Differentiation of *Candida dubliniensis* from *Candida albicans* on Rosemary Extract Agar and Oregano Extract Agar

Érico Silva de Loreto, Patrícia Pozzatti, Liliâne Alves Scheid, Deise Santurio, Janio Moraes Santurio, and Sydney Hartz Alves*

Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

Candida dubliniensis is a recently described pathogenic species which shares many phenotypic features with *Candida albicans* and therefore, may be misidentified in microbiological laboratories. Because molecular methods can be onerous and unfeasible in routine mycological laboratories with restricted budgets such as those in developing countries, phenotypic techniques have been encouraged in the development of differential media for the presumptive identification of these species. We examined the colony morphology and chlamydospore production of 30 *C. dubliniensis* isolates and 100 *C. albicans* isolates on two new proposed media: rosemary (*Rosmarinus officinalis*) extract

agar (REA) and oregano (*Origanum vulgare*) extract agar (OEA). These substrates are traditionally used as spices and medicinal herbs. In both of these media, all *C. dubliniensis* isolates (100%) showed rough colonies with peripheral hyphal fringes and abundant chlamydospores after 24 to 48 hr of incubation at 25°C. In contrast, under the same conditions, all isolates of *C. albicans* (100%) showed smooth colonies without hyphal fringes or chlamydospores. In conclusion, REA and OEA offer a simple, rapid, and inexpensive screening media for the differentiation of *C. albicans* and *C. dubliniensis*. J. Clin. Lab. Anal. 22:172–177, 2008. © 2008 Wiley-Liss, Inc.

Key words: *Candida*; yeasts; identification; chlamydospores; rosemary; oregano

INTRODUCTION

Candida dubliniensis is an emerging opportunistic species of chlamydospore and germ tube positive yeast described by Sullivan et al. (1), which is phenotypically and genotypically similar to *Candida albicans* and *Candida stellatoidea*. Although previously associated with oral candidiasis in human immunodeficiency virus (HIV)-infected patients (1), there is a growing number of reports describing *C. dubliniensis* isolation in diabetic patients (2), cystic fibrosis patients (3) and cancer patients (4). It has also been identified as a cause of systemic diseases (4), pulmonary infection (5) and endocarditis (6).

C. dubliniensis is usually isolated in mixed cultures with *C. albicans* and/or other yeasts evidencing good growth in the Sabouraud dextrose agar at 30–37°C and forming white-creamy colonies, similar to *C. albicans*. As does *C. albicans*, the *C. dubliniensis* species emits a germ tube when incubated in serum at 37°C for 2 hr, producing pseudohyphal and chlamydospores in media such as cornmeal agar.

Usually the isolates of *C. dubliniensis* differ from *C. albicans* isolates in their production of abundant chlamydospores, forming pairs or triplets attached to pseudohyphae (1), while *C. albicans* isolates rarely present chlamydospores to the pairs or triplets. This identification criterion is conflicting and subjective and should not be used as the only differentiation test (7).

However, under certain environmental conditions, such as growth on Staib and Pal's agar (8,9), the isolates of *C. dubliniensis* produce rough colonies and abundant chlamydospores while *C. albicans* produce smooth colonies with no rough colonies or chlamydospore formation, characteristics that can be used as specific species markers for the identification of *C. dubliniensis*.

*Correspondence to: Sydney Hartz Alves, Rua Andradas 1985/201, Santa Maria (RS), Brazil 97010-033. E-mail: hartzsa@ccs.ufsm.br

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These media are considered to be good for discriminatory tests but are not reliable media for differentiating between *C. dubliniensis* and *C. albicans* in some studies (10–13).

C. dubliniensis seems to be a minor constituent of the normal human flora and the human oral cavity appears to be the preferred human location. In spite of this, it may in some cases cause systemic infections, although it does so far less frequently than *C. albicans*, which suggests that *C. dubliniensis* presents a reduced virulence, as it has been obtained in comparative studies using a mouse model of systemic infection (14).

The majority of *C. dubliniensis* clinical isolates examined has exhibited high susceptibility to commonly used antifungal agents. However, isolates with significantly reduced susceptibility to fluconazole have been recovered from acquired immunodeficiency syndrome (AIDS) patients previously treated with this azole; on the other hand, susceptible isolates can quickly become resistant by subculturing the organisms on medium supplemented with progressively increasing concentrations of drugs (15).

