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NOTES

Tobacco Agar, a New Medium for Differentiating *Candida dubliniensis* from *Candida albicans*

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Isolates of *Candida dubliniensis* may be misidentified as *Candida albicans* in microbiological laboratories if only the germ tube and/or the chlamydospore test is used for identification to the species level. In this study, we have evaluated the efficacy of tobacco agar for the differentiation of *C. dubliniensis* from *C. albicans*. On this medium at 28°C, all 30 *C. dubliniensis* isolates produced yellowish-brown colonies with hyphal fringes and abundant chlamydospores, whereas 54 *C. albicans* isolates formed smooth, white-to-cream-colored colonies with no chlamydospore production. This medium provides a simple tool for presumptive differentiation of *C. dubliniensis* from *C. albicans*.

Candida dubliniensis is a newly described species that was first isolated from oral cavities of human immunodeficiency virus (HIV)-infected individuals (19). Although initially found to be principally associated with oral carriage and infection, recent reports indicate that this species is capable of causing a variety of clinical conditions, including candidemia, both in HIV-infected and in non-HIV-infected individuals (7, 9, 10, 14, 20). Since *C. dubliniensis*, like *Candida albicans*, forms germ tubes and chlamydospores, it may be misidentified in routine diagnostic laboratories (12). While a number of useful tests based on phenotypic characteristics, such as discrimination based on colony color on CHROMagar Candida medium, growth at 42 to 45°C, and assimilation profiles, have been developed (8, 10, 15, 16), they are not completely reliable (10). PCR-based molecular methods offer the most reliable alternative for definite discrimination of *C. dubliniensis*, but these tests are labor-intensive and may not be readily available (2, 5, 6).

In 1999, Staib and Morschhauser (18) reported that on Staib's Niger seed (*Guizotia abyssinica*)-creatinine agar, *C. dubliniensis* isolates formed rough colonies and abundant chlamydospores but that *C. albicans* isolates grew as smooth colonies without chlamydospores. Those authors concluded that under appropriate growth conditions, the formation of rough colonies and chlamydospores can be used as species-specific markers for the identification of *C. dubliniensis*. Subsequently, Al-Mosaid et al. (3) evaluated the efficacy of Staib agar and caffeic acid-ferric citrate agar for discrimination between *C. dubliniensis* and *C. albicans* and confirmed the usefulness of only Staib agar for this purpose. Those authors inferred that differentiation between these two species was best achieved on the basis of colony morphology (97.7% efficacy). In 2003, three additional publications confirmed the usefulness of Staib agar (11) and sunflower (*Helianthus annuus*) seed agar (1, 4) for this purpose. On the basis of the formation of hyphal fringes alone, Al-Mosaid et al. (4) were able to differentiate with 100% accuracy 128 *C. dubliniensis* isolates on sunflower seed agar. Niger seed agar and sunflower seed agar media were initially developed to differentiate *Cryptococcus neoformans* from other yeasts, as it forms brown colonies on these media (13, 17). Taking a clue from a very recent publication by Tendolkar et al. (21) showing that *C. neoformans* also develops brown colonies on tobacco agar, we thought it would be interesting to test this medium for the differentiation of *C. dubliniensis* and *C. albicans*. The results of the study are presented in this communication.

Twenty-eight local clinical isolates and two reference strains (CD 36 and CBS 7987) of *C. dubliniensis* were studied in comparison with 4 reference strains and 50 randomly selected clinical isolates of *C. albicans*. The identities of the test isolates were established by using the germ tube test, the chlamydospore test on cornmeal-Tween 80 agar, and the Vitek 2 ID-YST system (BioMerieux, Marcy l'Etiole, France). The identities of *C. dubliniensis* and *C. albicans* isolates were further confirmed by seminested PCR amplification of their rRNA genes with species-specific primers corresponding to unique sequences within the internally transcribed spacer 2 regions of *C. dubliniensis* and *C. albicans* and/or by direct sequencing of the internally transcribed spacer 2 regions (2, 5). In addition, five clinical isolates each of *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei* and two reference strains of *Cryptococcus neoformans*, ATCC 90112 (serotype A) and CBS 1930 (serotype B), were also tested for colony characteristics on tobacco agar. The method used for the preparation of tobacco agar was the same as that described by Tendolkar et al. (21), except that we used cigarette tobacco instead of tobacco leaves. Briefly, 50 g of tobacco from commercially available cigarette brands (Marlboro; tar, 8 mg; nicotine, 0.6 mg; Philip Morris Products SA, Richmond, Va.) was mixed with 1 liter of distilled water. The mixture was boiled for 30

