

Performance of Five Phenotypical Methods for Identification of *Candida* Isolates from Clinical Materials

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Abstract

Although *Candida albicans* is the most common etiologic agent of candidiasis, *C. dubliniensis*, has been emerged, as another pathogen resembles *C. albicans* in many phenotypic aspects and noted for its in vitro potential for fluconazole resistance. Since there was no evidence of any report about detection of this organism in Iran, this study was designed to use of five different tests for identification of *Candida* species with special reference to *C. dubliniensis* among 313 suspected *Candida* isolates in Tehran, capital of Iran. Overall, 199 (63.6%) *C. albicans* and 114 (36.6%) *Candida* spp. were identified. All 199 *C. albicans* isolates were found germ tube and chlamyospore positive. Different shades of green color colonies were yielded on CHROMagar *Candida* of which 23 (11.6%) showed dark green color indicative of *C. dubliniensis*. All but four *C. albicans* isolates grew well at 45 °C. These 4 isolates beyond to 23 dark green colony producers were suspected of being *C. dubliniensis*, later examined by API 20C AUX system. The results indicated that all 27 isolates were able to assimilate both xylose and α -methyl-D-glucoside, therefore these isolates were identified as *C. albicans*. Overall, *C. dubliniensis* had not been found in present study. It must be concluded that no single phenotypic test has proven to be highly effective, and the use of several tests may be necessary of these two closely related *Candida* species for definitive identification.

Keywords: *Candida albicans*, Phenotypic markers, *Candida species*, Iran

Introduction

Candida albicans is one of the most frequent isolated yeasts in clinical laboratories. Different studies have shown that this organism can account for up to 75% of the yeasts recovered from site of infection (1, 2). The isolation of *C. albicans* has been associated with infections, as well as colonization, in both immunocompromised and immunocompetent patients (1-4). This species is usually identified by its ability to produce germ tubes and chlamyospores. However a new species of *C. dubliniensis* has recently been described that is very similar to *C. albicans* in many characteristics, especially germ tube formation and chlamyospore production. Since *C. albicans* strains share these char-

acteristics, it is likely that some *C. dubliniensis* strains have been and will continue to be identified in clinical laboratory as *C. albicans* (5, 6). Evidence for the inducibility of stable fluconazole resistance in vitro in *C. dubliniensis*, strains may indicate an emerging pathogen for immunocompromised patients receiving long-term fluconazole prophylaxis (7, 8).

Since literature review showed no evidence of any report about detection and isolation of this organism in Iran so far, therefore this study was designed to evaluate the performance of various conventional and new methods in identification and detection of clinical *Candida* isolates with special reference to *C. dubliniensis* in Tehran, capital of Iran.

Materials and Methods

Patients and specimens Totally 313 patients were included in the present study. One hundred eighty four of patients were female and 129 were male and ranging in age from 0 to 90 years. The conducted clinical specimens were as follows: 19 oral swabs, 12 vaginal discharges, 39 sputa, 3 bronchoalveolar lavage, 2 cerebrospinal fluid, 5 biopsied tissue, 5 urine, 1 stool, 227 skin and nail scrapings.

Organisms The 313 *Candida* isolates recovered from patients at separate clinical evaluations between June 2001 and December 2004 were studied. In addition to the patient isolates, the *C. albicans* NCPF 3939, NCPF 3302, NCPF 3242, and *C. dubliniensis* NCPF 3108 were studied as reference and control strains. All these yeasts were maintained on sabouraud glucose agar (SGA) and incubated at 28 °C for 2 to 3 d.

Chlamyospore formation (CF) Each of the following methods was performed in triplicate on the same day to provide estimates of within-day variation. Briefly, to determine chlamyospore production cornmeal-tween 80 agar (CM-T80, E. Merck, Germany) plates were streaked and stabbed with a 48 h old yeast colony, covered with sterile cover slip, incubated at 28 °C for 2-3 d. Chlamyospore production was examined after staining with lactophenol cotton blue.

Germ tube test Germ tube test (GTT) was performed by inoculating 2 ml fresh-pooled normal human serum with small portion of a single fresh colony of yeast and incubating at 37 °C for 3h and subsequently observed for germ tube production.

