

## Adherence to HeLa cells, typing by killer toxins and susceptibility to antifungal agents of *Candida dubliniensis* strains

Adesão a células HeLa, tipagem pelas toxinas “killer” e sensibilidade a antifúngicos de cepas de *Candida dubliniensis*

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**Abstract:** The aim of this study was to evaluate the adherence capability to HeLa cells, the susceptibility to killer toxins and the *in vitro* susceptibility to antifungal agents (eTest® method – AB Biodisk, Solna, Sweden) of 9 *Candida dubliniensis* isolates recovered from HIV+ and AIDS patients. The adherence test was strongly positive for strain ATCC 777 and positive for all other strains. Typing by killer toxins revealed two different biotypes among the 9 isolates studied: 888 and 688. Only biotype 688 (ATCC 777) was susceptible to the K2 toxin. There was a significant inverse correlation between adherence and killer toxin susceptibility ( $r = -0.8525$  -  $p = 0.0035$ ). No strains presented resistance to fluconazole, itraconazole, ketoconazole, voriconazole, flucytosine or amphotericin-B. With the exception of ATCC 777, all the other isolates presented similar behavior.

**Descriptors:** *Candida*; Cell adhesion; Acquired immunodeficiency syndrome; HIV.

**Resumo:** O objetivo do presente trabalho foi avaliar o comportamento de cepas de *Candida dubliniensis* recuperadas de pacientes HIV+ e com AIDS por meio da pesquisa de capacidade de adesão a células HeLa, susceptibilidade a toxinas “Killer” e resistência *in vitro* a antifúngicos (eTest® AB Biodisk, Solna, Suécia). O ensaio de adesão foi fortemente aderente para a amostra padrão ATCC 777, e aderente para os demais isolados. Os testes de tipagem das amostras frente às cepas-padrão produtoras de toxinas “Killer” mostraram dois biótipos diferentes dos 9 isolados estudados: 888 e 688. Somente o biótipo 688 (ATCC 777) de *C. dubliniensis* foi sensível à toxina K2. Houve correlação inversa significativa entre adesão e sensibilidade a toxinas “killer” ( $r = -0,8525$  -  $p = 0,0035$ ). Em relação à pesquisa de resistência a antifúngicos, as amostras de *C. dubliniensis* foram sensíveis ao fluconazol, itraconazol, cetoconazol, voriconazol, à flucitosina e anfotericina B. Com exceção da amostra ATCC 777, todas as demais mostraram comportamento similar.

**Descritores:** *Candida*; Aderência celular; Síndrome de imunodeficiência adquirida; HIV.

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## Introduction

*Candida dubliniensis* is a newly described species which is closely related phylogenetically to *Candida albicans*. It was first identified as a new species in 1995, in Dublin, Ireland.<sup>19</sup>

Numerous researches are attempting to identify more detailed characteristics of *C. dubliniensis* and similitudes and differences between the two related species.<sup>4,5,8,9,13,14</sup>

*C. dubliniensis* may be isolated from numerous sites but has been frequently isolated from HIV+ patients.<sup>5,6,21</sup> Notwithstanding the researches studying virulence factors of *C. dubliniensis*, mainly those isolated from such patients, some properties as adherence and killer toxin resistance have not been well established, opening a perspective to obtain new data to better explain the biological behavior of this new species of *Candida*.

Adherence to host epithelial cells is the first step in yeast colonization. It occurs due to the ability of the yeast to recognize extra cellular proteins such as laminin, collagen and fibrin, present on the surface of the mucosal host cells.<sup>2,5,7,11</sup>

Killer toxins are low molecular weight glycoproteins produced by 9 *Hansenula* and *Pichia* species. They act producing pores on the cytoplasmic membrane, modifying its permeability, resulting in the death of the susceptible yeasts.<sup>1,16</sup>

On the other hand, literature has stressed the importance of some aspects of the resistance of *C. dubliniensis* to the drugs commonly used to treat candidosis, mainly in AIDS patients.<sup>3,10,12,17,18</sup>

The aim of this study was to assess adherence, resistance to killer toxins and susceptibility to antifungal agents of *Candida dubliniensis* strains isolated from HIV+ and AIDS patients.

## Material and Methods

The Ethical Committee in Research, School of Dentistry, University of São Paulo, approved this investigation (protocol n. 189/03).

Nine strains of *Candida dubliniensis* were utilized:

- Two isolates (ATCC 777 and ATCC 778 from AIDS patients) furnished by Prof. Claudete R. Paula, from ICB-USP, São Paulo, Brazil.

- Two isolates (CD07 and CD 14 from AIDS patients) furnished by Prof. Arnaldo Colombo, from UNIFESP, São Paulo, Brazil.
- Five strains (013, 038, 058, 096, and 107) were isolated from the oral mucosa of HIV+ patients from the “Casa da AIDS”, FM-USP, São Paulo, Brazil.