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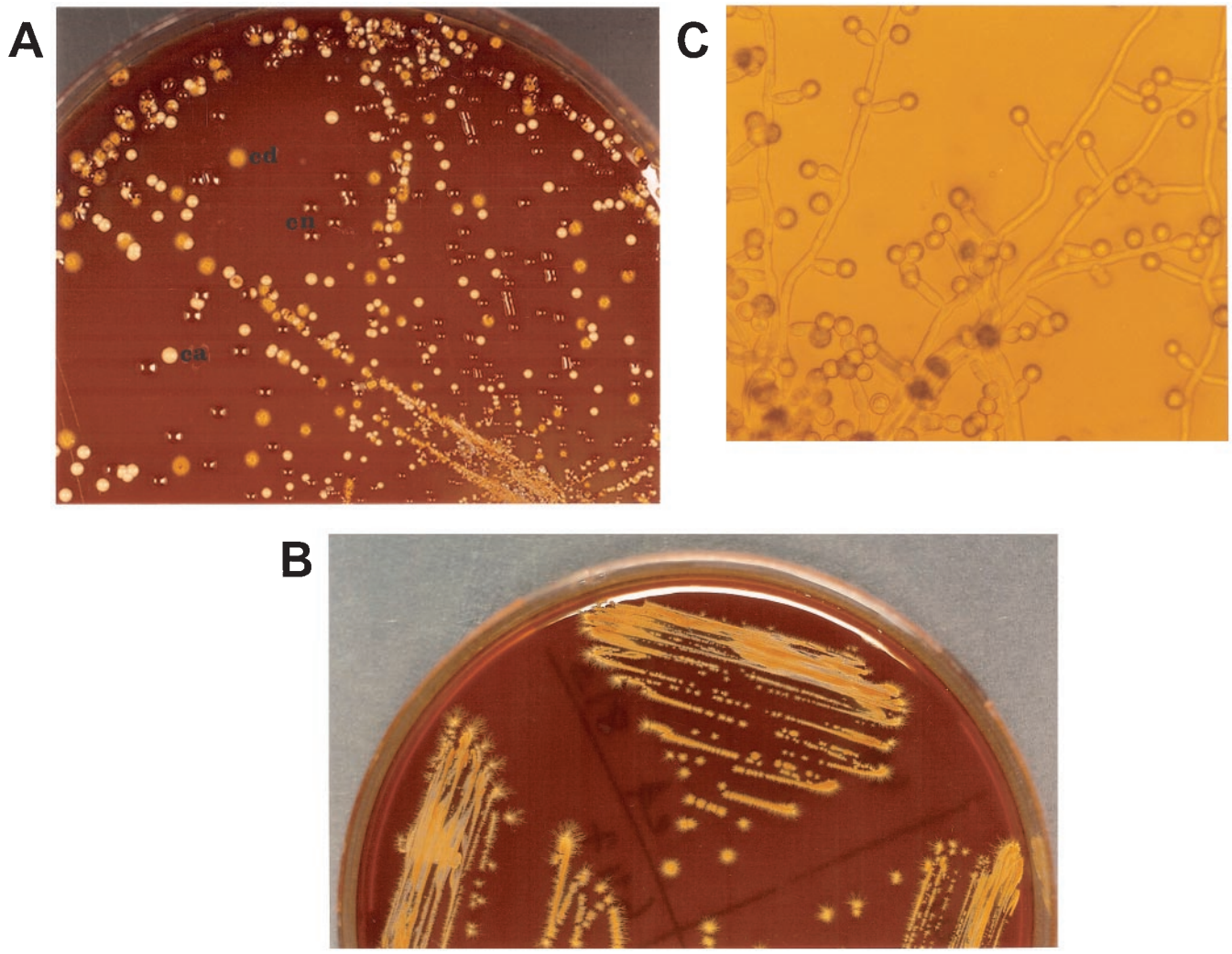

