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Phenotypic and genotypic detection of *Candida albicans* and *Candida dubliniensis* strains isolated from oral mucosa of AIDS pediatric patients

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ABSTRACT

The aim of this study was to assess a collection of yeasts to verify the presence of *Candida dubliniensis* among strains isolated from the oral mucosa of AIDS pediatric patients which were initially characterized as *Candida albicans* by the traditional phenotypic method, as well as to evaluate the main phenotypic methods used in the discrimination between the two species and confirm the identification through genotypic techniques, i.e., DNA sequencing. Twenty-nine samples of *C. albicans* isolated from this population and kept in a fungi collection were evaluated and re-characterized. In order to differentiate the two species, phenotypic tests (Thermotolerance tests, Chromogenic medium, Staib agar, Tobacco agar, Hypertonic medium) were performed and genotypic techniques using DNA sequencing were employed for confirmation of isolated species. Susceptibility and specificity were calculated for each test. No phenotypic test alone was sufficient to provide definitive identification of *C. dubliniensis* or *C. albicans*, as opposed to results of molecular tests. After amplification and sequencing of specific regions of the 29 studied strains, 93.1% of the isolates were identified as *C. albicans* and 6.9% as *C. dubliniensis*. The Staib agar assay showed a higher susceptibility (96.3%) in comparison with other phenotypic techniques. Therefore, genotypic methods are indispensable for the conclusive identification and differentiation between these species.

KEYWORDS: *Candida dubliniensis. Candida albicans*. Children. HIV/AIDS Phenotypic and genotypic methods.

INTRODUCTION

Oral candidosis is the most frequent manifestation in Human Immunodeficiency Virus (HIV)-infected children, with prevalence varying from 20% to 72%. Infection usually persists for a long time, it is punctuated by periods of remission, and is frequently resistant to antifungal conventional therapies. These manifestations may also occur in healthy infants in the first six months of life[1].

Oral colonization by *Candida* spp. has been thoroughly investigated in HIV-positive adults, but the number of studies with HIV-positive children is still small[2].

Candida albicans is the most frequent species related to oral fungal infections in adults and children and it is the most virulent species of the genus. However, *Candida dubliniensis*, a species recently described by Sullivan *et al.*[3], has attracted considerable attention due to its almost exclusive association with HIV+/ AIDS individuals. *Candida dubliniensis* has been isolated from erythematosus candidosis, mainly from the oropharyngeal mucosa of HIV+/AIDS patients[3,4]. Nevertheless, few studies on *Candida dubliniensis* infection among the pediatric population have been conducted so far[5,6]. Al-Ahmad *et al.*[7] studied *C. dubliniensis* in caries-free and caries-active healthy children in relation to the oral microbiota and found an association between caries-active children and the presence of *C. dubliniensis*.

Since its characterization in 1995, *C. dubliniensis* has attracted the attention of researchers because this yeast presents similar phenotypic and genotypic characteristics to *C. albicans*. *C. dubliniensis* differs from *C. albicans* with respect to frequency of isolation, pathogenic characteristics, and resistance to antifungal drugs, as well as by presenting higher capacity of adherence to oral mucosa cells[8]. The similarities between these two species hinder their prompt differentiation and may lead to results that underestimate their prevalence.

Phenotypic tests are useful for presumptive identification of *C. dubliniensis,* but they do not provide definitive identification; in contrast, molecular methods provide conclusive identification, but they are difficult to perform, expensive, and require specific equipment[9].

Definitive identification of *C. dubliniensis* is still a problem in routine laboratories because phenotypic and genotypic characteristics of isolated samples are indispensable for final characterization. Reports on the incidence of this yeast by reference laboratories are needed for a better understanding of the epidemiology of this new species[10]. Most importantly, strains of *C. dubliniensis* present higher chance of developing antifungal resistance to "azoles" in comparison with strains of *C. albicans.* Moreover, a phenotype resistant to fluconazole can be induced *in vitro* after exposure to the drug[11].