All the strains were maintained in Sabouraud-dextrose agar (Difco™) containing Chloranphenicol (200 µg/ml).

All the isolates were re-evaluated according to the identification yeast protocol of the ICB-USP (Mycology section) and the results were confirmed by testing the culture in CHROM-Agar *Candida* (CHROMagar Company, Paris, France), at 42°C in Sabouraud agar.<sup>13,14</sup>

The adherence test was done according to the protocol of the Cell culture section of the Adolfo Lutz Institute<sup>15</sup> and consisted in obtaining a monolayer of HeLa cells washed in PBS solution (pH 7.2). A PBS solution (pH 7.2) was added to a yeast suspension of 1 to 2 × 10<sup>15</sup> cells/ml. Monitoring was performed through a Neubauer Chamber. Each cell culture containing the monolayer of HeLa cells was previously washed in PBS solution (pH 7.2). A volume of 2.5 ml of yeast cells was aseptically added to the cell culture and incubated for 1 hour and 30 minutes (37°C). The plates were fixed in formalin (5%) and the cells were dyed by the Gram method. The test was repeated three times.

The following criteria were adopted for cell counting:

- Yeasts with buds smaller than the mother cell were considered as one cell.
- An hyfal cell was considered as one cell.
- Areas with discontinuous monolayer cells were discharged during the count.

The adhered cells count was randomly done in 5 fields of 100 cells for each plate (100 X). To evaluate the results, an arithmetical average of the counts was calculated. The strains which presented an average count of more than 40 cells in 100 HeLa cells were considered strongly adherent. Those which presented an average count between 10 and 40 cells in 100 HeLa cells were considered adherent

**Table 1** - Values ( $\mu\text{g/ml}$ ) of Sensitivity (S) and Resistance (R) of antifungal drugs.

Amphotericin	Fluconazole	Fluocytosine	Itraconazole	Ketoconazole	Voriconazole
S < 1	S $\leq$ 8	S $\leq$ 4	S $\leq$ 0.125	S $\leq$ 8	S $\leq$ 4
R $\geq$ 1	R $\geq$ 64	R $\geq$ 32	R $\geq$ 1	R $\geq$ 32	R $\geq$ 16

**Table 2** - Adherence of the *C. dubliniensis* isolates to HeLa cells.

Strains	ATCC777	ATCC778	CD 07	CD14	096	058	107	038	013
Adherence* Average	53.53	26.93	26.53	14.13	32.27	19.20	27.93	13.93	21.20
Standard deviation	$\pm$ 5.15	$\pm$ 8.25	$\pm$ 6.46	$\pm$ 3.87	$\pm$ 5.65	$\pm$ 1.55	$\pm$ 4.30	$\pm$ 2.54	$\pm$ 1.44

\*Adherence level (average after three counts). Strongly adherent: 40 yeasts in 100 HeLa cells. Adherent: 10 to 40 yeasts in 100 HeLa cells. Weakly adherent: less than 10 yeasts in 100 HeLa cells.

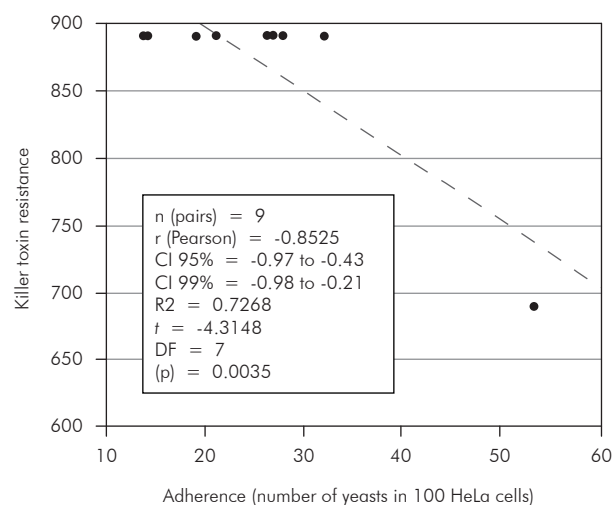
and those with less than 10 cells were considered weakly adherent.

The killer toxins test was done according to Polonelli *et al.*<sup>16</sup> (1983), in which the yeasts and the standard cells grow in modified Sabouraud dextrose agar (SDA) (at 25°C for 48 hours). The yeasts were suspended in 1 ml of sterile distilled water, to obtain a suspension with a turbidity equivalent to the turbidity of a 0.5 McFarland scale, and mixed in 20 ml of modified SDA containing methylene blue. Then the pattern strains killer producers (K1 to K9 – Parma University) were inoculated onto the agar medium. The cultures with an inhibition zone around the pattern strains were considered susceptible. The triplet codes were mounted according to the sensitivity or resistance of the studied yeast to each killer toxin.

