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CANDIDA DUBLINIENSIS IN A BRAZILIAN FAMILY WITH AN HIV 1- INFECTED CHILD: IDENTIFICATION, ANTIFUNGAL SUSCEPTIBILITY, DRUG ACCUMULATION AND STEROL COMPOSITION

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ABSTRACT

This study investigated the prevalence of *C. dubliniensis* in a Brazilian family with an HIV - infected child. A total of 42 oral isolates were obtained from eight family members. The identification of *C. dubliniensis* was performed by polymerase chain reactions (PCR) using primers against a specific sequence of the *C. dubliniensis* cytochrome *b* gene. Only the HIV-infected child and his grandmother were colonized by *C. dubliniensis.* In this study *C. dubliniensis* isolated from the HIV-infected child exhibited high susceptibility for azoles tested with MICs of 0.125 and 0.5 µg/mL for voriconazole and fluconazole, respectively. Accumulation of [^{3}H] fluconazole in *C. dubliniensis* isolated from the HIV-infected child was slightly reduced in comparison to the reference susceptible strain. *C. dubliniensis* isolates had significantly lower ergosterol levels in comparison to *C. albicans* reference strains.

Key words: *Candida dubliniensis*, antifungal agents, sterol composition

INTRODUCTION

C. dubliniensis is closely related to *C. albicans* phylogenetically and is found commonly around the world (23,26). In previous investigations *C. dubliniensis* was isolated from the oral cavities of 27% of human immunodeficiency virus (HIV)-infected subjects and 32% of AIDS patients with oral candidosis (4,18). *C. dubliniensis* has also been recovered from the oral cavities of asymptomatic and symptomatic immunocompetent individuals, although to a much lesser extent. *Candida dubliniensis* was first described in South America by Rodero *et al.* (20). Transmission of genetically indistinguishable strains of *C. albicans* between HIV-infected adult partners has been reported previously (14). Little is known about the transmission of *C. dubliniensis* between children and within families (14). The majority of *C. dubliniensis* clinical isolates tested to date are susceptible to several antifungal agents (13). This study investigated the prevalence of *C. dubliniensis* in a Brazilian family with an HIV - infected child.

MATERIALS AND METHODS

Subjects

One HIV-infected child who acquired HIV vertically is being monitored at the Pediatric Immunodeficiency Outpatient Service, **Medical School, State University of Campinas**. The child's parents had deceased from HIV infection disease. The oral flora from the HIV-infected child and his family members was investigated. A total of 42 oral isolates were obtained from eight family members (Table 1).

Identification

All isolates (n=42) were identified according to the standard technique (21). They were primarily cultured at 30ºC for 48 hours

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Table 1. Characteristics of the eight family members with an HIV-infected child.

Name	Kindred	Age (years)	Race	Gender
LPS	HIV child	5	white	Male
MLA	grandmother	55	white	Female
LA	aunt	25	white	Female
PA	uncle	20	white	Male
CA	uncle	21	white	Male
CHA	cousin	2	white	Male
CAMA	cousin	7[a]	white	Female
JA	cousin	6	mulatto	Female

[a]months.

on chromogenic agar (CHROMagar® *Candida*- France), (1) prior to growth on corn meal agar (DIFCO, USA), supplemented with 1% tween 80, at 25ºC for 7 days for chlamydospore production. Germ tube formation was performed using calf serum (GIBCO BR, USA) at 37ºC for 2 to 4 hours. In addition the isolates were tested with *Candida* Check kit® (Iatron laboratories, INC, Japan) (28) and ID32C® analytic profile index strip (bioMerieux, Marcy l'Etoile, France). Differential growth test at 45ºC was used to distinguish *Candida albicans* from *C. dubliniensis*. All isolates were stored on potato dextrose broth (PDA Difco, MO, USA) containing 25% glycerol at -80ºC and tested for molecular biotyping at a later time.

Candida dubliniensis isolates

C. dubliniensis isolates of IFM 48184 (F6583 isolated from a Japanese patient), 48313 (CBS 7978), 48314 (CBS 7988) and 49192 (S-34 isolated from a Brazilian patient) were used as references, and isolates 73 and 390 were obtained from the Brazilian HIV-infected child during 1998 and 1999 (23). The tested isolates were 2-MLA, 3-MLA (grandmother) and 3-LPS (Brazilian HIV-infected child), collected during 2000 and 2001. The Brazilian HIV-infected child received highly active antiretroviral therapy (HAART) including nelfinavir, zidovudina and 3TC. The HIV-infected child received homecare from the grandmother.