Considering the limited number of studies among the pediatric population and the need to establish methods for the prompt identification of *C. dubliniensis*, which may contribute to an efficient therapy, the aim of this study was to assess a collection of yeasts to verify the presence of *C. dubliniensis* among strains isolated from the oral mucosa of AIDS pediatric patients which were initially characterized as *C. albicans* by the traditional phenotypic method, as well as to evaluate the main phenotypic methods used in the discrimination between the two species and confirm the identification through genotypic techniques, i.e., DNA sequencing.

MATERIALS AND METHODS

Samples and Patients

Twenty-nine strains from collections were included in this study. They had been previously identified as *C. albicans* through the traditional phenotypic method, isolated from the oral mucosa of AIDS pediatric patients in the Infectious Diseases outpatient clinic and daycare hospital of the Children Institute, *Hospital das Clínicas*, *São Paulo*, Brazil. Strains belong to the fungi collection of the Laboratory of Pathogenic Yeasts, Biomedical Science Institute II, *University of São Paulo* (USP), and were kept lyophilized and in mineral oil. The research was approved by the Research Ethics Committee of USP in accordance with the Declaration of Helsinki (1975).

Phenotypic re-characterization

Yeasts were studied according to the traditional phenotypic method described by Kurtzman *et al.*[12].

Quality control

Control strains ATCC64548 (*C. albicans*) and 777 (*C. dubliniensis*) were included in all tests performed in this study.

Differentiation between *C. albicans* and *C. dubliniensis*

Phenotypic Techniques

Thermotolerance

All the *C. albicans* strains were cultivated in *Sabouraud* dextrose agar (Difco- Detroit, EUA) to test their ability to grow at 45 ºC. Yeast growth under these conditions indicates the presence of *C. albicans*, whereas the absence of growth indicates presence *C. dubliniensis*[13].

Chromogenic medium

Strains were seeded in CHROMagar *Candida* medium (Microbiology, EUA) aiming to obtain specific coloring for the two species according to the manufacturer: light green for *C. albicans* and dark green for *C. dubliniensis*.

Microculture in Staib Agar

Microcultures in Staib Agar were prepared according to Staib and Morschhauser[14]. Readings were performed under optical microscope and the presence of clamydoconidium in bunches would lead to *C. dubliniensis*.

Culture in Tobacco agar

Culture in tobacco agar was conducted according to Khan *et al.*[15]. Microscopic examination would show the development of clamydoconidiums and hyphae, typical of *C. dubliniensis*.

Table 1 - Phenotypic and genotypic identification of isolates previously identified through phenotypic methods as *C. albicans*

Species (n)	Phenotypic Identification					Genotypic Identification
	Temperature 45 ºC	Chromogenic Medium	Staib agar	Tobacco agar	Hypertonic Medium	
C. albicans	24	25	27	23	24	27
C. dubliniensis	05	04	02	06	05	02

Hypertonic Broth

Isolates were seeded in hypertonic *Sabouraud* broth. The growth in this broth would suggest the presence of *Candida albicans* strain because *C. dubliniensis* does not grow in medium with such high concentration of NaCl[16].

Genotypic Technique

The genotypic technique was used as a standard in the identification of *Candida albicans* and *dubliniensis*. DNA extraction of the total yeasts was performed according to the commercial protocol PrepManTM Ultra Sample Preparation reagent Quick Reference Card (Applied Biosystems, EUA). Amplification was performed using primers that amplify the fragments of the ITS region of the rDNA[17]. For the sequencing of the ITS region, the *forward* ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and *reverse* ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') primers[18] were also used. The sequencing agreement was obtained by comparison with sequences available in *GenBank* with the aid of the BLASTn software.

Statistical analysis

Susceptibility and specificity measures were used to evaluate the performance of classification of Thermotolerance tests, Chromogenic medium, Staib agar, Tobacco agar, and Hypertonic medium. In this analysis, DNA sequencing was considered as gold standard. The tests were used to classify samples of *C. albicans* and *C. dubliniensis*.