*In vitro* susceptibility to antifungal drugs was determined by the eTest® method (AB BIODISK, Solna, Uppland, Sweden), using RPMI-1640 Agar (GIBCO™ - Invitrogen, Carlsbad, Ca, USA) + MOPS (GIBCO™ - Invitrogen, Carlsbad, Ca, USA). The results were compared to the eTest® Application Sheet (EAS006 AB BIODISK 2005 - 04 M0000145 MF0104) (AB BIODISK, Solna, Uppland, Sweden) for the values of Sensitivity (S) and Resistance (R) (Table 1).

### Statistical analysis

The statistical method employed was Pearson's Correlation (Biostat 3.0™) between the variables adherence and killer toxins (9 pairs; DF = 7; CI = 95-99%).

**Graph 1** - Pearson's linear correlation for adherence versus killer toxin resistance.

## Results

The adherence results are presented in Table 2.

All the isolates were adherent (ATCC 777 was strongly adherent and all other were adherent).

The killer test revealed two biotypes (8 strains of the 888 biotype and 1 strain of the 688 biotype). Strain ATCC 777 was susceptible to the K2 toxin. All the others were resistant to all killer toxins. There was a significant inverse correlation between adherence and killer toxins ( $r = -0.8525$  -  $p = 0.0035$ ). This means that for higher adherence values, there were smaller values of resistance to killer toxins (Graph 1).

All isolates were susceptible to the tested antifungal agents and the eTest® results are shown in Table 3.

**Table 3** - eTest results ( $\mu\text{g/ml}$ ) for the nine *C. dubliniensis* isolates.

Strains	Amphotericin	Fluconazole	fluocytosine	Itraconazole	Ketoconazole	Voriconazole
CD 07	< 0.002	0.008	0.047	0.002	< 0.002	< 0.002
CD 14	0.004	0.002	0.047	0.004	< 0.002	< 0.002
ATCC 777	< 0.002	< 0.002	0.125	0.002	< 0.002	< 0.002
ATCC 778	0.016	0.008	1.0	0.25	0.012	0.012
107	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002
038	0.032	0.016	0.25	0.047	0.008	0.006
013	< 0.002	0.003	0.125	0.003	< 0.002	< 0.002
058	0.0023	0.016	0.50	0.047	0.016	0.012
096	0.016	0.016	0.25	0.016	0.002	0.023

Resistance MIC values:  $\geq 1 \mu\text{g/ml}$  for itraconazole;  $\geq 64 \mu\text{g/ml}$  for fluconazole;  $\geq 32 \mu\text{g/ml}$  for ketoconazole;  $\geq 16 \mu\text{g/ml}$  for Voriconazole;  $> 1 \mu\text{g/ml}$  for amphotericin B;  $\geq 5 \mu\text{g/ml}$  for Fluocytosine.

## Discussion

We observed that isolate ATCC 777 was strongly adherent and this fact did not occur in relation to all the other isolates which were simply adherent. None of the strains were weakly adherent. Similar results were obtained for *C. albicans* isolates,<sup>15,16</sup> but it was not possible to compare them with *C. dubliniensis* results due to the absence of literature concerning this feature.

In our research we found two killer biotypes: 888 (88.88%) and 688 (11.11%). Silva<sup>18</sup> (1999) found that strains of *C. albicans* from HIV+ patients were resistant to K2 and K3 killer toxins while strains from healthy individuals were susceptible to all the killer toxins. We obtained a different behavior of *C. dubliniensis* when compared to that of *C. albicans* strains. It is a remarkable fact that our research is the first to correlate adherence and resistance to killer toxins for *C. dubliniensis* isolates. The comparison was always done in relation to *C. albicans* strains due to the scarcity of literature results on adherence and killer toxin resistance involving *C. dubliniensis*. We believe that the inverse correlation between adherence and killer toxin susceptibility reached in our results deserves more accurate studies to be confirmed.

The eTest<sup>®</sup> method was assessed and proved to be an acceptable alternative to the reference methods. All of the isolated strains of *C. dubliniensis* showed

susceptibility to the tested antifungal agents. Our results on resistance to antifungal agents differed from those found in the literature, mainly in respect to itraconazole and fluconazole, which are commonly used drugs for the management of candidosis in AIDS patients.<sup>3,10,12,20</sup>

## Conclusions

1. The ATCC 777 isolate of *C. dubliniensis* was susceptible to the K2 toxin. All the other 8 isolates were resistant to all the killer toxins notwithstanding their origin (HIV+ and AIDS patients), configuring a behavioral difference related to HIV+ and AIDS patients, *C. albicans* carriers.
2. The *C. dubliniensis* strains tested for antifungal drugs (eTest<sup>®</sup>) were all susceptible to the azoles, to fluocytosine and to amphotericin B.
3. Except for the pattern isolate ATCC 777, which presented a distinct behavior, all the other *C. dubliniensis* isolates showed similar behavior.

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