious Diseases Institute of Turin University's culture collection isolated from AIDS patients, and sixteen from the Institute of Microbiology of Messina University, isolated from bird droppings (BD), were transferred onto malt agar slants and incubated at 25°C for 5 days. After this time period, the following tests were performed:

Variety and serology of the strains. In order to differentiate the two *Filobasidiella neoformans* varieties the *C. neoformans* AIDS strains were tested on Canavanine-Glycine–Bromothymol blue agar (CGB) Petri dishes at 25°C for up to 5 days [4]. The serotype of the AIDS strains was determined by a slide agglutination test with specific monoclonal antibodies for capsular polysaccharide Crypto-check kit (Iatron Laboratories Inc., Japan).

API-ZYM system. A loopful ($1x10^8$ cell/ml) was inoculated into 200 ml of sterile 2% malt extract liquid medium and shaken on a gyrotary 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 sterile malt extract liquid medium and then tested for their enzymatic activity by using the semi-quantitative API-ZYM system (Biomérieux, France) according to the manufacturer's instructions. For this purpose each *C. neoformans* strain was inoculated into 5 ml ($1x10^6$ cell/ml) of physiologic saline solution (0.9%). The optical density (OD) at 550 nm of each suspension was about 0.1. Sixty-five microlitres of each inoculum was dispensed into each of the 20 API-ZYM strip wells and incubated at 37°C in a thermostat for 4 h in the apposite API-ZYM chamber humidified with 5 ml of distilled water. After this incubation period a drop of each of ZYM A (Biomérieux - France) and ZYM B (Biomèrieux, France) reagents were added to each of the twenty wells.

The color reaction was read after 5 min, according to the API-ZYM system's reading color-scale (Table 1), 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 API-ZYM substrate metabolized by the strains. Each strain was tested in triplicate.

Phospholipase production. Determination of phospholipase production was performed essentially according to Polak [5] using the egg-yolk plate method of Price *et al.* [6]. The inoculated plates were incubated at 37°C. After 6 days of incubation, the diameter of the colony (a) and that of the colony plus its precipitation-zone (b) was measured. Phospholipase activity was expressed by Pz = a/b. Thus, a high Pz value means low production of phospholipase. The average Pz value was obtained with three separate samples of each strain.

Protease production. Determination of protease production was performed according to Aoki *et al.* [2], the test medium consisted of agar plates containing bovine serum albumin (BSA), 60 ml of a solution containing 0.04 g $MgSO_4.7\ H_2O$, 0.5 g K_2HPO_4, 1 g NaCl, 0.2 g dried yeast extract, 4 g glucose and 0.5 g BSA (Fraction V, Sigma, USA) was prepared, the pH was adjusted to 3.5 with 1 N HCl. The solution was sterilized by filtration and mixed with 140 ml of melted agar; 20 ml of this medium were poured into each Petri dish and 10 µl of cells suspended in 2.5 ml of sterile physiological saline solution were inoculated in each Petri dish (four inocula were placed in each Petri dish) and incubated at 37°C for 7 days. The diameter of the zones around the colonies was considered as a measure of protease production. Protease activity (Pz), according to the method of Price *et al.* [6], was measured in terms of the ratio of the diameter of the colony plus the precipitation zone, thus a low Pz signified a high production of the enzyme, i.e., high virulence, while a high Pz indicated low production of the enzyme, i.e., low virulence [7]. The average Pz value was obtained with three separate samples of each strain.

Phenoloxidase activity. Phenoloxidase production after six days, was tested at 37°C in Pal's medium [8] which contains the extract from seeds of *Helianthus annuus* instead of *Guizotia abyssinica*. Phenoloxidase activity was scored as follows according to the color intensity of the medium: 3+ high activity; 2+ low activity; 1+ very low activity; and 0 no activity. Phenoloxidase activity of each strain was tested in triplicate.

Statistical analysis. Statistical analysis of the enzymatic activities of AIDS and BD *C. neoformans* strains was performed by using the Mann-Whitney test.

Table 1. Interpretation of the enzymatic tests (API-ZYM system).