Colony color on CHROMagar Candida A 48-h-old single colony from a SGA medium plate culture of each isolate to be tested was separately streaked with a sterile wire loop onto CHROMagar Candida (Company LTd-France) plates (CA) and incubated at 37°C for 48h. Plates were checked for growth after 24 h of incubation and read for visual colony color af-

ter 48h of incubation. Colony color and morphology were recorded for each isolate.

Growth at 37 °C, 42 °C and 45 °C Each isolate was subcultured on SGA and incubated at 37 °C, 42 °C and 45 °C for 48 h. The growth of isolates at 42 °C and 45 °C were scored as 1) none or poor and 2) not reduced in comparison to that of 37 °C.

Carbohydrate assimilation The API 20C AUX system (Bio Merieux, Marcy, 1 Etoile, France), a commercial kit for the evaluation of the assimilation of 19 carbon sources, was used according to the manufacture's instructions. Since 48 to 72 h of incubation is recommended, test strips were incubated for both times to allow a comparative evaluation of the assimilation patterns. Numerical profiles were constructed from the reaction patterns and were used to obtain identification results with the analytical profile index.

Results

Overall, 199 (63.6%) of 313 *Candida* isolates displayed the typical fewer chlamyospores on longer hyphae and the contiguous chlamyospores were rarely present in pairs or triplets (Fig.1). Moreover, all 199 above mentioned isolates were GTT positive (Fig.2). All of these strains were later identified as *C. albicans* using API 20C AUX system. Other 114 isolates were identified as: 85(27.2%) *C. parasilosis*, 15(4.8%) *C. tropicalis* 2(0.6%) *C. lusitaniae*, 4(1.3%) each of *C. inconspicua* and *C. guilliermondii*, 1(0.3%) each of *C. ciferri*, *C. lipolytica*, *C. kefyr* and *C. famata* by using the same method.

All of 199 *C. albicans* isolates showed growth on CA and also yielded different shades of green colonies after 48 h as follows: green, 68; light-green, 34; dark-green, 23; light-green center with dark edge, 28; and blue-green, 46. All studied *C. albicans* strains grew well at 37 °C, 42 °C, whereas no growth at 45 °C was found for 4 (2%) of isolates. The GTT, CF, sugar assimilation test, and CA test were further repeated for those 4 strains beyond 23 dark

green colored colonies which were suspected of being *C. dubliniensis* (Fig. 3). Finally after judgment of their phenotypic characteristics, the suspicious isolates were reconfirmed to be *C. albicans*. When the *C. albicans* type strains were examined as control for characteristic features, following results were obtained: *C. albicans* NCPF 3242, NCPF 3302 and NCPF 3939 grew well at 37 °C, 42 °C and 45 °C. Besides, produced germ tubes and fewer chlamydospores on longer hyphae, green colonies on indicative medium (Fig. 4), and were able to assimilated D-Xylose (XYL) and α -Methyl-D-glucoside (MDG). Although *C. dubliniensis* NCPF 3108 tested was positive for GTT and produced chlamydospores which were attached in the characteristics triplet or pair arrangement at the end of short hyperbranching pseudohyphae but it was not able to grow at 45 °C and unable to assimilate XYL and MDG when tested with API 20C AUX system. The *C. dubliniensis* NCPF 3108 also produced dark green color when plated on CA.

Moreover, all *C. tropicalis* isolates (n=15) developed a distinctive dark blue or blue-gray color after 48 h of incubation on CA (Fig.5). Nearly all isolates of *Candida* species tested gave colonies with colors described as ranging from white, through pink, pinkish purple, and purple after 48 h of incubation on CA at 37 °C (Table 1, Fig.6).

Table 1: Growth and colony colors of 313 yeast isolates on CHROMagar Candida after 48 h at 37 °C.

Species	Total No. of isolates	Range of colony colors described
<i>C. albicans</i>	199	Different shads of green
<i>C. parapsilosis</i>	85	White, pale pink, violate, whitish-pink
<i>C. tropicalis</i>	16	Dark blue, blue-gray
<i>C. lusitaniae</i>	1	Pink
<i>C. inconspicua</i>	4	Pink
<i>C. guilliermondii</i>	4	Pink, purple, pinkish-purple
<i>C. ciferri</i>	1	Pink, purple
<i>C. lipolytica</i>	1	Purple
<i>C. kefyr</i>	1	Pink, purple
<i>C. famata</i>	1	White, light- pink, pink.

