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Brief Communication

SUNFLOWER SEED HUSK AGAR: A NEW MEDIUM FOR THE DIFFERENTIATION OF *CANDIDA DUBLINIENSIS* FROM *CANDIDA ALBICANS*

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Abstract

A sunflower (*Helianthus annuus*) seed husk agar medium has been developed and evaluated for differentiation of *Candida dubliniensis* from *Candida albicans* on the basis of colony morphology and chlamydospore production. All *C. dubliniensis* isolates (n=40) produced rough colonies with hyphal fringes and abundant chlamydospores whereas 101 of 105 (96.2%) *C. albicans* isolates produced smooth colonies with no evidence of chlamydospore production. Since this medium is free from oil droplets, chlamydospores can be examined with greater clarity by Dalmau plate technique. This medium provides a simple and cost-effective tool for the presumptive differentiation of *C. dubliniensis* from *C. albicans* and is particularly suited for clinical microbiology laboratories where biochemical or molecular methods for the differentiation of these two species are not available.

Key words: *Sunflower, C. dubliniensis, C. albicans, rough colonies, chlamydospores*

Candida dubliniensis is a newly described species of yeast and is phylogenetically closely related to *Candida albicans*.[1,2] Although initially implicated in oral candidiasis in HIV-infected and AIDS patients, it is capable of causing a variety of clinical conditions including vaginal and bloodstream infections.[3,4] Since *C. dubliniensis* and *C. albicans* have phenotypic similarities, such as ability to form germ tubes and chlamydospores, their differentiation in routine clinical microbiology laboratories is problematic and may lead to misidentification.[5] During the last five years several tests based on phenotypic characteristics have been developed to distinguish these two species.[6-11] In 1999, Staib and Morschausser[6] extended the diagnostic usefulness of Staib's bird seed (*Guizotia abyssinica*) agar,[12] originally used as a differential medium for *Cryptococcus neoformans*, to distinguish *C. dubliniensis* from *C. albicans*. *Candida dubliniensis* isolates formed rough colonies with abundant chlamydospores on this medium, whereas *C. albicans* isolates formed smooth colonies with no chlamydospore production. These observations were subsequently confirmed by Al-Mosaid *et al*.[7] and Lees and Barton.[8] Recently, sunflower (*Helianthus annuus*) seed agar has also been found useful for distinguishing *C. dubliniensis* from *C. albicans* on the basis of similar morphologic characteristics as have been observed on niger seed agar.[9-11] In this communication, we report that husk of sunflower seeds can be used as a substitute for whole seeds in the medium without compromising its efficacy.

Materials and Methods

Sunflower seed husk medium

The sunflower seeds were purchased from open market and the husk was separated manually. The medium was prepared as follows: 50 gram sunflower seed husk was pulverized in domestic grinder for 3-4 minutes. The pulverized husk was boiled for 30 minutes with 1 litre of distilled water and filtered through several layers of gauze. To husk extract so obtained were added 15 gram agar (Difco Laboratories, Detroit, USA) and 10 gram glucose. The volume was made up to 1 litre and pH adjusted to 5.5 before autoclaving at 15 pounds pressure for 15 minutes.

Reference strains and test cultures

Two reference strains (CBS 7987 and CD 36) and 38 local clinical isolates of *C. dubliniensis* (sputum-17, vagina-8, endotracheal secretions-8, urine-3, and one each from bronchoalveolar lavage and catheter tip), and three reference strains (ATCC 90029, ATCC 2091, ATCC 90028) and 102 clinical isolates of *Candida albicans* were included in the study. All the reference strains and test isolates were freshly subcultured and tested for germ tube formation in pooled human serum and for chlamydospore production on cornmeal agar (Becton Dickinson, France) supplemented with 1% Tween 80 (CM-T) by Dalmau plate technique.[13] Their identity was further confirmed by Vitek 2 ID-YST card and/or ID 32C system (bioMerieux, France) as well as by semi-nested PCR (snPCR) using species-specific primers corresponding to unique sequences within the internally transcribed spacer 2 (ITS 2) of *C. dubliniensis* and *C. albicans* and/or by direct sequencing of ITS 2. [14,15]

Dalmau plate culture

All the isolates forming germ tubes and chlamydospores on CM-T were streaked on sunflower husk (SSH) agar for studying colony morphology and chlamydospore production. Using a marker pen, the plate was divided into two halves,

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and each half on the bottom of the plate was labeled with the culture number. With a sterile inoculating needle, two streaks, approximately 1.5 cm long, were made on the plate one centimetre apart without digging into the medium. Only a very small quantity of the culture was streaked. After flaming and cooling the needle, an S-shaped streak was made across the two streaks made earlier. Coverslip (22 X 22 mm) was flame sterilized, cooled and placed over the streak marks. The plates were incubated at 28^0 C and examined under microscope using low (10 X) and high power (40 X) at 24, 48 and 72 h intervals. All the isolates showing fringed colonies with chlamydospores were re-examined for reproducibility by directly inoculating SSH plates by streaking.