N°	Enzyme assayed	Substrate	pH	Result	
				Positive	Negative
1	Control			No colour or colour of the sample if it has an intense coloration	
2	Phosphatase alcaline	2-naphtyl phosphate	8.5	violet	No colour or colour of the control if the strip has been exposed to an intense light source after addition of the reagents. - Very pale yellow if the strip has not beenexposed to an intense light.
3	Esterase (C4)	2-naphtyl butyrate	6.5	violet	
4	Esterase Lipase (C8)	2-naphtyl caprylate	7.5	violet	
5	Lipase C14	2-naphtyl myristate	7.5	violet	
6	Leucine arylamidase	L-leucyl-2-naphtylamide	7.5	orange	
7	Valine arylamidase	L-valyl-2-naphtylamide	7.5	orange	
8	Cystine arylamidase	L-cystyl-2-naphtylamide	7.5	orange	
9	Trypsin	N-benzoyl-DL-arginine-2-naphtylamide	8.5	orange	
10	Chemotrypsin	N-glutaryl-phenylalanine-2-naphtylamide	7.5	orange	
11	Phosphatase acid	2-naphtyl phosphate	5.4	violet	
12	Naphtol-AS-BI-phosphohydrolase	Naphtol-AS-BI-phosphate	5.4	blue	
13	α-galactosidase	6-Br-2-naphtyl-αD-galactopyranoside	5.4	violet	
14	β-galactosidase	2-naphtyl-βD-galactopyranoside	5.4	violet	
15	β-glucuronidase	Naphtol-AS-BI-βD glucuronide	5.4	blue	
16	α-glucosidase	2-naphtyl-αD-glucopyranoside	5.4	violet	
17	β-glucosidase	6-Br-2-naphtyl-βD-glucopyranoside	5.4	violet	
18	N-acetyl-β-glucosaminidase	1-naphtyl-N-acetyl-βD-glucosaminide	5.4	brown	
19	α-mannosidase	6-Br-2-naphtyl-αD-mannopyranoside	5.4	violet	
20	α-fucosidase	2-naphtyl-αL-fucopyranoside	5.4	violet	

Table 2. Parameters of classification for phospholipase, protease and phenoloxidase production and enzymatic activity assayed by the API-ZYM system.

	Negative	Weak	Moderate	Strong
Phospholipase (Pz)	1	0.70–0.99	0.50–0.69	< 0.50
Protease (Pz)	1	0.70–0.99	0.50–0.69	< 0.50
Phenoloxidase (activity)	0+	1+	2+/3+	4+
API-ZYM (activity)	0	1/2	3/4	5

RESULTS

Variety and serology of the strains. All the AIDS and BD *C. neoformans* strains tested on the CGB agar belonged to the variety *C. neoformans neoformans*. All the BD strains collected from separate sites were serotype A, while serotype D was prevalent among the AIDS strains. Only two AIDS strains, CN7 and CN13, resulted serotype C and D, respectively.

Phospholipase activity. In the 19 AIDS strains assayed, phospholipase activity was positive for 18 strains and their Pz ranged between 0.271 and 0.980 (Pz average = 0.63, standard deviation = 0.199). Only one strain, CN7, did not show phospholipase activity (Table 3). On the contrary, in the 16 B.D. strains only eight of them were positive for phospholipase activity and their Pz ranged between 0.1 and 0.948 (Pz average = 0.68, standard deviation = 0.27) (Table 4).

Table 3. PZ values obtained from phospholipase, protease and phenoloxidase in AIDS *Cryptococcus neoformans* strains (n = 19).

Strains	Phospholipase 37°C	Protease 37°C	Phenoloxidase
CN 5	0.517	0.45	+
CN 6	0.689	0.708	++
CN 7	1	0.8	+
CN 8	0.56	1	0
CN 9	0.271	0.91	0
CN 10	0.65	1	0
CN 13	0.408	1	+++
CN 14	0.583	1	+
CN 16	0.98	0.58	+++
CN 17	0.98	0.7	+
CN 18	0.56	0.56	+++
CN 19	0.626	0.9	+++
CN 20	0.62	0.78	++
CN 21	0.921	0.43	+++
CN 22	0.596	0.69	+++
CN 25	0.5	0.9	++
CN 19015	0.35	0.86	+++
AIDS I	0.73	0.5	+++
AIDS II	0.8	0.63	+++
Average Pz± standard deviation	0.650 ± 0.211	0.76 ± 0.19	

Protease activity. Fifteen AIDS *C. neoformans* strains showed positive protease activity with Pz values between 0.43 and 0.91 (Pz average = 0.69, standard deviation = 0.16) (Table 3). Four strains did not show protease activity (Table 3). In the 13 BD strains with positive protease activity the Pz values ranged between 0.34 and 0.94 (Pz average = 0.66, standard deviation = 0.19) (Table 4). Only three BD *C. neoformans* strains (CN 40M, CN 74M and CN 13(4)) were negative for protease production (Table 4).

