Differences in extracellular enzymatic activity between *Candida dubliniensis* and *Candida albicans* isolates

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Summary

Twenty-six *Candida dubliniensis* and 27 *Candida albicans* oral strains isolated from patients infected by the human immunodeficiency virus (HIV) were tested for germ tube production and 21 extracellular enzymatic activities. Assessment of the enzymatic profile was performed by using the API-ZYM commercial kit system (bioMérieux, France), which tests 19 different enzymes. Protease activity was expressed during the first days of incubation by 100% of the strains studied and resulted higher than phospholipase activity in the *C. dubliniensis* and *C. albicans* strains tested. The API-ZYM profile of the *C. dubliniensis* and *C. albicans* strains differs with respect to the number and percentage of the enzymes considered, as well as with the intensity of the substrate metabolized by the strains, in particular for the enzymes nº 8 (cystine-arylamidase), nº 12 (naphtol-AS-BI-phosphohydrolase) and nº 16 (α-glucosidase). These enzymes may be useful to differentiate *C. dubliniensis* and *C. albicans* together with other phenotypic characteristics proposed in the literature. No relationship among protease, phospholipase and other extracellular enzymatic activities was observed in *C. dubliniensis*. The average percentage of strains filamentation after 4 h was between 32 and 42%.

Key words

Filamentation, *Candida dubliniensis*, *Candida albicans,* Extracellular enzymatic activities

Diferencias en la actividad enzimática extracelular entre cultivos de *Candida dubliniensis* y *Candida albicans*

Resumen

Las actividades de 21 enzimas extracelulares y la producción de tubos germinales fue estudiada en 26 cepas de *Candida dubliniensis* y 27 cepas de *Candida albicans* aisladas de la cavidad oral de pacientes infectados por el virus de la inmunodeficiencia humana. Diecinueve actividades enzimáticas fueron estudiadas con la prueba API-ZYM (bioMérieux, Francia).
La actividad proteasa en los primeros días de incubación resultó mayor que la actividad de la fosfolipasa en todas las cepas de *C. dubliniensis* y de *C. albicans* estudiadas. La actividad de las enzimas nº 8 (cistin-arilamidasa), nº 12 (naftol AS-BI-fosfohidrolasa) y nº 16 (α-glucosidasa) ensayadas con el API-ZYM mostró diferencias en el numero y el porcentaje de las cepas de *C. dubliniensis* y de *C. albicans*. Estos enzimas podrían ser útiles para diferenciar *C. dubliniensis* de *C. albicans* junto a otras características fenotípicas.
No se ha demostrado ninguna relación en *C. dubliniensis* entre la actividad de la proteasa, fosfolipasa y otros enzimas extracelulares. El porcentaje de filamentación de las cepas de *C. dubliniensis* y de *C. albicans* estudiadas después de 4 h varió entre el 32 y el 42%.

Palabras clave

Filamentación, *Candida dubliniensis*, *Candida albicans,* Actividades enzimáticas extracelulares

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The recently described *Candida dubliniensis* species is prevalently isolated in the oral cavities of immunocompromised patients [31-34]. The yeast has also been isolated in other areas of the body such as lungs, vagina, blood, sputum, gastrointestinal tract and faeces [9,19,20, 28,31,33]. Although *C. dubliniensis* differs from *Candida albicans* in both genotypic and phenotypic characteristics [5,23-25,31,33,34], its close relationship with *C. albicans*, has sometimes led to the misidentification of isolates of *C. dubliniensis* as *C. albicans* [22,33]. Phenotypic methods for the rapid identification of *C. dubliniensis* may be less reliable than genotypic methods [25], but the later are expensive, complex, laboratory intensive and require special equipment [9,14,23,29,31,33].

The purpose of this study was to assess whether or not a group of extracellular enzymatic activities and the germ tube formation can be used for the rapid identification of *C. dubliniesis*.