FIG. 1. (A) Tobacco agar showing mixed growth of *C. dubliniensis*, *C. albicans*, and *Cryptococcus neoformans* at 28°C. Note the yellowish-brown colonies of *C. dubliniensis* with hyphal fringes (cd), the white-to-cream-colored colonies with no hyphal fringes of *C. albicans* (ca), and the brown colonies (white spots are due to light reflection) of *C. neoformans* (cn) after 72 h of incubation. (B) Characteristic yellowish-brown, rough colonies with peripheral hyphal fringes of three additional isolates of *C. dubliniensis* on tobacco agar. (C) Chlamydospores of *C. dubliniensis* on tobacco agar after 48 h of incubation (magnification, ×280).

min and then filtered through several layers of gauze. To this filtrate, 20 g of agar was added, and the volume was made up to 1 liter. The pH of the medium at this point was 5.4. It was autoclaved at 121°C for 15 min. Twenty milliliters of medium was poured into each petri plate (90-mm diameter). All the test isolates were freshly subcultured on Sabouraud dextrose agar (Difco), and tobacco agar plates were streaked with a small amount of inoculum from the isolated colonies. The culture plates were incubated at 28°C and observed daily up to 96 h for colony characteristics, such as surface topography (rough or smooth), formation of hyphal fringes at the periphery, and color. Colonies were also observed directly at low-power (×10) and high-power (×40) magnifications for the formation of hyphal fringes and chlamydospores. In addition, tobacco agar prepared from two other commercial brands of cigarettes (Wills Navy Cut [ITC Ltd., Calcutta, India] and Charminar [VST Industries Ltd., Hyderabad, India]) was also used for comparison.

On tobacco agar, all 30 isolates of *C. dubliniensis* produced rough, yellowish-brown colonies with peripheral hyphal fringes after incubation for 48 to 72 h at 28°C (Fig. 1A and B). In addition, all the isolates formed abundant chlamydospores on the peripheral hyphal fringes after 24 to 48 h of incubation at 28°C (Fig. 1C). In contrast, on this medium, all *C. albicans* isolates showed smooth, white-to-cream-colored colonies without hyphal fringes or chlamydospores even after extended incubation for up to 10 days. These observations were reproducible when experiments were repeated on three different occasions. Like *C. albicans*, none of the isolates of *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, or *C. krusei* formed yellowish-brown colonies or chlamydospores on tobacco agar and thus were indistinguishable from each other. However, microscopic examination of *C. krusei*, *C. tropicalis*, and *C. parapsilosis* colonies after 48 h of incubation showed peripheral hyphal fringes in five, three, and one of the isolates, respectively. No peripheral hyphal structures were seen in *C. glabrata* colonies. Two ref-

erence strains of *C. neoformans* produced dark-brown colonies on this medium when they were examined individually and in mixed cultures with *C. dubliniensis* and *C. albicans* (Fig. 1A). Similar observations were made when tobacco agar prepared from other commercial brands (Wills Navy Cut and Charminar cigarettes) were used. Although all initial experiments were performed on freshly prepared media, no appreciable difference in results was obtained with media stored for 7 days. Likewise, no difference in phenotypic characteristics was observed when tobacco agar plates were incubated at 30°C. However, when plates were incubated at 37°C, all 30 *C. dubliniensis* isolates formed rough and fringed colonies, with considerable reduction in the intensity of yellowish-brown pigmentation. Chlamydospores were formed by only two of the isolates, and their numbers were slight. None of the isolates of *C. albicans* formed fringed colonies or chlamydospores at this temperature. Our results suggest that all germ tube- and/or chlamydospore-positive yeast isolates may be tested on tobacco agar to differentiate between *C. dubliniensis* and *C. albicans*.

In conclusion, based on phenotypic characteristics, i.e., the development of yellowish-brown rough colonies, the formation of hyphal fringes around colonies, and the production of abundant chlamydospores, tobacco agar offers an additional simple means for differentiating *C. dubliniensis* from *C. albicans* with 100% accuracy.

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