Phenoloxidase activity. From the 19 AIDS strains, three were negative for phenoloxidase activity and the majority of the strains (12) showed moderate phenoloxidase activity (++ or +++) (Table 3). Two of the 16 BD

Table 4. PZ values obtained from phospholipase, protease and phenoloxidase in *Cryptococcus neoformans* bird dropping strains (n = 16).

Strains	Phospholipase 37°C	Protease 37°C	Phenoloxidase
CN 21M	0.93	0.74	+++
CN 30M	0.948	0.79	0
CN 40M	0.7	1	0
CN 74M	0.1	1	+++
CN 79M	0.8	0.94	+++
CN 1(10)	1	0.559	++
CN 12(2)	1	0.8	++
CN 12(8)	1	0.421	++
CN 13(4)	1	1	++++
Can 1	1	0.83	+++
Can 4	1	0.7	++++
CN 3UB	0.7	0.65	+
CN 3UD	0.7	0.34	++
CN 3UE	1	0.46	++
Cn 7rcg1	1	0.47	+++
Cn 2pac	0.571	0.82	++
Average Pz ± standard deviation	0.90 + 0.15	0.72 + 0.22	

C. neoformans strains were negative for phenoloxidase production and the others strains showed medium activity (++ or +++) (Table 4).

API-ZYM test. As presented in Table 6, all of the AIDS *C. neoformans* strains showed enzymatic activity for the enzymes number 3 (esterase C4) and 4 (esterase lipase C8). Most of the AIDS strains tested showed good enzymatic activity for the enzymes 6 (leucine arylamidase), 11 (phosphatase acid), 12 (naphthol-AS-BI-phosphohydrolase) and 17 (β glucosidase). In contrast, no enzymatic activity was observed for the enzymes 2 (phosphatase alcaline), 8 (cystine arylamidase), 9 (trypsin), 10 (chymotrypsin), 13 (α galactosidase), 15 (β glucuronidase), 19 (α mannosidase) and 20 (α fucosidase).

In the BD strains, no enzymatic activity was observed for enzymes 2 (phosphatase alcaline), 5 (lipase C14), 8 (cystine arylamidase), 9 (trypsin), 10 (chymotrypsin), 13 (α galactosidase), 14 (β galactosidase), 15 (β glucuronidase), 19 (α mannosidase) and 20 (α fucosidase). All of the BD strains showed enzymatic activity for enzyme number 3 (esterase). The majority of the BD strains showed good enzymatic activity for enzymes 4 (esterase lipase), 6 (leucine arylamidase), 11 (phosphatase acid), 12 (naphthol-AS-BI-phosphohydrolase), 16 (α glucosidase) and 17 (β glucosidase) (Table 5).

From the results obtained, the following correlations could be deduced:

a) Correlation between protease and phospholipase activity in the AIDS strains. Among the 19 *C. neoformans* AIDS strains 2 showed a correlation between moderate phospholipase and protease activity (Pz = 0.5-0.69).

b) Correlation between protease and phospholipase activity in the BD strains. In the BD *C. neoformans* strains, five showed a correlation between weak or negative protease and phospholipase activity (Pz = 0.7-1).

c) Correlation between phospholipase and phenoloxidase activity in the AIDS strains. Among the 19 AIDS *C. neoformans* strains seven presented a correlation between moderate phospholipase and phenoloxidase activity (Pz = 0.5-0.69).

d) Correlation between phospholipase and phenoloxidase activity in the BD strains. No correlation was observed between phospholipase and phenoloxidase production in the BD *C. neoformans* strains.

e) Correlation between protease and phenoloxidase activity in the AIDS strains. Five of the AIDS *C. neoformans*

Table 5. Enzymatic activity of the *Cryptococcus neoformans* BD strains (n = 16).