Materials and methods

Fungal strains and culture conditions. Twenty-six *C. dubliniensis* isolates from the Universidad del País Vasco (Spain) and 27 *C. albicans* from the Infectious Disease Institute of Torino University (Italy) were used in this study. They were originally isolated from the oral cavity of patients infected by the HIV and were identified by using conventional methods. Identification of *C. dubliniensis* was performed by both phenotypic and genotypic methods including the reactivity with an antiserum specific for *C. dubliniensis*, the production of chlamydospores on casein agar and PCR [2,5,6,21]. All the strains where transferred onto fresh malt agar slants and incubated at 37 °C for three days. After this period of time, the *C. dubliniensis* and the *C. albicans* strains were tested for the production of germ tubes and extracellular enzymatic activities.

Protease activity. Proteinase production was assayed according to Aoki *et al*. [3]. The test medium consisted of agar plates containing bovine serum albumin. Sixty millilitres of a solution containing 0.04 g $MgSO_4 \cdot 7H_20$; 0.5 g K_2HP04; 1 g NaCl; 0.2 g dried yeast extract; 4 g glucose and 0.5 g bovine serum albumin (Fraction V, Sigma, USA). 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. Twenty ml of this medium were poured into each Petri dish and 10 µl of *C. dubliniensis* and *C. albicans* cells suspended in 2.5 ml of sterile physiological saline solution were inoculated in each Petri dish (four strains were tested in each Petri dish) and incubated in thermostat at 37 °C for two, four, seven and nine days. After this incubation period, the dishes were stained with Coomassie Brillant Blue G-250. The diameter of the unstained zones around the colonies was considered as a measure of proteinase production [3]. Protease activity (expressed as Pz) of the *C. dubliniensis* and *C. albicans* strains was measured in terms of the ratio of the diameter of the colony to the diameter of the proteolytic unstained zone [27]. When the Pz = 1, no protease activity was detected in the strains. Thus, low Pz means high production of the enzyme. The Pz of three separate samples of each *C. dubliniensis* and *C. albicans* strain was measured to obtain the average Pz reported in table 1.

Phospholipase activity. Phospholipase production was assayed according to Polak [26]. The test medium consisted of malt agar containing 1 M sodium chloride, 0.05 M calcium chloride and 2% egg yolk enrichment 50% (4 ml in 100 ml of agar). The *C. dubliniensis* and *C. albicans* strains were tested in Petri dishes filled with 20 ml agar and 0.5 ml of water. Ten microliters of thick suspension of each *C. dubliniensis* and *C. albicans* strain were placed in the center of the plate after the agar had set. Measurement and calculation of the zone of phospholipase activity (Pz) was performed according to the method described by Price *et al*. [27]. Phospholipase activity after two, four, seven and nine days of incubation at 37 °C of both *C. dubliniensis* and the C. *albicans* strains, was measured in terms of the ratio of the diameter of the colony to the diameter of the zone of precipitation [27]. Thus, low Pz means high production of the enzyme. The Pz of three separate samples of each *C. dubliniensis* and *C. albicans* strain was measured to obtain the average Pz reported in table 1.

Detection of enzymatic activities by the API-ZYM. After five days of incubation at 25 °C, a loopful (1 x 10^8 cells/ml) of each *C. dubliniensis* and *C. albicans* 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. The cells were centrifugally washed three times in the same sterile malt liquid medium and then tested for their enzymatic activity by using the semi-quantitative API-ZYM system, according to manufacturer's instructions. Each yeast strain was inoculated into 5 ml of physiologic saline solution (0.9%) sodium chloride at a concentration of 1 x 10^6 cell/ml. The optical density at 550 nm of each suspension was about 0.1. Sixty five microlitres of each inoculum were dispensed into each of the 20 API-ZYM strip microtubes and incubated at 37 °C in thermostat for 4 h in the apposite API-ZYM chamber humidified with 5 ml of distilled water. After the incubation period, a drop of ZYM A and ZYM B reagents was added to each of the twenty wells.