This problem emphasizes the need for developing simple methods of differentiation between *C. albicans* and *C. dubliniensis* because these species share many phenotypic characteristics, such as the production of chlamydo spores on rice-agar-Tween and of germ tubes in serum as well as having similar biochemical patterns (1). Consequently, several genotypic diagnostic assays and a commercial method (Bichro-Dublin Fumouze[®]; Fumouze Diagnostics, Asnieres, France) have been developed to aid in the correct identification of *C. dubliniensis* (16,17). However, they can be expensive in routines of microbiological laboratories with restricted budgets such as those in developing countries.

The objectives of the present study were to examine the colony morphology and chlamydo spore production of *C. albicans* and *C. dubliniensis* on two new proposed media: rosemary (*Rosmarinus officinalis*) extract agar (REA) and oregano (*Origanum vulgare*) extract agar (OEA) and then to evaluate their use as a simple technique for differentiating between *C. albicans* and *C. dubliniensis*.

MATERIALS AND METHODS

Culture

The aqueous extracts of rosemary and oregano were obtained after 2 hr of boiling in the Clevenger apparatus in order to remove essential oils (200 g of the entire fresh plant per liter of distilled water). After filtration through several layers of gauze, 200 mL of each extract were incorporated into 800 mL of a basal medium (dextrose 1%; creatinine 0.078%; agar 2%, and 800 mL of

distilled water), which was autoclaved at 121°C/15 min. Immediately after that, Petri dishes were filled with 25 mL of the medium. After solidification, a single colony freshly subcultured on Sabouraud dextrose agar was streaked, inoculated separately, and incubated for 24 to 48 hr at 25°C. Niger seed agar and Pal's agar were prepared as previously indicated (8,9) and used as reference media for all isolates used in the study.

Strains Tested

A total of 133 clinical yeast isolates were used in this study, including: *C. albicans* (n = 100 isolates), *C. dubliniensis* (30), *C. pelliculosa* (1), *C. haemulonii* (1), and *C. krusei* (1); as well as six reference strains: *C. albicans* ATCC 28367, *C. albicans* ATCC 44373, *C. dubliniensis* CBS 7987, *C. glabrata* ATCC 2201, *C. lusitanae* ATCC 66035, and *C. parapsilosis* ATCC 90018.

All the clinical isolates of *C. dubliniensis* were previously identified by randomly amplified polymorphic deoxyribonucleic acid (DNA) (RAPD), using the primers CDU (5'-GCG ATC CCC A-3') (1) and B-14 (5'-GAT CAA GTC C-3') (18). *Candida* strains were obtained from patients with different clinical forms of candidiasis including oropharyngeal candidiasis, vulvovaginal candidiasis, candidemia, intertriginous, and nail involvement. The majority of *C. dubliniensis* isolates were obtained from AIDS patients with oropharyngeal candidiasis; two isolates were from vulvovaginal candidiasis (AIDS patients) and one strain was isolated from the normal flora of the sputum of a non-AIDS patient. These strains were isolated from different hospital services located in six Brazilian cities. All strains were maintained in the fungal stock collection of the Laboratório de Pesquisas Micológicas (LAPEMI), Universidade Federal de Santa Maria (Santa Maria City, Brazil).

Phenotypic Analysis

Initially, the macroscopic appearance of the colonies on the plates was observed. Then the microscopic characteristics were evaluated with objective 10 × and 40 × magnifications in order to detect the presence or absence of hyphal fringes and chlamydo spores. All the plates, including the reference media, Staib agar, and Pal's agar, were inspected daily, for an observation period of 8 days.

RESULTS

In 24-hr intervals, colonies were examined microscopically for the presence or absence of hyphal fringes and chlamydo spores. All the isolates tested grew well on proposed and reference media. On REA and OEA agar,

Fig. 1. Observation from the optic microscope of the morphology of the colonies in the oregano agar. **A,B:** *C. dubliniensis* (100 and 400 $\times$). **C,D:** *C. albicans* (100 and 400 $\times$).

all 30 isolates and the reference strain of *C. dubliniensis* (100%) produced rough colonies with hyphal fringes and abundant chlamydospores on the peripheral hyphal fringes, easily visualized with an optic microscope after incubation for 48 hr at 25°C (Fig. 1), and it was unnecessary to use coverslips to generate microaerophilic conditions.