BD	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
CN 21M			2	1							1	1				1	3				
CN 30M			3	1		5	1					1									
CN 40M			3			2					2	4									
CN 74M			2	1		1					1	1									
CN 79M			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 4			1	1							1	1				1		1			
CN 3UB			1	1							2										
CN 3UD			2	1							1										
CN 3UE			2	2							2					2	1				
Cn 7rcg1			2	1								1									
Cn 2pac			2	1								2									

Table 6. Enzymatic activity of the *Cryptococcus neoformans* AIDS strains (n = 19).

AIDS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
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				

strains showed a correlation between moderate protease and phenoloxidase activity (Pz = 0.5-0.69).

f) Correlation between protease and phenoloxidase activity in the BD strains. No correlation was observed between phenoloxidase and protease activity in the *C. neoformans* BD strains.

The results obtained for phospholipase, protease and phenoloxidase activity in the AIDS and BD *C. neoformans* strains can be resumed:

The greatest part of the AIDS *C. neoformans* strains (n = 13, 68.4%) presented strong or moderate phospholipase production, while for the B.D. strains the higher percentage (n = 15, 93.8%) showed weak or negative phospholipase production.

On the other hand, protease activity was similar for BD and AIDS strains with weak or negative protease activity (AIDS = 12, 63.1%, BD = 10, 62.5%).

The highest percentage of AIDS strains (n = 13, 68.4%) and BD strains (n=11, 68.75%) showed moderate phenoloxidase production. Furthermore, two (12.5%) strains of BD *C. neoformans* showed strong phenoloxidase production while in the AIDS strains no strong phenoloxidase production was observed.

Considering the results obtained for phospholipase, protease, phenoloxidase and the enzymatic activity determined with the API-ZYM system, the following correlations could be made:

•*Phospholipase activity*. In the AIDS strains no correlation was observed between the enzymatic activity of enzymes 3 (esterase) and 4 (esterase lipase) and phospholipase production, because at low or medium Pz values of the strains did not correspond to high (3 or 4) API-ZYM enzymatic activity (Tables 3-6). On the other hand, an inverse correlation in the AIDS strains was observed between enzyme 6 (leucine arylamidase) and phospholipase production, because in general a low Pz (high phospholipase production) of the strains corresponded to a negative or low activity of enzyme 6 (leucine arylamidase). In the BD *C. neoformans* strains no correlation was observed between phospholipase production and the enzymatic activity tested by the API-ZYM system.

•*Protease production*. In AIDS strains CN10, CN13 and CN14 the high activity of enzyme 6 (leucine arylamidase) corresponded to negative protease production in the AIDS *C. neoformans* strains (Tables 3-6). On the other hand, no correlation was observed between the activity of the enzymes studied by API-ZYM and protease production by the *C. neoformans* BD strains (Tables 4-5).

•*Phenoloxidase production*. In the 19 AIDS *C. neoformans* strains, eight showed a correlation between moderate phenoloxidase production (3+) and weak enzymatic activity of enzyme 6 (leucine arylamidase) (1-2) (Tables 3-6). No correlation was observed for the other enzymes and phenoloxidase production. In the BD *C. neoformans* strains, no correlation was observed between phenoloxidase and the enzymes tested by the API-ZYM system (Tables 4-5). Although the phenoloxidase activity of the CN8, CN9, CN10 AIDS and CN30M, CN40M, BD

C. neoformans strains resulted negative (Tables 3-4) all these strains were confirmed to be *C. neoformans* var. *neoformans*.

Statistical analysis. Statistical analysis between the AIDS and the BD *C. neoformans* strains performed by the Mann-Whitney test resulted significant for enzymes 4 (esterase lipase C8) and 6 (leucine arylamidase) P= 0.04 and P= 0.05, respectively. The same test was not significant for enzymes 3 (esterase C4) P= 0.872, 11 (Phosphatase acid) P= 0.136 and 12 (naphtol-AS-BI-phosphohydrolase) P= 0.585.

The Mann-Whitney test for phosholipase activity between the AIDS and the BD *C. neoformans* strains resulted very significant (P=0.003). On the contrary, the test was not significant for protease and phenoloxidase activities (P=0.654 and P=0.597) respectively.

DISCUSSION

Yeast enzymatic activity could be a useful method to help the identification and discrimination of different *C. neoformans* strains isolated from immunocompromised patients or from the environment. The techniques involved are rapid, inexpensive and do not require a great deal of experience. Further research in this field would be very useful to better understand *C. neoformans's* physiology and biochemistry. On the contrary, molecular biologic or genetic techniques, which are useful tools for the identification of different *C. neoformans* strains, are expensive and require very experienced technicians [9-17].