RESULTS

According to traditional methods of identification previously performed, all the strains studied were *C. albicans*.

Table 1 describes the identification suggested by phenotypic markers used in this study for differentiation between *C. albicans* and *C. dubliniensis.* After amplification and sequencing of specific regions of the 29 studied strains, 27 (93.1%) of the isolates were identified as *C. albicans* and two (6.9%) as *C. dubliniensis*.

Table 2 shows the performance measures of susceptibility and specificity testing of *C. albicans* and *C. dubliniensis*.

The phenotypic characteristics observed in the two samples molecularly identified as *C. dubliniensis* are described in Table 3.

Table 2 - Performance of susceptibility and specificity testing to *C. albicans* and *C. dubliniensis*

Test	Susceptibility	Specificity
Thermotolerance	81.5%	0.0%
Chromogenic medium	88.9%	50.0%
Staib agar	96.3%	50.0%
Tobacco agar	81.5%	50.0%
Hypertonic medium	88.9%	100.0%

DISCUSSION

The prevalence of *C. dubliniensis* has been widely discussed in the specific literature worldwide with varied results. Research on the incidence of *C. dubliniensis* is needed for a better understanding of its epidemiology, mainly in South America where its frequency is not well-known[10].

Table 3 - Phenotypic characteristics of the two strains molecularly identified as *C. dubliniensis*

Genotypic Identification	Phenotypic Identification				
	Temperature 45 ºC	Chromogenic medium	Staib agar	Tobacco agar	Hypertonic medium
C. dubliniensis	Presence of growth	Light-green	Presence of clamydoconidiums	Absence of clamydoconidium	Absence of growth
C. dubliniensis	Presence of growth	Dark-green	Absence of clamydoconidium	Presence of clamydoconidiums	Absence of growth

In this area, the prevalence of *C. dubliniensis* isolates seems to be lower than the that found in European countries or in the USA[19,20]. However, in Argentina, *C. dubliniensis* was found in 12.9% of the oropharyngeal isolates of HIV-positive patients[21]. Authors consider their results similar to those found in Ireland (18- 32%), but different from those in Brazil (2.8%), where two (5.4%) isolates of *C. dubliniensis* were identified among 37 strains isolated from the oral erythematous candidiasis from HIV-positive and HIV-negative patients previously identified as *C. albicans*[10]. Our findings confirm the data found in the literature. Two (6.9%) out of a total of 29 strains of the oral mucosa initially identified as *C. albicans* were genotypically characterized as *C. dubliniensis*.

In the present study, we consider that rapid identification of *C. dubliniensis* is relevant because it allows professionals to choose the most appropriate medicine to treat patients, as the species proved resistant to fluconazole.

Routine discrimination between *C. albicans* and *C. dubliniensis* has been a problem in microbiological diagnostic laboratories and the description of phenotypic tests for differentiation between these two species in the literature has increased in the past ten years[22]. Phenotypic tests provide a suggestive identification of the focal species according to its behavior under a given set of conditions. However, although these methods are simple and inexpensive, they are normally time-consuming and/or demonstrate, in most cases, low ability to discriminate the species[23]. All phenotypic tests conducted in the present study showed suggestive strains of *C. dubliniensis*, in addition to the strains confirmed through the molecular test.

The thermotolerance test at 45 °C is considered easy to perform and is frequently used in association with other phenotypic tests. However, this test presents low specificity according to some authors considering that *C. albicans* may be incorrectly identified as *C. dubliniensis*[24]. In our study, five (17.2%) of the isolates did not grow at 45 °C, suggesting the presence of *C. dubliniensis.* Conversely, the two isolates molecularly identified as *C. dubliniensis* grew under this temperature. After studying eight isolates of *C. dubliniensis,* Mähnss *et al*.[22] also observed some growth under these conditions. Nevertheless, other authors did not find any strain of *C. albicans* or *C. dubliniensis* with unusual behavior[25,26], as it occurred in the study by Spolidorio *et al*.[27], in which all specimens of *C. dubliniensis* were not able to grow at 45 °C.