The color reaction was read after 5 min according to the API-ZYM reading-color scaler, which ranges from 0 to 5, where 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. According to the API-ZYM color scale, the *C. dubliniensis* and *C. albicans* strains were grouped into 3 classes: 1-3 low color scale, 3-4 moderate color scale and 4-5 high color scale. The enzymatic activity assayed by the API-ZYM kit with each strain was used to calculate the average percentage of the strains with the same or with different enzymatic patterns (Table 2). Each strain of *C. dubliniensis* and *C. albicans* was tested in triplicate.

Germ tube production. The percentage of germ tube production was calculated according to Mc Ginnis [18] and Kwong-Chung and Bennet [23]. Briefly, 10^6 cells/ml from each *C. albicans* and *C. dubliniensis* strain were inoculated into 3 ml of bovine serum albumin contained in test tubes of 12 x 75 mm and incubated in thermostat for 4 h at 37 °C. After 2, 3 and 4 h of incubation at 37 °C, a drop of the yeast suspension was poured on a clear microscope slide and covered with a cover glass. The presence of germ tubes was observed with an optical microscope at 400x. Fifty cells from each strain were counted and the number of germinated cells was recorded.

Statistical analysis. The Mann-Whitney test was used to compare the phospholipase and protease activities (expressed as Pz), and the intensity of the color reaction scale, as well as the average percentage of the enzymatic activity of the other enzymes. The chi-square test was used to compare both the germ tube production between the *C. dubliniensis* and *C. albicans* strains after 4 h of incubation and the average percentage of the enzymes tested by the API ZYM kit.

Results

Protease activity. All the 26 *C. dubliniensis* and the 27 *C. albicans* strains tested in the 9-day experiment produced this activity (Pz < 1). Protease activity was very well observed after two days of incubation at 37 °C in both the *C. dubliniensis* and *C. albicans* strains tested (Table 1A). Their average Pz ranged between 0.4 and 0.43 in the *C. dubliniensis* and between 0.4 and 0.47 in the *C. albicans* strains after nine days of incubation (Table 1A). There were no statistically significant differences in protease activity expressed as Pz during the nine days of incubation in the group of *C. dubliniensis* strains. Similar results were observed when the protease activity was compared among the *C. albicans* tested.

Phospholipase activity. All the 26 *C. dubliniensis* and the 27 *C. albicans* strains tested for phospholipase activity after nine days of incubation produced this activity (Pz < 1). Phospholipase activity was not clearly observed after two days of incubation at 37 °C in both the *C. dubliniensis* and *C. albicans* strains. In fact, 17/26 (65%) of the *C. dubliniensis* and 15/27 (55 %) of the *C. albicans* strains did not show this activity after two days of incubation.

During the nine days of the experiment, the average Pz ranged between 0.96 and 0.75 in the *C. dubliniensis* strains and between 0.86 and 0.79 in the *C. albicans* strains (Table 1B). As observed with the protease activity, there were no statistically significant differences in phospholipase activity expressed as Pz during the nine days of incubation in the group of *C. dubliniensis* strains nor among the *C. albicans* tested. However, comparison between protease and phospholipase production differed between the *C. dubliniensis* and *C. albicans* strains. The enzymatic activities were significantly different on days 2, 4, 7 and 9 ($p \leq 0.01$).

API-ZYM enzymatic activities. The *C. dubliniensis* and *C. albicans* strains tested showed 10 enzymes with an activity ≥ 1 nmoles (Table 2). Average values of the enzymatic activity ranged from 1.03 to 4.9 in *C. dubliniensis* and from 1.1 to 4.96 nmoles in *C. albicans* (Table 2). The major enzymatic activities detected were shared by both *Candida* species. Only the enzyme nº 12 (naphtol-AS-BI-phosphohydrolase), showed moderate color scale (3.57 nmoles) in the *C. dubliniensis* strains and low color scale (2.14 nmoles) in the *C. albicans* strains (Table 2), the difference being statistically significant ($p = 0.02$). Interestingly, although they were in the same color scale group, the enzyme nº 8 (cystine-arylamidase) showed statistically significant differences ($p = 0.004$) between *C. dubliniensis* (2.07 nmoles) and *C. albicans* (1.1 nmoles) (Table 2).