Further evidence to support the study of yeast enzymatic activity are the very different results obtained by Chen *et al.* [18] and ours on the different *C. neoformans* strains tested with the API-ZYM kit. In fact, 10 of the enzymatic activities among the 19 of the API-ZYM kit resulted different. In particular enzymes 2, 5, 7, 8, 10 and 15 resulted negative in our AIDS and BD strains and positive in those of Chen *et al*.[18]. Moreover, the study of the enzymatic activity of the 35 *C. neoformans* strains examined showed a typical enzymatic *C. neoformans* profile, which is different from that of the other medically important yeasts [19,20].

Phospholipase activity specifically according to recent literature [21,22] and to the results obtained, seems to be more related to *in vitro C. neoformans* virulence. The phospholipase activity of enzyme number 4 (esterase lipase C8) and number 6 (leucine arylamidase) are statistically significant, according to the Mann-Whitney test. It would be useful to identify and discriminate the different *C. neoformans* strains to determine their virulence.

The new and different enzymatic *C. neoformans* activities shown by the API-ZYM on the strains tested, in particular in enzymes 3, 4, 5, 11, 12 and 17, according to Chen *et al.* [18] reveal that there are several new extracellular enzymatic activities in tissue invasion and may also indicate a relationship between *C. neoformans* and its virulence. For this reason more detailed studies would be very useful on these new extracellular *C. neoformans* activities.

Little attention has been paid to the recently confirmed proteolitic activity of *C. neoformans* [2,23,24]. Although the results obtained in this study, did not prove to be very useful to separate *C. neformans* AIDS and BD strains, it would be of great interest to research whether the extracellular protease activity of *C. neoformans* is associated, as in *C. albicans*, with its virulence and pathogenicity [5,18,25-30].

The results obtained regarding the inverse relationship, only observed among the AIDS *C. neoformans* strains, between high phospholipase production and low enzymatic activity of enzyme 6 (leucine arylamidase) and between low protease activity and high activity of enzyme 6 are very interesting. More detailed studies in this field is necessary to discover if this inverse correlation is a valid and useful criterium to discriminate pathogenic from environmental *C. neoformans* strains. Previous findings indicate that phenoloxidase activity is related to *C. neoformans* virulence [31-33].

According to the results obtained *in vitro* using Pal's medium it was not possible to evaluate a relationship between the different *C. neoformans* strains tested and their virulence. For this purpose the assay of phenoloxidase activity, would be more useful according to the enzymatic methods described by Kwong-Chung *et al.* [31]. This is because not all of the strains tested developed the typical brown pigment although these strains were confiremed to be *C. neoformans* var. *neoformans*. Those in which the pigment was manifested were difficult to discriminate their intensity due to human error (the inability to discriminate amongst the different pigments).

The same problem occurs when using media that contain *G. abyssinica* seeds or media that contains caffeic acid (data not reported). In contrast to that reported in the literature, it seems that not all *C. neoformans* strains are able to produce brown pigment by using these media [8,34-37]. We can hypothesize that, while the testing methods have remained unchanged, the *C. neoformans* strains have undergone phenotypic or genetic changes.

According to the results obtained, no relationship was observed among any of the enzymatic activities recorded such as phospholipase, protease, phenoloxidase and those of the API ZYM kit. This means, according to Jacobson *et al*. [32], that each enzymatic activity has its own pathway, which could in *C. neoformans* be separately related to its virulence. Nor was there any relationship among the enzymatic activities studied in *C. neoformans* that were not already known. *C. neoformans* virulence virulence factors i.e. capsule, growth at 37°C and melanine activity. The activity of protease and phenoloxidase can be altered under *in vitro* conditions and this could influence a discrimination between AIDS and BD*C. neoformans* strains.

According to Franzot *et al.* [23] it is possible to hypotesize that *C. neoformans* undergoes rapid changes *in vitro*, which may induce new genotypic and phenotypic characteristics and interfere with its enzymatic activity. For this reason careful attention to storage and working laboratory conditions shoud be mandatory.

The serotype A of the BD *C. neoformans* strains indicate than in Italy two different serotype A and D can be prevalent. The first in the northern and central part of Italy, the second in the second part. According to Criseo *et al.* [38] geographical and climatological conditions can play an important role in the diffusion of *C. neoformans* serotypes.

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