Confirmation of *C. dubliniensis* by polymerase chain reactions (PCR)

The isolates were cultured on potato dextrose agar (PDA Difco, MO, USA) slants at 30ºC temperature for 48 hours. Extraction of DNA was performed using a DNA extracting kit; Gen Toru Kun for yeasts (TaKaRa, Ohtsu, Japan). PCR was performed by amplifying a specific sequence of the *C. dubliniensis* cytochrome b gene (2,23). The primers used were Cdub-F (5'-TTCTCTGTAAGTAATCCTACAATACAGCGT-3') and Cdub-R (5'-ACAATTGATGGAGGTGTCACCATTGGGTTT-3'). A positive result was indicated by the presence of a 305 base product after resolution by electrophoresis in 1% agarose. Comparison of DNA fingerprinting by random amplified polymorphic DNA (RAPD) patterns. The isolates were analyzed by RAPD patterns generated by PCR using 10 pmole of the primer R28M (5'-ATGGATCSSC). An annealing temperature of 35ºC was used with Taq DNA polymerase (2,23).

Antifungal susceptibility tests

Susceptibility tests were performed using the broth microdilution method according to the National Committee for Clinical Laboratory Standards (15). Antifungal agents used in this study included amphotericin B (AMPH) (Bristol-Myers Squibb), nystatin (NYS) (Bristol-Myers Squibb), fluconazole (FLCZ) (Pfizer Pharm. Inc. Japan), voriconazole (VOR) (Pfizer Pharm. Inc. UK), miconazole (MCZ) (Mochida Pharm. Inc. Japan), itraconazole (ITCZ) (Janssen-Kyowa Co., Ltd., Japan), ketoconazole (KTZ) (Janssen-Kyowa Co., Ltd., Japan), and clotrimazole (CTZ) (Sigma, St. Louis, Mo., USA). The minimal inhibitory concentration (MIC) for azoles was defined as the lowest concentration of antifungal agent at which 80% inhibition of growth occurs compared with that of the growth control well. And the breakpoints for amphotericin B and nystatin were defined as 100% inhibition compared with that of the growth control well.

Accumulation of [^{3}H] fluconazole in *C. dubliniensis* isolates

Accumulation of [^{3}H] fluconazole in *Candida* cells was determined by a filter-based assay adapted from Sanglard *et al.* (22). All experiments were repeated on three separate occasions. Overnight cultures were grown in YNB containing 2% glucose at 30ºC, 200 rpm to a density of 10^9 cells per mL. The cells were centrifuged at 4,000 rpm for 5 minutes and the pellet resuspended in YNB medium to the original cell density. A total of 20 µL of [^{3}H] fluconazole (0.154 kBq), with a specific activity of 37 kBq/mmole, was added to 1 mL of the cell suspension. The cells were incubated at 30ºC with shaking at 200 rpm. Samples of 100 µL were withdrawn at fixed time intervals, and mixed with 0.5 mL of cold YNB medium containing 20 µM unlabelled fluconazole placed in a Spin-X nylon membrane microfiltration unit (pore size 0.45 µm - Costar, Cambridge). The cells were isolated by centrifugation at 9,000 rpm for 30 s and then washed with the unlabelled fluconazole-YNB medium three times. Liquid scintillant was added and the radioactivity within the cells was measured using a liquid scintillation analyzer (2500 TR-TRI carb, Packard Bioscience Company). In a separated experiment cells were exposed to a subinhibitory concentration of sodium azide (NaN3; 0.01 mM) (13) to establish whether [^{3}H] fluconazole accumulation was an active energy- dependent process.

Sterol analysis

C. dubliniensis cells were grown to saturation in YNB medium containing 2% glucose. Cells (10^8 cells/mL) were

harvested by centrifugation for 5 min at 3000 rpm, transferred to glass tubes and resuspended in 5 mL of methanol, 3 mL of 60% KOH and 2 mL of 0.5% (w/v) Pyrogalol dissolved in methanol. Additionally 50 µL of 1 mg/mL cholesterol was added to the control sample. The samples were saponified by heating at 90ºC for 2 hours. Sterols were extracted using two 5 mL aliquots of hexane followed by evaporation to dryness with N_2. The isolated sterol fractions were resuspended in 100 µL of toluene and heated at 60ºC for 1 h for silylation with 20 µL of bis (trimethylsily) trifluoride (BSTFA). The sterol samples were analyzed by gas chromatography/mass spectrometry using split injections at a ratio of 20:1. Sterol identification was determined by comparison of retention times and mass spectra (5,6).