The enzymes tested were grouped, according to the average percentage of the *C. dubliniensis* and *C. albicans* strains tested (Table 3). The results were similar for both yeast species, but differences were observed for the enzymes nº 8 (cystine-arylamidase), nº 16 (α-glucosidase) and nº 19 (α–mannosidase) (Table 3). Only differences in cystine-arylamidase were statistically significant ($p = 0.004$). Enzymes which presented high color scale API ZYM intensity, showed also a high average percentage of activity (Tables 2 and 3).

Germ tube production. The greatest majority of the *C. dubliniensis* 24/26 (92%) and *C. albicans* 26/27 (96%) strains produced germ tubes during the 4 h of incubation at 37 °C. The average germ tube production of the *C. dubliniensis* and *C. albicans* strains after 2 and 3 h of incubation resulted similar (Table 1C). Only after 4 h, the germination of *C. dubliniensis* was higher than that of *C. albicans*, but the difference was not statistically significant ($p = 0.45$) (Table 1C).

Table 1. Protease and phospholipase activity and germ tube (GT) percentage production by *Candida dubliniensis* and *Candida albicans.* Average values.

Candida dubliniensis (26 strains)										
A				B				C		
2	4	7	9	2	4	7	9	2	3	4
00:40	00:40	00:41	00:43	0,96	0,81	0,8	0,75	17.5%	26%	42%
Candida albicans (27 strains)										
A				B				C		
2	4	7	9	2	4	7	9	2	3	4
00:40	00:48	00:48	00:47	0,86	0,84	0,81	0,79	18%	25%	32%

A = Protease (Pz) activity in the four days studied; B = Phospholipase (Pz) activity in the four days studied; C = Percentage of germ tube.

Table 2. *Candida dubliniensis* and *Candida albicans* extracellular enzymatic activities color reaction scale (nmoles) according to the API-ZYM color reaction scale Average values.

	Color reaction scale 1 to 3 n moles		
Candida dubliniensis	26 strains	*Candida albicans*	27 strains
ENZYME AVERAGE VALUES		ENZYME AVERAGE VALUES	
Esterase C4	02:30	Esterase C4	02:00
Esterase Lipase C8	2,8	Esterase Lipase C8	02:20
Valine Arylamidase	1,46	Valine Arylamidase	1,48
Cystine Arylamidase	2,07	Cystine Arylamidase	1,11
Alpha glucosidase	1,03	NaphtholASBIPhosphohydrolase	2,14
		Alpha glucosidase	1,55
	Color reaction scale 3 to 4 n moles		
NaphtholASBIPhosphohydrolase	3,57	N AcetylB glucosaminidase	3,48
N AcetylB glucosaminidase	3,07		
	Color reaction scale 4 to 5 n moles		
Phosphatase alcaline	4,6	Phosphatase alcaline	4,4
Leucine arylamidase	4,7	Leucine arylamidase	4,96
Phosphatase acid	4,9	Phosphatase acid	4,85

Table 3. Percentage of enzymatic activity (API-ZYM) in 26 *Candida dubliniensis* and 27 *Candida albicans* strains.