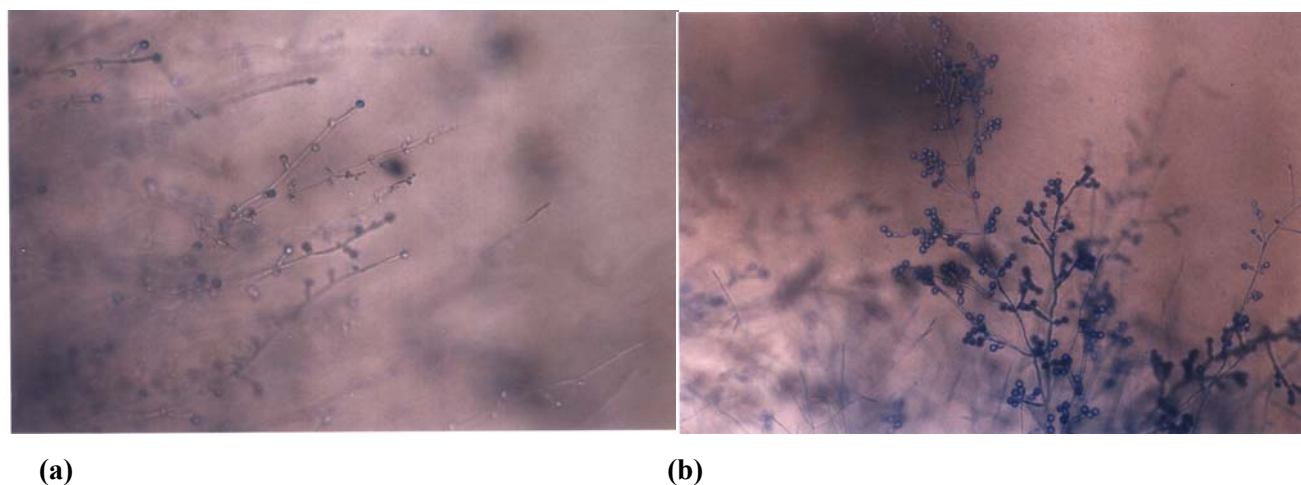


Fig. 1: Chlamydospore production on CM-T80 plates at 28 °C after 3 d (a) Chlamydospores and pseudohyphae produced by *C. albicans*. (b) Production of abundant chlamydospores by few *C. albicans* isolates in our study similar to *C. dubliniensis*: The terminal pairs and triplet arrangement is shown.