RESULTS AND DISCUSSION

HIV-infected patients often suffer severe forms of oropharyngeal candidosis, mainly caused by *Candida albicans*, although over the last decade the reported incidence of infections caused by other *Candida* species has increased significantly (16). *Candida dubliniensis* is a recently identified yeast, (23,24,26) mostly isolated in HIV-infected individuals with oral candidosis. *C. dubliniensis* has also been recovered from the oral cavities of asymptomatic and symptomatic immunocompetent individuals, although to a much lesser extent. Transmission of genetically indistinguishable strains of *C. albicans* between HIV-infected adult partners has been reported previously (14). However, little is known about the transmission of the isogenic *C. dubliniensis* strain between children and within families. The aim of this study was to investigate the presence of *C. dubliniensis* among Brazilian family members.

Candida dubliniensis was first described in South America by Rodero *et al.* (20). Oral flora from one Brazilian HIV-infected child and his family members was investigated. A total of 42 oral mucosa isolates were obtained from eight family members between September 2000 and January 2002 (Table 1). Yeast isolates were identified by classical methods. These included chromogenic agar culture, chlamydospore production, germ tube formation, *Candida* Check kit®, ID32C® profiled; and the temperature test was also used to distinguish *Candida albicans* from *Candida dubliniensis* by its differential growth at 45ºC. The confirmation of *Candida dubliniensis* identification was performed using molecular biotyping. The oral flora of the family members showed high diversity with several non-*albicans* isolates (Fig. 1), particularly the HIV-infected child was carrier of several C*andida* species. *Candida dubliniensis* was isolated only in the HIV-infected child and in his grandmother. The grandmother has repeatedly refused to be HIV tested. The colony formation unit (CFU) quantification was higher for *Candida dubliniensis* (CFU=100) in the HIV-infected child than in his grandmother (CFU <30). No one presented symptomatic oropharingeal candidosis at the moment of the oral examination.

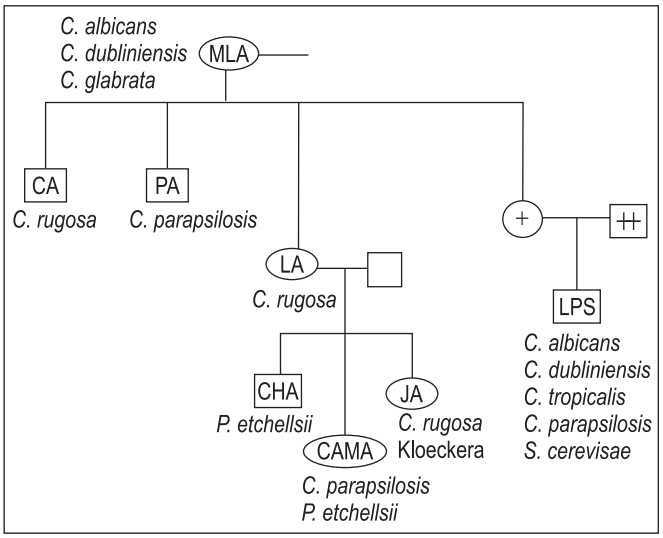

Figure 1. Diagram of the species identified amongst the isolates from the family members of the HIV-infected child. 0 LPS (HIV-infected child); + (HIV-infected mother) and ++ (HIV-infected father) both are deceased. Kindred of the HIV-infected child (MLA, CA, PA, LA, CHA, JA and CAMA).

Higher *Candida* species diversity was observed in the HIV-infected child (Fig. 1).