Candida dubliniensis		*Candida albicans*	
Percentage activity	Enzymes n°	Percentage activity	Enzymes n°
100%	2,3,4,6,11	100%	2,3,4,6,11,12
96,50%	8,12,18	88%	16,18
88%	7	85%	7
80%	5	59%	8
65%	16		
15%	19		
No activity	9,10,13,14,15,17,20	No activity	5,9,10,13,14,15,17,19,20

API-ZYM enzyme n°assays
Numbers: 1 Control, 2 Phosphatase alcaline, 3 Esterase (C4), 4 Esterase Lipase (C8), 5 Lipase (C14), 6 Leucine arylamidase, 7 Valine arylamidase, 8 Cystine arylamidase, 9 Trypsin, 10 Chimotrypsin, 11 Phosphatase acid, 12 Naphthol-AS-BI-phosphohydrolase, 13 alpha galactosidase, 14 Beta galactosidase,
15 Beta glucuronidase, 16 alpha glucosidase, 17 Beta glucosidase, 18 N-acetyl-beta-glucosaminidase, 19 alpha mannosidase, 20 alpha fucosidase

Discussion

Although *Candida* pathogenicity and virulence is complex and multifactorial [12], evidence is accumulating about the role of proteases and phospholipases [4,10-13]. A similar role may be suspected for other extracellular enzymes such as acid phosphatase (nº 11), alkaline phosphatase (nº 2), leucine-arylamidase (nº 6), esterase C4 (nº 3) and esterase-lipase C8 (nº 4) detected in this study. These enzymes may allow *C. dubliniensis* and *C. albicans* to penetrate into the host tissues (in particular buccal and vaginal tissues) and disrupt and digest more rapidly the cell membranes composed of proteins and lipids [4,8,10,16,30].

The extracellular enzymatic API-ZYM profile of the *C. dubliniensis* and *C. albicans* strains differed with respect to the number of the enzymes considered, and in particular for the enzymes nº 8 (cystine-arylamidase) and nº 16 (α-mannosidase). These enzymes may be useful to differentiate *C. albicans* and *C. dubliniensis,* together with other phenotypic characteristics such as the color of the colonies on CHROMagar Candida, growth at 42-45 °C [31,33]. According to their high reaction color scale and high percentage of activity, the enzymes nº 8 (cystine-arylamidase) and nº 12 (naphthol-AS-BI-phosphohydrolase) could be also used in the differentiation of *C. dubliniensis* and *C. albicans*. However, this was not the case with enzyme nº 17 (β-glucosidase), since none of the *C. albicans* from *C. dubliniensis* strains tested showed this activity. This result is in disagreement with previously published results suggesting that *C. albicans* can be differentiated from *C. dubliniensis* because the later does not show β-glucosidase activity [1,22,24,31,35].

Kurnatowska [16] found that hydrolase activity in *C. albicans* was characterized by 14 enzymes. However, in our study this enzymatic activity was detected in only ten enzymes: nº 2 (phosphatase alcaline), nº 3 (esterase C4), nº 4 (esterase-lipase C8), nº 6 (leucine-arylamidase), nº 7 (valine–arylamidase), nº 8 (cystine-arylamidase), nº 11 (acid phosphatase), nº 12 (naphthol-AS-BI-phosphohydrolase), nº 16 (α-glucosidase) and nº 18 (N-acetyl-β-glucosaminidase). The enzymatic activities detected by Kurnatowska [16] but not detected in this study included nº 14 (β-galactosidase), nº 17 (β-glucosidase), nº 19 (α-mannosidase) and nº 20 (α-fucosidase). The reasons for these differences are presently unknown but they may be due to differences in enzymatic activities among *C. albicans* strains.

The differences in enzymatic activity observed in *C. dubliniensis* and *C. albicans* may be a useful method to differentiate the two *Candida* species, and also for epidemiological studies. In agreement with previously published results obtained in *C. albicans* and *Cryptococcus neoformans*, no relationship was observed among protease and phospholipase activities and those of the API-ZYM kit [7,36,37].

The results presented in this study confirm that germ tube production in *C. dubliniensis* and *C. albicans* is not a relevant test to rapidly differentiate the two species [15,31, 32,34]. Only after 4 h *C. dubliniensis* strains produced a higher percentage of filamentation than *C. albicans* strains, but differences were not statistically significant ($p = 0.45$).

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