Aiming to facilitate the isolation and presumptive identification of yeasts, many culture media containing chromogenic substrates have been developed and used to investigate the colonization and infection by *C. dubliniensis*, considering that the dark green color of its colonies in these media is considered specific for this species[28].

In this study, four (13.7%) isolates showed dark-green color suggestive of *C. dubliniensis*. However, only one of these isolates was confirmed to belong to this species. Sahand *et al*.[29] observed that 12 (12.2%) out of the 98 studied strains presented dark-green color, but they were later identified as *C. albicans*[29]. This result differs from that obtained in the study by Kirkpatrick *et al*.[30], in which 23 out of 63 strains presented dark-green color, but 16 (69.5%) were later identified as *C. dubliniensis.*

According to Tintelnot *et al*.[31], strains subcultured at 37 °C in chromogenic medium may lose their ability to show specific coloring. This may explain the mismatch between chromogenic medium coloring and the genotypic results observed in this study.

In recent years, many plant-extract and seed-based culture media have been used as phenotypic markers to distinguish *C. albicans* from *C. dubliniensis*. Staib agar (or Niger agar) and Tobacco agar are some examples[14,15].

In the present study, we observed that two (6.9%) strains presented clamydoconidiums when microcultured in Staib agar, and one of them was molecularly confirmed as *C. dubliniensis*. The other genotypically identified *C. dubliniensis* strains did not produce such structures. Al Mosaid *et al.*[32] reported a significant percentage of isolates of *C. dubliniensis* (14.6%) that were not able to produce clamydoconidiums in this medium. According to Tintelnot *et al*.[31], the presence of abundant chlamydospore formation is considered to have low predictive value to differentiate these species. However, Pasligh *et al*.[33] state that the use of these phenotypic methods, after scanning with chromogenic medium, is a simple, low-cost, highly susceptible technique for differentiation between these species in the laboratorial routine. In our study, the phenotypic technique using Staib agar showed higher susceptibility (96.3%) in comparison with other techniques.

When cultured in Tobacco agar, six (20.7%) strains presented abundant formation of clamydoconidiums, characteristics suggestive of *C. dubliniensis*. One of these strains was identified as *C. dubliniensis* using the molecular method, but the other *C. dubliniensis* strains did not present clamydoconidiums in this medium. Notably, Khan *et al*.[15] concluded that this medium is 100% efficient because all *C. dubliniensis* isolates tested had produced abundant clamydoconidiums after incubation. Other authors have also suggested that this is a presumptive method used for identification of *C. dubliniensis*[11,34], although they never obtained total efficacy in this medium.

In Sabouraud agar medium supplemented with 6.5% NaCl, five strains did not present any growth. Only two of these strains were identified as *C. dubliniensis*, whereas the others were *C. albicans*. In a study conducted by

Oliveira *et al.*[35], all the 39 strains identified as *C. albicans* presented growth in this medium; likewise in the study developed by Chowdhary *et al*.[36], in which all the 84 strains identified as *C. albicans* presented growth.

In this study, none of the phenotypic tests alone proved to be highly effective for a conclusive identification of *C. dubliniensis* or *C. albicans*. No test showed 100% susceptibility and only the hypertonic medium showed 100% specificity. In the study performed by Pineda *et al.*[34], despite the fact that none of the tests used was 100% susceptible or specific, the authors considered that at least three different phenotypic tests should be used to properly distinguish the two species.

The phenotypic techniques used in our study suggested the presence of *C. dubliniensis* among the studied strains. However, there was no coherence between the tests to reach a conclusive identification. Furthermore, none of these methods showed full agreement with the genotypic method.

The results of the identification obtained through phenotypic tests were discussed in relation to the findings of the genotypic identification, which is considered the gold standard.

Therefore, there is still the need to use genotypic methods to achieve conclusive identification and differentiation between species because most of the phenotypic tests show ambiguous results, as demonstrated herein.

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CONFLICT OF INTERESTS

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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