At the phenotypic level of analysis a number of traits are distinguishable between the majority of *C. albicans* and *C. dubliniensis* isolates (27). However, conclusive differences must be assessed at the genetic level (25). Using the classical identification methods it was not possible to identify some isolates tested. The primary culture of *Candida* isolates on CHROMagar showed a dark green colony, rough appearance and smaller size in comparison with *C. albicans* colonies. The tested isolates that were identified as *C. dubliniensis* by PCR appeared as 305 base pair bands on the gel (Fig. 2). The reference isolates had independent band patterns after the RAPD-PCR. Isolates 73, 390, 2-MLA, 3-MLA and 3-LPS had identical RAPD band patterns (Fig. 3), indicating that the clinical follow-up of *C. dubliniensis* might have the same genotype. The other Brazilian *C. dubliniensis* (S-34) had different genotype. The genotypic coincidence among *C. dubliniensis* isolates from the same family member revealed that the grandmother was probably contaminated through the HIV-infected child (Fig. 3). Further epidemiological studies for his environment, such as neighbors, classmates and relatives might be requested. Recently Milan and collaborators (12) reported that *Candida* spp. colonization was 33% of the AIDS household contacts in contrast with 14% of the HIV-negative control (11,12). *C. albicans* was the most frequently isolated species. Our findings also reveal that transmission through the family members is possible and perhaps

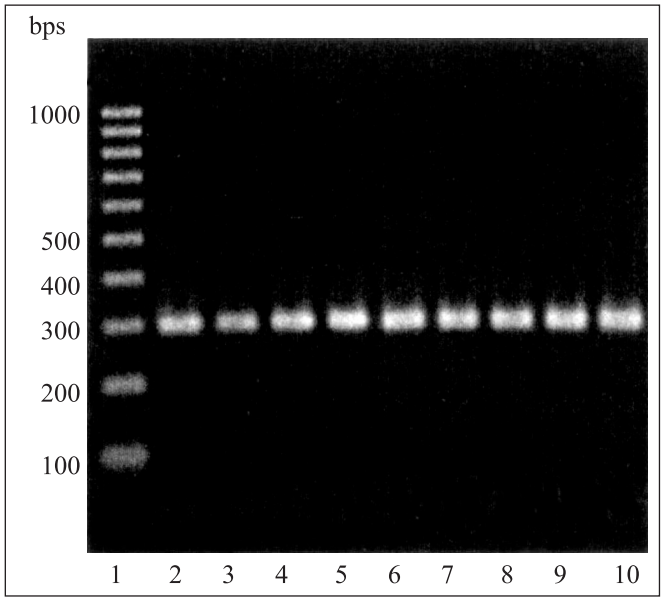

Figure 2. The gel image of amplification of *C. dubliniensis* specific gene in cytochrome *b*. Marker, 1 line; 2, IFM 48184; 3, IFM 48313; 4, IFM 48314; 5, IFM 49192; 6, 73; 7, 390; 8, 2-MLA; 9, 3-MLA; and 10, 3-LPS.

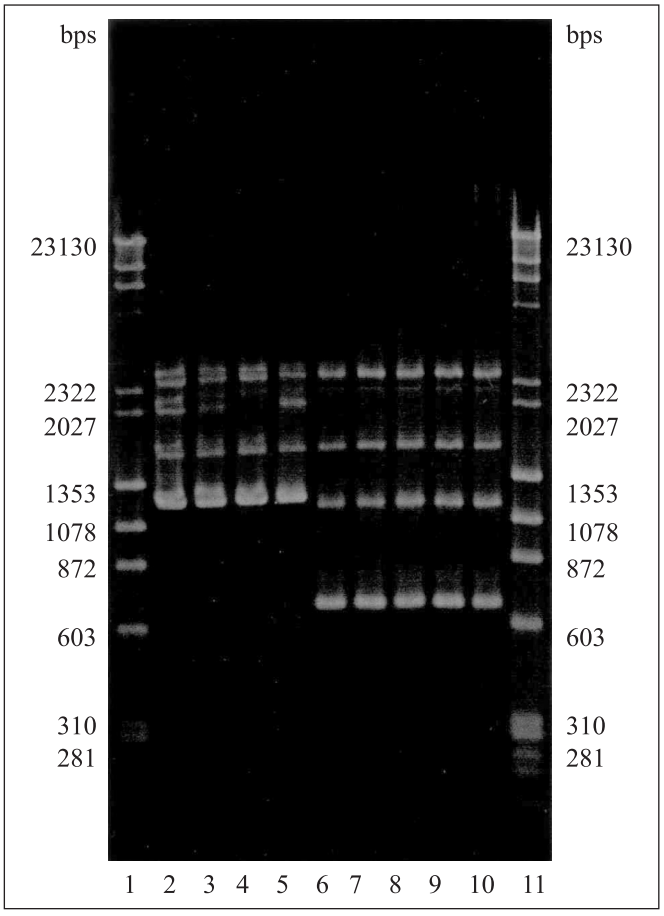

Figure 3. The gel image of RAPD fingerprinting patterns of *C. dubliniensis* isolates. 1, marker; 2, IFM 48184; 3, IFM 48313; 4, IFM 48314; 5, IFM 49192; 6, 73; 7, 390; 8, 2-MLA; 9, 3-MLA; 10, 3-LPS; and 11, marker.

