Article/Artigo

Differentiation between *Candida albicans* and *Candida dubliniensis* using hypertonic Sabouraud broth and tobacco agar

Diferenciação entre *Candida albicans* e *Candida dubliniensis* usando caldo *Sabouraud* hipertônico e ágar Tabaco

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ABSTRACT

Introduction: Opportunistic fungal infections in immunocompromised hosts are caused by *Candida* species, and the majority of such infections are due to *Candida albicans*. However, the emerging pathogen *Candida dubliniensis* demonstrates several phenotypic characteristics in common with *C. albicans*, such as production of germ tubes and chlamydospores, calling attention to the development of stable resistance to fluconazole *in vitro*. The aim of this study was to evaluate the performance of biochemistry identification in the differentiating between *C. albicans* and *C. dubliniensis*, by phenotyping of yeast identified as *C. albicans*. **Methods:** Seventy-nine isolates identified as *C. albicans* by the API system ID 32C were grown on Sabouraud dextrose agar at 30°C for 24-48h and then inoculated on hypertonic Sabouraud broth and tobacco agar. **Results:** Our results showed that 17 (21.5%) isolates were growth-inhibited on hypertonic Sabouraud broth, a phenotypic trait inconsistent with *C. albicans* in this medium. However, the results observed on tobacco agar showed that only 9 (11.4%) of the growth-inhibited isolates produced characteristic colonies of *C. dubliniensis* (rough colonies, yellowish-brown with abundant fragments of hyphae and chlamydospores). **Conclusions:** The results suggest that this method is a simple tool for screening *C. albicans* and non-*albicans* yeast and for verification of automated identification.

Keywords: Biochemistry identification. Phenotypic characteristics. *Candida albicans*.

RESUMO

Introdução: Infecções fúngicas oportunistas em hospedeiros imunocomprometidos são causadas por espécies de *Candida*, cuja maioria das infecções se deve a *Candida albicans*. Entretanto, o patógeno emergente *Candida dubliniensis* demonstra várias características fenotípicas em comum com *C. albicans*, tais como produção de tubo germinativo e clamidósporos, solicitando atenção por desenvolver resistência *in vitro* estável ao fluconazol. O objetivo do presente estudo foi avaliar a performance da identificação bioquímica na diferenciação entre *C. albicans* e *Candida dubliniensis*, analisando fenotipicamente leveduras previamente identificadas como *C. albicans*. **Métodos:** Setenta e oito isolados identificados como *C. albicans* pelo sistema API ID 32C foram cultivados em ágar *Sabouraud* dextrose a 30°C por 24-48h e em seguida inoculados em caldo hipertônico *Sabouraud* e agar tabaco. **Resultados:** Nossos resultados mostraram que 17 (21,5%) isolados tiveram o crescimento inibido no caldo hipertônico *Sabouraud*, característica fenotípica inconsistente para *C. albicans* neste meio de cultura. Entretanto, os resultados observados em ágar tabaco mostraram que somente 9 (11,4%) dos isolados inibidos produziram colônias características de *C. dubliniensis* (colônias rugosas, marrom-amarelada com fragmentos de hifas e abundantes clamidósporos). **Conclusões:** Os resultados obtidos sugerem que este é um instrumento simples para triagem entre leveduras de *C. albicans* e não-*albicans*, bem como confirmação de identificação automatizada.

Palavras-chaves: Identificação bioquímica. Características fenotípicas. *Candida albicans.*

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INTRODUCTION

The increased incidence of opportunistic fungal infections has been well documented, especially during the last two decades. The main factor contributing to this increase is growing number of immunocompromised patients[1]. Opportunistic fungal infections are caused above all by *Candida albicans* and other *Candida* species[2].

C. albicans is a human pathogenic fungus capable of infecting both the skin and mucous membranes, often causing severe systemic infections in immunocompromised hosts[3]. Distinctive features of this species are morphological conversion in the body of the host yeast blastoconidia and hyphae true, and germ tube formation[4]. *C. dubliniensis* is a yeast that has been isolated in various geographical regions and is associated with superficial and systemic infections in immunocompromised individuals, especially those infected with HIV, who need of attention due to their ability to develop resistance to fluconazole *in vitro*[5]. This species has several morphological, phenotypic, biochemical and virulence characteristics that are similar to those of *C. albicans*, hampering the presumptive identification[6].

The most accurate method for identifying of *C. dubliniensis* and discriminating it from *C. albicans* is based on PCR[7]. Carbohydrate assimilation for yeast identification is widely used in laboratories due to the speed. However, systems such as API 20C AUX and Vitek YBC have not been entirely specific in differentiating of *C. albicans* and *C. dubliniensis* and thus require additional phenotypic tests[8].

Several phenotypic tests differentiating to differentiate between the two species have been described, such as discrimination by color on CHROMagar, growth at 42 and 45°C[9], and produce fluorescence in Sabouraud methylene blue (for isolates of *C. albicans*) and abundant production of chlamydospores on cornmeal agar with Tween-80 (by isolates of *C. dubliniensis*)[10].