Results

The observations on colonial morphology and chlamydospore formation by *C. dubliniensis* and *C. albicans* on SSH agar and CM-T agar are presented in the table and depicted in figures 1-3. Dalmau plate cultures of all the 40 *C. dubliniensis* isolates including two reference strains produced rough colonies with hyphal fringes and abundant chlamydospores on SSH agar after 24 to 48 hours of incubation at 28^0 C. In contrast, 101 (96.2%) *C. albicans* isolates including three reference strains produced smooth colonies with no evidence of chlamydospore formation on this medium. Of the four isolates which showed fringed colonies, only two produced scanty chlamydospores. The results were reproducible when the experiment was repeated. On CM-T agar, all the *C. dubliniensis* isolates and *C. albicans* isolates produced smooth colonies and chlamydospores, and no discernible differences were observed in the colony morphology of the two species.

Discussion

The phenotypic characteristics of *C. albicans* to form chlamydospores and germ tubes have long been used to differentiate it from other *Candida* species. However, with the recognition of *C. dubliniensis* as a separate species,[2,3] which

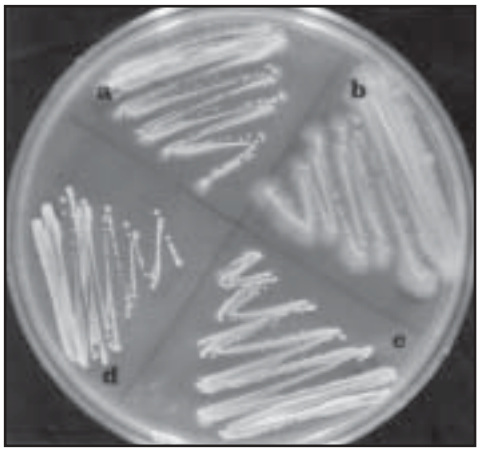

Figure 1: Sunflower seed husk plate showing growth of three stains of *C. dubliniensis* (a-CBS 7987, b-848/02, c- 239/03) and a clinical isolate of *C. albicans* (d- 6/03) after 3 days of incubation at 28 ˚C. Note: rough colonies with hyphal fringes of *C. dubliniensis* and smooth colonies of *C. albicans*

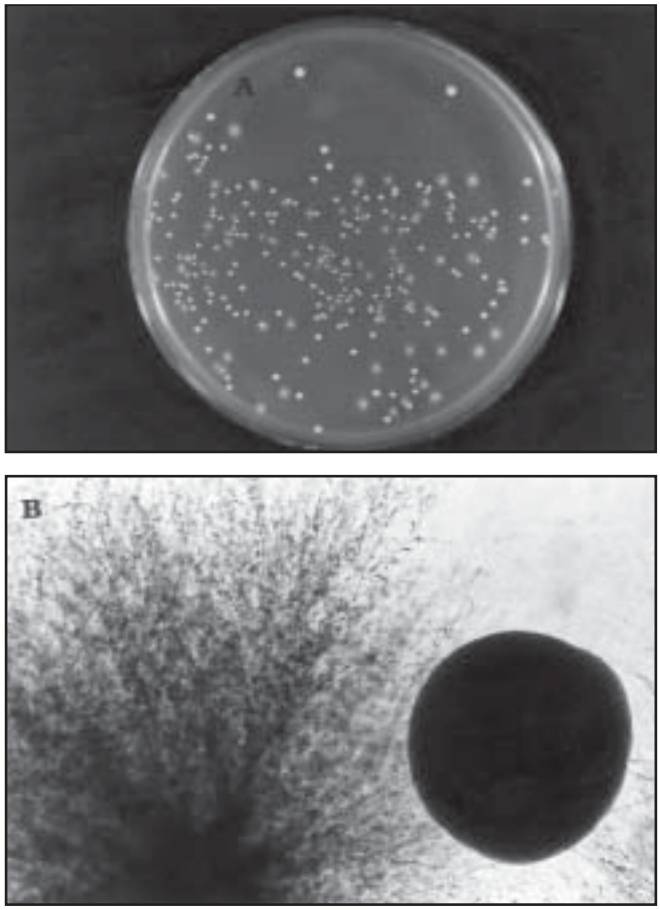

Figure 2: (A) Mixed culture of *C. dubliniensis* (CD-36, type strain) and *C. albicans* (ATCC 90028) on sunflower seed husk agar showing rough colonies with hyphal fringes of *C. dubliniensis* and small smooth colonies of *C. albicans* after 2 days of incubation at 28 ˚C. (B) microscopic view of a portion of *C. dubliniensis* colony showing hyphae with chlamydospores (left side), and a *C. albicans* colony with entire margin (right side). Magnification x 100.