represents a previously under appreciated factor in families with or without HIV infection. Moreover, the asymptomatic members, who have not received antifungal therapy, may also be colonized with resistant *Candida* species. The transmission of *C. dubliniensis* among siblings, parents and relatives may be the exchange of contaminated fomites, which commonly occurs in the sharing of food, utensils, and toys.

MICs for each antifungal agent were determined and all *C. dubliniensis* isolates investigated were susceptible to the antifungal drugs tested (Table 2), with the exception of nystatin. *C. dubliniensis* isolates exhibited MIC values of 0.125 µg/mL for voriconazole. *C. dubliniensis* isolates from Brazil in a previous study (10) reported high susceptibility for azoles and similar results were described in the literature (13,18,19). Non-*dubliniensis* isolates from the other members of the family were susceptible to all drugs tested (data not shown).

Table 2. MICs for antifungal drugs in *C. dubliniensis* isolates.

Drug	3-LPS MIC (µg/mL)	2-MLA	IFM 48313
VOR	0.125	0.125	0.125
FLCZ	0.5	0.125	0.25
ITCZ	1	1	0.125
KTZ	0.03	0.03	0.03
MCZ	2	2	2
CLTZ	0.06	0.06	0.06
AMPH	2	2	2
NYS	16	4	8

Few studies have reported the sterol composition in *C. dubliniensis* isolates (19). As it is known enzymes of the ergosterol biosynthetic pathway are important targets of several classes of antifungals used to treat *Candida* infection (5,8,9). In this study sterol profile of *C. dubliniensis* isolates (3-LPS, 2-MLA and IF 48313) showed percentages of 45.4%, 50% and 49.5% of accumulated ergosterol, respectively. In contrast to azole-susceptible *C. dubliniensis* isolates reported in another study in which the ergosterol profile showed 65%, 56% or 60% accumulation (19). *C. dubliniensis* isolates from the Brazilian HIV-infected child (3-LPS) presented several intermediate sterols (Table 3). Interestingly other intermediate sterols were cholesta-

8,24-dienol (14%) and ergosta dienol (11%) in the 3-LPS isolate. It has previously been reported that altered membrane composition can affect the function of efflux pumps and susceptibility to azole antifungal agents (8,9). Sterol composition has been extensively investigated for *C. albicans* but very little is known about the sterol profile in *C. dubliniensis*. In this report *C. dubliniensis* isolates showed a reduced ergosterol level in comparison to *C. albicans* reference strains (ATCC 90028 and 28516). The ergosterol level has shown values up to 80% to both *C. albicans* reference strains (ATCC 90028 and 28516), these results are similar to that described previously (3,9). The sterol profile in this work suggests an interesting difference between *C. dubliniensis* and *C. albicans*, in regarding that both species are closely related phylogenetically further investigation about the sterol profiles in other *C. dubliniensis* isolates is required.

Table 4. Sterol profile of *C. dubliniensis clinical* isolates (3-LPS, 2-MLA).

Sterol Profile	Total sterol fraction (%)				
	3-LPS	2-MLA	IFM[a] 48313	ATCC[b] 90028	ATCC[b] 28516
Cholesta-8,24-dienol	14	13.1	19.1	-	4
Ergosterol	45.4	50	49.5	93.7	80.2
Ergosta-7,22-dienol	3.4	2.8	3.7	-	0.6
Ergosta-dienol	10.9	10.4	9.6	-	3.7
Methylfecosterol	1.5	-	1.8	-	3
Obtusifoliol	4.7	5.6	6.1	-	2.5
Eburicol	0.3	-	-	-	
4,4-dimethylcholesta-dienol	6.6	5.8	-	-	
Unidentified	12.3	12.3	9.6	6.3	6

[a]*C. dubliniensis* and [b]*C. albicans* reference strains.