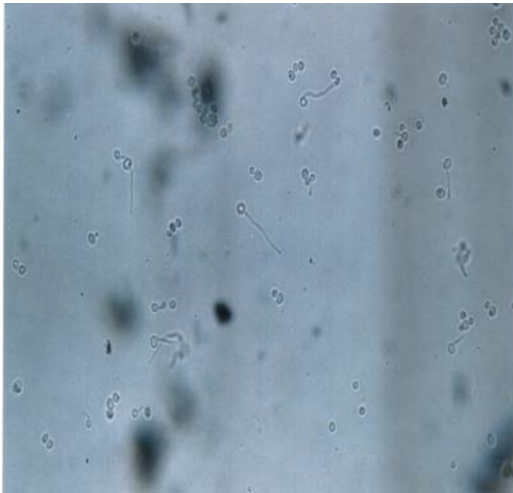


Fig. 2: Germ tube production in blood serum at 37 °C after 3 h

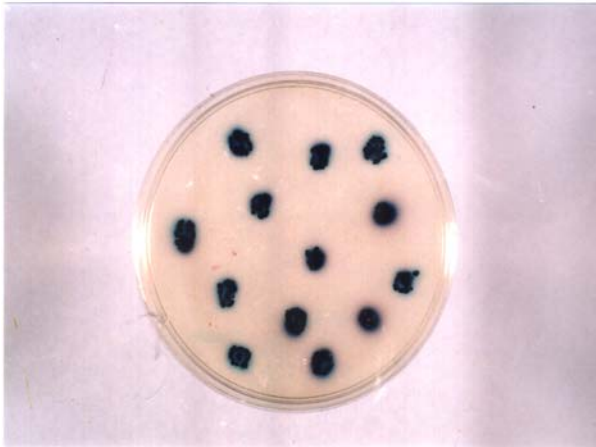


Fig. 3: Dark green color colonies of *C. albicans* on CA

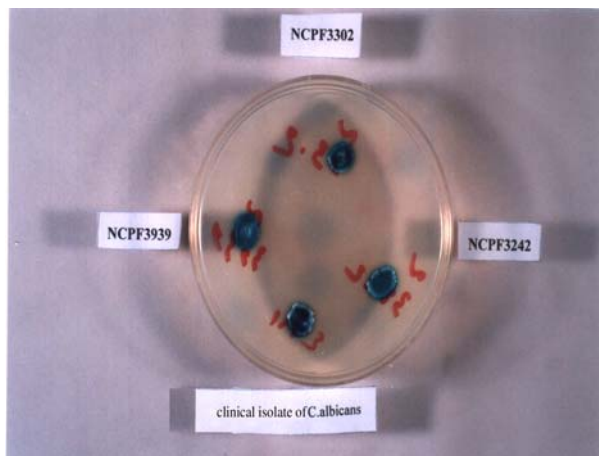


Fig. 4: Colonies of *C. albicans* control strains and a clinical isolate on CA at 37 °C after 48 h

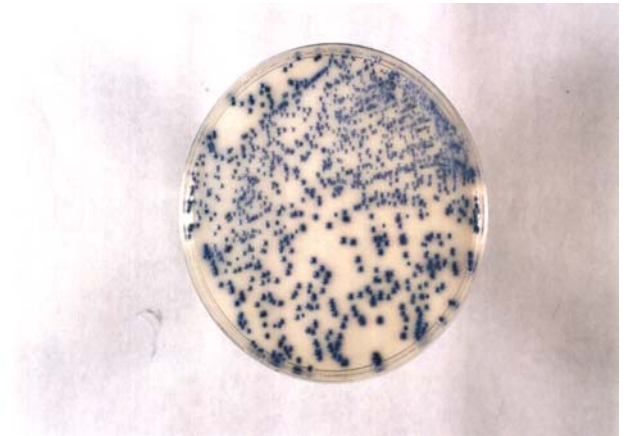


Fig. 5: Typical colonies of *C. tropicalis* on CA at 37 °C after 48 h

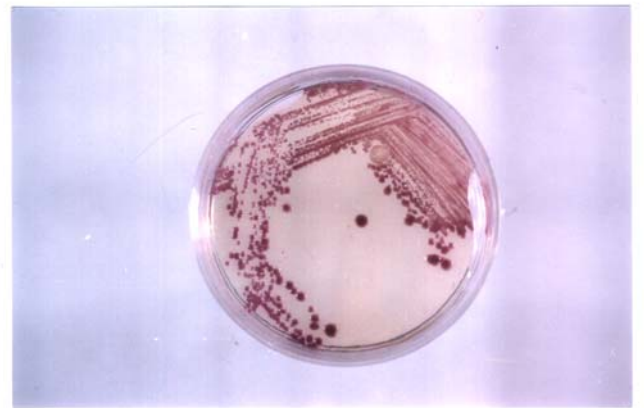


Fig. 6: *Candida* spp. colonies on CA at 37 °C after 48 h

Discussion

In recent years the number of serious opportunistic yeast infections, particularly in immunocompromised patient has been dramatically increased (9). Although *C. albicans* was the most frequent isolated yeast pathogen, but the emergence of *Candida* species other than *C. albicans* as important agents of infection was a concern in several investigations (10-12). The ability to differentiate readily between *C. albicans* and other *Candida* species in the routine diagnostic laboratory remains a technical problem. Various reports of the recently described yeast species *C. dubliniensis* indicated a worldwide occurrence of this fungus, which is phylogenetically closely related to *C. albicans* (5, 10-16). Evidence for the inducibility of stable fluconazole resistance in vitro in *C. dublinien-*