Hypertonic Sabouraud broth[11] and tobacco agar[12] culture media have been described to distinguish between these species with high reproducibility and simplicity at low cost, results using this media agreed with those of molecular identification. In hypertonic Sabouraud broth (Sabouraud dextrose broth with 6.5% NaCl), *C. dubliniensis* displays no growth, and its micromorphological aspects on tobacco agar is distinguishable from that of *C. albicans*.

In this study, we evaluated the performance of carbohydrate assimilation (API ID 32C) and phenotypic identifications in the differentiation between *C. albicans* and *C. dubliniensis* of yeasts previously identified as *C. albicans*.

METHODS

Isolates

A total of 79 yeast isolates identified as *C. albicans* by the API ID 32C system (bio Merieux, France) were studied. The analyzed isolates belong to the culture collection of the Mycology Laboratory of the Instituto Evandro Chagas. The yeasts were stored in Malte Extrat broth medium and 15% glycerol at -70°C, and were grown on Sabouraud dextrose agar (Difco, Detroit, Mich.) and maintained at 30°C for 24-48h for testing. All were isolates from culture of wide spectrum of clinical presentation of patients (hospital/outpatient) suspected of fungal infection (skin, sputum, bronchoalveolar lavage and hemoculture), in the period of 2005 to 2009. *C. albicans* (ATCC 90028) and *C. dubliniensis* (was obtained from the *Escola Paulista de Medicina* Yeast Collection, Mycology Laboratory, São Paulo, Brazil.) strains were used as control both for hypertonic Sabouraud broth and tobacco agar.

API ID 32C system

According to the instructions of the manufacturer (bio Merieux, France), 250µL of the yeast suspension was inoculated and homogenizated in an ampule of API C medium. An aliquot (135µL) of this suspension was dispensed into each well of the strip and incubated at 28°C for 24-48h. Strip was read using the *mini API*® instruments and identification was obtained using the database (V2.0). After automatic reading the results transmitted to the computer was interpreted by the identification software.

Hypertonic Sabouraud broth

Hypertonic Sabouraud broth was produced as described by Alves et al[11]. Briefly, isolates from a 48h culture on Sabouraud agar plates were suspended in sterile distilled water (0.5 Mc Farland). Aliquots (20µL) of each suspension were added to tubes containing 1ml of sterile Sabouraud broth supplemented with 6.5% NaCl and then statically incubated for 96h. The cultures were then visually examined for the detection of fungal growth at 24h intervals. A positive test result was defined as the absence of any visible colony growth of in the test tube after 96h of incubation. In this case, *C. albicans* but not *C. dubliniensis* is able to grow in an excess of NaCl.

Tobacco agar

Tobacco was produced with agar as described previously[12]. Briefly, 50g of tobacco from commercially available tobacco (*Fumo Extra Forte* LTDA, Brazilian Industry, Manaus, State of Amazonas) was mixed with 1L of distilled water. The mixture was boiled for 30min and then filtered through several layers of gauze. To this filtrate, 20g of agar was added, and the volume was adjusted made up to 1L. The pH of the medium at this point was 5.4. It was autoclaved at 121°C for 15min and poured into each Petri plates. Yeast isolates were freshly subcultured on Sabouraud dextrose agar (Difco, Detroit, Mich.), and the tobacco agar plates were streaked with a small amount of inoculum from the isolated colonies. The culture plates were incubated at 28°C, and the macro and micromorphological characteristics were observed daily up to 96h. The isolates were observed under a light microscope (Carl Zeiss) at lower (10X) and higher magnification (40X) to identify fragments of hyphae and chlamydospores produced by *C. dubliniensis*.

RESULTS

In this study, 79 yeasts previously identified as *C. albicans* by the API ID 32C system were tested for salt tolerance (hypertonic Sabouraud broth) and growth on tobacco agar. Seventeen isolates (17/79; 21.5%) were growth inhibited in hypertonic Sabouraud broth, initially suggesting that they represented *C. dubliniensis* as described by Alves et al in 2002[11]. However, eight of the growth-inhibited isolates (8/79; 10.1%) showed morphotype and micromorphology on tobacco agar typical of *C. albicans*. Smooth colonies of a whitish-cream color developed, and microscopy revealed globose yeasts with or without one bud, eliminating the possibility of identification as *C. dubliniensis*.

Colonial and micromorphological aspects suggestive of *C. dubliniensis* on tobacco agar were observed for nine isolates (9/79; 11.4%) inhibited in hypertonic Sabouraud broth. This presented rough/brownish-yellow colonies constituted of pseudohyphae and hypae with chlamydospores. **Table 1** summarizes the results obtained on tobacco agar for isolates inhibited in hypertonic Sabouraud broth, and the values indicated the percentage of identified as *C. albicans* by API ID 32C.