Additionally because these *C. dubliniensis* clinical isolates showed different ergosterol amount we decide to investigate if the alteration in the function of efflux pumps could be presented in these isolates. To determine if alterations in cellular permeability to fluconazole could be different in both *C. dubliniensis* isolates, from HIV-infected child (3-LPS) and his grandmother (2-MLA), cells were incubated in the presence of [^{3}H] fluconazole and the intracellular accumulation of this compound was determined. These isolates were exposed to [^{3}H] fluconazole and intracellular fluconazole levels were determined at several time intervals. The two clinical isolates were found to differ with regard to fluconazole accumulation. The isolate from the HIV-infected child was found to accumulate half the amount of [^{3}H] fluconazole than the isolates from grandmother and IF 48313 reference strain (Table 4) however no significant difference. It is also true that in the reference strain (IF 48313 reference strain) ATP-dependent pumps are operating, however no effect was observed when sodium azide was added. However, the isolates from the HIV-infected child and grandmother were found to accumulate approximately 1.5 times more [^{3}H] fluconazole in the presence of sodium azide. [^{3}H] fluconazole accumulation observed in the isolates from the HIV-infected child and grandmother indicate that the efflux of fluconazole from these two strains was an active, energy-dependent process, as sodium azide inhibits ATP formation required for active transport. The most frequent molecular mechanism of azole resistance recently described has been the upregulation of efflux pumps (17). Reports have demonstrated resistance in *C. dubliniensis* isolate and its ability to rapidly develop resistance to fluconazole. This characteristic may partially explain the emergence of this species. Several studies have investigated the multiplicity of mechanisms involved in resistance to azole antifungal agents (7,13,17). Three *Candida albicans* proteins, namely the ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p, respectively and the major facilitator protein Mdr1p were shown to be major mediators of azole resistance. These two super families of active multidrug transporters play an important role in decreasing the intracellular fluconazole concentration of fluconazole-resistant *C. albicans* isolates by a mechanism of active drug efflux pump. Drug-efflux-mediated resistance mechanisms in yeasts provide a further therapeutic target for the future. Therefore the differences in the membrane sterol composition may influence the basic biology of these two closely related species, including virulence factors and antifungal drug targets, which requires further investigation.

Table 3. Accumulation of [^{3}H] fluconazole (dpm/min) in presence of NaN3 in clinical *C. dubliniensis* isolates.

Strains	[^{3}H] fluconazole (MD ± SD)	[^{3}H] fluconazole + NaN_3 (MD ± SD)
3-LPS	46 ± 11.5	70.3 ± 20.7
2-MLA	94.6 ± 26.1	179.4 ± 64.7
IFM 48313	96.2 ± 17.5	107.7 ± 45.5

The acquisition of *C. dubliniensis* and resistant strains by an asymptomatic HIV-infected patient has important clinical implications (29) and may result in the new presentation of oral candidosis refractory to initial azole therapy. Therefore risks for intrafamilial transmission should be included in infection control programs.

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RESUMO

Candida dubliniensis em uma família brasileira com uma criança infectada pelo vírus HIV: identificação susceptibilidade a antifúngicos, acúmulo de fluconazol e composição de esteróis

O presente estudo investigou a prevalência de *C. dubliniensis* em uma família brasileira com uma criança infectada pelo vírus HIV. Um total de 42 isolados orais foram obtidos de 8 membros da família. A identificação de *C. dubliniensis* foi realizada por polymerase chain reactions (PCR) usando primers contra a sequência específica para o gene *C. dubliniensis* cytochrome *b*. Apenas a criança infectada pelo vírus HIV e a avó estavam colonizados por *C. dubliniensis.* Neste estudo *C. dubliniensis* isolado da criança infectada pelo vírus HIV exibiu alta susceptibilidade para azoles com concentração mínima inibitória de 0.125 and 0.5 μg/mL para voriconazole and fluconazole respectivamente. Acúmulo de [^{3}H] fluconazol intra-celular foi ligeiramente reduzido em *C. dubliniensis* isolado da criança infectada pelo vírus HIV em comparação com a cepa referência sensível ao fluconazole. Isolados de *C. dubliniensis* neste estudo apresentaram níveis significantemente reduzidos de ergosterol da membrane celular em comparação com *C. albicans.*

Palavras-chave: *Candida dubliniensis*, agentes antifúngicos, composição de esterol

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