sis strain, may indicate an emerging pathogen for immunocompromised patients receiving long-term fluconazole prophylaxis (7, 8, 16, 17). This situation has necessitated that clinical laboratories become more proficient in their ability to isolate and identify yeasts of medical importance as rapidly and accurately as possible. The detection and identification of microorganisms strongly depend on the availability of easy-to-perform screening and cost-effective methods. Several methods for identification of *C. dubliniensis* and discrimination from *C. albicans* have been reported which include the formation of dark green colonies on CA (11, 16, 18, 19) no or strictly reduced growth at 45 °C (16, 20) and lack of ability to assimilate XYL and MDG (20-23). In addition, *C. dubliniensis* formation often shows characteristic chlamydospore (5) and may differ from *C. albicans* in its distinctive carbohydrate assimilation pattern. In our laboratory, we routinely screen clinical yeast isolates by initially plating the samples on SGA at 30°C, and later screening the chlamydospore formation on CM-T80 and GTT for identification of *C. albicans* from other *Candida* species. The type of chlamydospore formation was found variable in both *C. albicans* and *C. dubliniensis* and therefore, it is not reliable for differentiation of these two species (14, 22). Although the GTT offers a simple rapid, easy-to-perform and well-known most helpful test for identification of *C. albicans* microbiology laboratories without adequately trained mycology personal, but data from several studies (1, 15, 24-27) showed that false positive or negative results could be easily occurred due to increased incubation time and heavy inoculums respectively. This, coupled with time needed for detailed microscopic examination, may make this test liable to error in busy laboratory. It has also been reported that up to 5% of *C. albicans* isolates are germ tube negative (1, 21-24). In contrary to those studies, the yeast isolates, which were later identified as *C. albicans* in this study, were all GTT positive and chlamydospore producer. Although type strain *C.*

dubliniensis NCPF 3108 showed the typical abundant production of chlamydospores, some of the *C. albicans* isolates produced chlamydospores similar in number and arrangement to those produced by *C. dubliniensis*, as also reported previously by other investigators (6, 24, 28). Therefore, this morphological feature was relied on for the initial identification of *C. dubliniensis*. Recent reports (19, 29, 30) have described the use of CA, a new chromogenic medium, as an effective medium in identifying the *C. albicans* and other common yeast species. Although according to manufacturer's instruction, the CA media were used within 2 wk of preparation but our study indicated that CA could not be used satisfactory as a sole indicative medium. In particular, *C. albicans* colonies grew in a variety of green colors ranging from light green to dark green. A dark green color on CA was described as a potential phenotypic marker for *C. dubliniensis*. Since the phenomenon was found in 23 of our *C. albicans* isolates, the shade of dark green proved to be unreliable for discrimination between the *C. albicans* and *C. dubliniensis*. These results are in agreement with Tintelnot et al. (18) and Odds et al. (30). Moreover, use of an incubation temperature of 45 °C for 48 h as recently proposed (32) in order to obtain more clear-cut discrimination seems not to be reliable, since the no growth of some *C. albicans* strains were observed at that temperature in our study. Nevertheless, one should be aware that rare atypical *C. albicans* strains, e.g; the former *C. stellatoidea* type 1 are not able to grow at 45 °C (31, 32). Since the current API 20C AUX data-base does include assimilation profiles for *C. dubliniensis*, this system was used for identification and detection of this organism. The results showed that all four suspicious isolates and the *C. albicans* type strains NCPF 3939, NCPF 3302, NCPF 3242, were able to assimilate XYL and MDG with API 20C AUX system, but *C. dubliniensis* NCPF 3108 failed to assimilate both XYL and MDG.

In conclusion, we feel the API 20C AUX system is a useful commercial system for identification of yeasts from clinical specimens. CA must always be used in conjunction with other tests as GTT, CF, and carbohydrate assimilation. In the meantime, results from our laboratory suggest that no single phenotypic test has proven to be highly effective, and other tests may be necessary for definitive identification. Finally, it should be stressed that no *C. Dubliniensis* has been found among the studied yeast isolates in Tehran. Additional studies involving a broader variety of clinical specimens from different sites of infection, HIV positive patients and *Candida* spp. are certainly warranted.

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