DISCUSSION

Hypertonic Sabouraud broth and tobacco agar were developed to discriminate between *C. albicans* and *C. dubliniensis*, based on the different phenotypic characteristics of these two species. In these studies[11,12], the carbohydrate assimilation was used as a test standard to identify and correctly differentiate between the two species. However, our results suggest that there may be a bias in species identification during the discrimination by API ID 32C system. One reason for this is that the carbohydrate assimilation profile, which is foundation of the API ID 32C identification system, shows significant variations among strains of the same species; some authors have suggested that such tests have low discriminatory power[8,13]. Other trials have been conducted that identify *C. dubliniensis* by the absence of growth at 42°C and the activity of α-D-glucosidase, but false positives can be obtained because of atypical strains of *C. albicans* are capable of displaying these phenotypic aspects[14].

The isolates inhibited in hypertonic Sabouraud broth, with typical aspects of *C. albicans* (colonies white/smooth with single yeasts) on tobacco agar, may represent non-osmotolerant, non-*albicans* species (e.g., *C. glabrata*) that are mistakenly identified by carbohydrate assimilation[15,16]. It is known that *C. albicans* isolates after long-term storage can produce both morphological and enzymatic changes[17], by phenomenon known as phenotypic switching. This event is spontaneous, reversible and observed at high frequencies[18]. At despite of morphotyping on stored isolates is not recommended due to occurrence of switching, in our laboratory was not observed change

TABLE 1 - Complete description of tests to discriminate *C. dubliniensis* from *C. albicans* based on the lack of growth in hypertonic Sabouraud broth (HSB); morphotyping on tobacco agar of yeasts identified as *C. albicans* by API ID 32C.

	Culture medium				
		Tobacco agar			% of identification
Isolates	HSB	Macromorphology	Micromorphology	Source	by API ID 32C
10	-	Smooth/cream	Yeasts	BAL*	99.9
28	-	Smooth/cream	Yeasts	Sputum*	99.9
37	-	Rough/brown	Hyphae/chlamydospores	Anal mucosa**	99.9
47	-	Rough/brown	Hyphae/chlamydospores	Skin**	99.9
65	-	Rough/brown	Pseudohyphae	BAL*	89.4
68	-	Rough/brown	Pseudohyphae	Oral mucosa**	96.1
76	-	Rough/brown	Hyphae/chlamydospores	BAL*	99.9
86	-	Rough/brown	Chlamydospores	BAL*	99.9
91	-	Smooth/cream	Yeasts	Sputum*	99.9
102	-	Smooth/cream	Yeasts	Skin**	99.9
103	-	Smooth/cream	Yeasts	Sputum*	99.9
130	-	Rough/brown	Pseudohyphae	BAL*	99.9
139	-	Smooth/cream	Yeasts	BAL*	99.9
149	-	Smooth/cream	Yeasts	BAL*	99.9
156	-	Smooth/cream	Yeasts	BAL*	99.9
157	-	Rough/brown	Pseudohyphae	BAL*	99.9
199	-	Rough/brown	Pseudohyphae	BAL*	99.2
CA	+	Smooth/cream	Yeasts	Blood	99.9
CD	-	Rough/brown	Hyphae/chlamydospores	NI	98.9

CA: *Candida albicans,* **CD:** *Candida dubliniensis,* -: negative, +: positive, **BAL:** bronchoalveolar lavage, **NI:** No information.
*Hospital, **Outpatient.

of colony in culture medium of routine, such as Sabouraud dextrose agar, during this experiment. Only were obtained colonies smooth/white before of the proceeding of salt tolerance and submission at tobacco agar. Nevertheless, we cannot to exclude the hypothesis of that the cause from isolates inhibited in hypertonic Sabouraud broth and constituted of pseudohyphaes (n = 5) is the switching. Additionally, this study is one of few relates of that salt tolerance can be affected by phenotypic switching in isolates of *C. albicans* storage for long-term, but the inclusion of others parameters, such as molecular identification, is critical for to corroborate this result.

Tobacco agar was developed for the presumptive identification of *Cryptococcus neoformans*[19] and later to discriminate between isolates of *C. albicans* and *C. dubliniensis* with 100% accuracy[12], displays a rough colony on tobacco agar, although isolates of *C. krusei*, *C. tropicalis*, and *C. parapsilosis* also produce fragments of hyphae after 48h of incubation. This leads us to believe that the three isolates inhibited in hypertonic Sabouraud broth and with morphotyping in tobacco agar suggestive of *C. dubliniensis* need of confirmation using molecular tools based in species-specific primers.

Based on these results, the carbohydrate assimilation and phenotyping gave the same result in 78.5% of isolates for identifying *C. albicans*. However, the results of 21.5% of the isolates were outliers for biochemistry identification, suggesting a low discriminatory power between the two closely related species *C. albicans* and *C. dubliniensis*. Therefore, the low cost and minimal infrastructure necessary to carry out the methodology in our study make the application of hypertonic Sabouraud broth and tobacco agar an attractive strategy to inquire concerning identification for carbohydrate assimilation in the discrimination between these two species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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