In contrast to the rough colonies of *C. dubliniensis*, the appearance of all isolates of *C. albicans* (100%) was of smooth white-to-cream-colored colonies without hyphal fringe or chlamydospore formation. These studies were carried out three times on different days, with different batches of fresh plant.

On Staib and Pal's agar, used as reference media, the results were similar and all isolates of *C. dubliniensis* produced rough colonies and abundant chlamydospores, while *C. albicans* showed smooth colonies with no evidence of chlamydospore formation.

Similar to *C. albicans*, the isolates of *C. glabrata*, *C. lusitaniae*, and *C. haemulonii* did not demonstrate peripheral hyphal structures or chlamydospore formation on REA and OEA agar, while some peripheral hyphal structures were seen in *C. krusei* and *C. parapsilosis* isolates after incubation for 48 hr at 25°C. *C. pelliculosa* showed abundant peripheral hyphal structures (Fig. 2).

DISCUSSION

Infections caused by yeast have become more common in recent years due to AIDS and the rising number of immunocompromised patients. While

infections involving *C. albicans* are still the most common, the significance of other *Candida* spp. is increasing as they are more resistant to antifungal agents (19).

The rapid differentiation between *C. albicans* and *C. dubliniensis* isolates with differential media is of special importance because *C. dubliniensis* can quickly become resistant to fluconazole and is usually isolated in mixed cultures with *C. albicans* and/or other yeasts in plates with traditional media, such as Sabouraud dextrose agar (1,15).

This study aimed to evaluate the morphologic behavior of *C. albicans* and *C. dubliniensis* in the proposed media based on oregano and rosemary extracts agar, two very well known plants used in cooking and pharmaceutical sciences.

Some researchers have observed that the isolates of *C. dubliniensis* are incapable of growing at 42°C or 45°C, in contrast to *C. albicans* (1,20). However, keeping an incubator at 42°C or 45°C solely for the differentiation of *C. albicans* from *C. dubliniensis* can become economically impracticable, since most laboratories use incubators at 25°C and 35°C. On the other hand, some isolates of *C. albicans* were not able to grow at 42°C and 45°C (7). Moreover, poor growth at elevated temperatures is a feature that *C. dubliniensis* shares with *Candida stellatoidea*, which suggests that this test presents low specificity and that *C. albicans* could be falsely identified as *C. dubliniensis*.

In CHROMagar Candida agar (CHROMagar, Paris, France), the contrast between dark green (*C. dubliniensis*) with light-blue or blue-green colonies (*C. albicans*)

TABLE 1. Comparison of extracts used and the incubation temperature in the differentiation of *C. albicans* from *C. dubliniensis*

Extract	<i>C. albicans</i>			<i>C. dubliniensis</i>			<i>t</i> (°C)	Reference number
	Rough colonies (%)	Chlamydospore Formation (%)	n	Rough colonies (%)	Chlamydospore formation (%)	n		
Niger	0 ^a	0	11	100	100	14	30	8
Niger	0 ^a	0	166	97,7	85,4	130	30	10
Sunflower	0 ^a	0	124	100	93,75	128	30	9
Niger	1,4	—	422	88,8	—	9	37	11
Sunflower	0	0	53	100	100	25	28	30
Niger	68,1	1,1 ^a	94	100	66,7	6	30	12
Tobacco	0	0	54	100	100	30	28	22
Sunflower	3,8	0	105	100	100	40	28	31
Sesame	0	0	102	100	100	27	25	23
Rapeseed	1	0	102	100	100	27	25	23
Canary Grass	0	3	102	100	100	27	25	23
Millet	4	0	102	100	96	27	25	23
Linseeds	0	0	102	100	100	27	25	23

^aAfter 48 hr of incubation.

n, number of isolates tested; *t*, temperature of incubation.

can be difficult. Another problem with CHROMagar is the decline of differentiation with cultures already subcultured (21), where *C. dubliniensis* can exhibit the light green appearance of *C. albicans*. The use of the methyl-blue 93 Sabouraud dextrose agar has shown low specificity and presents the disadvantage that the fluorescent hydrolysis products can be diffused to the agar and to the neighboring colonies after 24 hr of incubation, besides the need for ultraviolet light (7).

The colonial morphology and chlamydospore production on Niger seed agar or sunflower seed agar are, in our experience, the best options and have been used broadly. However, variations in the composition of these agars, and the use of different proposed media, such as tobacco, sesame, and linseed media (22,23), have shown similar results (Table 1). On the other hand, caffeic acid-ferric citrate and casein agar are not advantageous if compared with these (10,24).