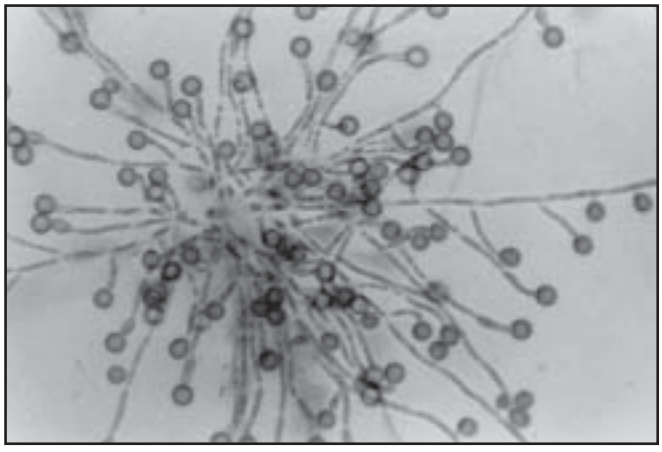

Figure 3: Dalmau plate culture on SSH agar showing chlamydospores of *C. dubliniensis*, strain 848/02, (magnification x 400).

Table: Observations on colony morphology and chlamydospore production on sunflower seed husk (SSH) agar and cornmeal-Tween 80 (CM-T) agar by *C. dubliniensis and C. albicans*

Morphologic characteristics	*C. dubliniensis*		*C. albicans*	
	SSH agar	CM-T agar	SSH agar	CM-T agar
Rough colonies with hyphal fringes	40/40* (100%)	0/40 (0%)	4/105 (3.8%)	0/105 (0%)
Chlamydospore production	40/40 (100%)	40/40 (100%)	2[a]/105 (1.9%)	105/105 (100%)

*Number positive/number tested, [a]showed only scanty chlamydospores

like *C. albicans* produces chlamydospores and germ tubes, several investigators have focused attention to develop simple and inexpensive methods for discriminating these two species in routine clinical microbiology laboratories. The tests that have been reported to be useful for this purpose include production of rough colonies and chlamydospores on Staib agar, [6-8] development of dark green colonies on CHROMagar *Candida*, [2,16] inability to grow at 45 ˚C, [17] and in 6.5% sodium chloride[18] by *C. dubliniensis* isolates. Recently, several commercial yeast identification systems based on carbohydrate assimilation profiles have also become available for the identification of *C. dubliniensis*.[3,4] In an extensive evaluation of commercial yeast identification systems which included API 20C AUX, ID 32 C, RapID yeast plus, Vitek YBC, and Vitek 2 ID YST, Pincus *et al.*[19] reported that assimilation of a-methyl-D-glucoside, trehalose, and D-xylose could be used for the differentiation of *C. dubliniensis*. However, the assimilation results were not consistent with different commercial assimilation systems used and varied with the incubation period.[19,20]

Several investigators have reported that *C. dubliniensis* isolates have the ability to produce chlamydospores more readily and abundantly than *C. albicans* on rice agar-Tween, Tween 80-oxgall-caffeic acid agar or cornmeal-Tween 80 agar[2, 3] and this morphologic characteristic could be used for differentiating the two species. However, identification of *C. dubliniensis* on the basis of abundance of chlamydospores or other phenotypic features have not been shown to be reproducible in some laboratories.[16] Moreover, assessment of relative abundance of chlamydospores is quite subjective in judgment, hence this feature alone may not serve as a dependable criterion for differentiation of *C. dubliniensis* from *C. albicans*. Recently, Staib and Morschhauser[6] reported that *C. dubliniensis* on Staib's *G. abyssinca* seed agar[12] under appropriate growth conditions forms rough colonies due to mycelial growth and abundant chlamydospores which may be used as a species-specific marker for identification of this species. In an extended evaluation of this medium, Al-Mosaid *et al*[7] found that 85.4% of the *C. dubliniensis* isolates (n = 130) while none of the *C. albicans* isolates (n = 166) produced chlamydospores on this medium. In contrast, while all the isolates of *C. albicans* produced smooth colonies, as many as 97.7% isolates of *C. dubliniensis* formed rough colonies. The authors concluded that morphological discrimination between the two species is best achieved on the basis of colony morphology rather than chlamydospore production. In a more recent study, Adou-Bryn *et al*[9] using a modified sunflower seed agar observed that all the 60 *C. dubliniensis* isolates produced rough colonies, whereas all the 47 *C. albicans* isolates produced smooth colonies. Efficacy of sunflower seed agar was subsequently confirmed by Al-Mosaid *et al*[10] and Khan *et al.*[11]

In conclusion, production of rough colonies and abundant chlamydospores by all the 40 isolates of *C. dubliniensis*, and of smooth colonies with lack of chlamydospores by over 96% isolates of *C. albicans*, suggests that SSH agar has a value in the presumptive differentiation of these two clinically important species. The low production cost, wider availability of the ingredients and simple composition make SSH medium ideally suited for routine use in clinical microbiology laboratories. Since the medium is free from oil droplets, it provides a clearer view of chlamydospores during microscopic examination, a problem that could be encountered with media prepared from the whole seeds.

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