Coaggregation with *Fusobacterium nucleatum* requires the maintenance of this anaerobic bacterium, in order to carry out the test (25). In Tween 80 opacity agar *C. dubliniensis* does not exhibit the halo around the colony, but some *C. albicans* isolates also failed to show this halo (26).

Differentiation based on the inability of *C. dubliniensis* to grow in hypertonic broth (NaCl 6.5%) requires a longer time (4 days) and some *C. albicans* isolates do not support this high salt concentration, reducing the specificity of the test (27).

Recently, a new phenotypic commercial method (latex agglutination), Bichro-Dublin Fumouze[®] based on monoclonal antibody (12F7-F2) was developed in order to detect *C. dubliniensis* antigens (17). This test

presents sensitivity above of 97% and specificity of 100%, including the identification of the slower isolates (genotype 4). It presents either lower cost to identification than that performed by polymerase chain reactions (PCR) (28,29). However, the use of this latex agglutination test can be onerous due to low prevalence of *C. dubliniensis* in the clinical mycology laboratory.

Due to these problems and other phenotypic similarities between the two species, the definitive identification of *C. dubliniensis* continues to be based on differential amplification of species-specific sequences using the PCR and real-time PCR (16). These and other genotyping techniques demonstrate indispensable elucidations that are specific, fast, and applicable to a great number of isolates.

The two media here proposed, REA and OEA, showed similar results and allowed the differentiation between *C. albicans* and *C. dubliniensis* isolates. All *C. albicans* (100%) showed colonies without hyphal fringe and blastospores were frequently present. All *C. dubliniensis* isolates (100%) grew as rough colonies producing hyphal fringes and abundant chlamydospores. Further studies including a larger number of isolates are necessary to confirm the present results. The results obtained with the proposed media were the same as those observed with Niger seed agar and sunflower seed agar, here used as reference media. If compared with Niger seed agar or sunflower agar, the disadvantage of the REA and OEA is the two hours period of boiling the vegetable material in order to remove the essential oils. On the other hand, the great advantage of REA and OEA is the limpid and translucent aspect of the media, favoring enormously the microscope

Fig. 2. Macroscopic morphology of *C. glabrata* (A) (100 $\times$), *C. lusitanae* (B) (400 $\times$), *C. pelliculosa* (C) (40 $\times$), and *C. parapsilosis* (D) (100 $\times$).

observations. When we added antibacterial agents and the media were employed as primary planting media, the screening of *C. dubliniensis* was very easy and preliminary observations of this technique showed excellent performance. Studies in this direction are in progress.

The morphologic characteristics of other *Candida* species on REA and OEA were varied (Fig. 2), without species characterization, which is in accordance with the morphologic variability observed by other researchers using similar media (9,22). Thus, the presence of smooth colonies or rough colonies on the proposed media does not allow to suggest the species. The formation of chlamydospores was observed only in *C. dubliniensis* isolates.

For the analysis of fungal structures, it is essential that the isolates tested exhibit a good growth. Isolates of *C. dubliniensis* presented good growth and in 24 hr it was already possible to see the pseudohyphae and *chlamydospore* formation, mainly in the edges of areas of dense growth. Obviously, these structures are more abundant when visualized at least after 48 hr and during the following days of incubation. Eventually, after 5 days of incubation, some isolates of *C. albicans* can present mycelia-like structures, characteristic of those that can also be observed in traditional media, after prolonged incubations periods. However, the formation of *chlamydospores* in *C. albicans* was not observed at any time of tested incubation.

It is noteworthy to emphasize that regardless of the extract used, the temperature was a determinative factor for the abundant production of chlamydospores in the *C. dubliniensis* isolates on REA and OEA. When plates

were incubated at 37°C, the formation of chlamydospores was varied and reduced, with peripheral hyphal fringes formation increased in some isolates. This fact could come to explain the inter-laboratory variation in the effectiveness presented by Staib and Pal's agar as well as others media (Table 1).

In conclusion, in a context where *C. dubliniensis* may be underreported in clinical samples because most of the identification methods used fail in the recognition of this species, we suggested the new media REA and OEA as a simple option for an inexpensive phenotypic screening between *C. albicans* and *C. dubliniensis*, being that definitive identification of *C. dubliniensis* still requires genotypic techniques.

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