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Evaluation of a Rapid Immunochromatographic Assay for Identification of *Candida albicans* and *Candida dubliniensis*

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Candida dubliniensis* was first established as a novel yeast species in 1995. It is particularly associated with recurrent episodes of oral candidosis in human immunodeficiency virus (HIV)-infected patients, but it has also been detected at other anatomical sites and at a low incidence level in non-HIV-infected patients. It shares so many phenotypic characteristics with *C. albicans* that it is easily misidentified as such. No rapid, simple, and commercial test that allows differentiation between *C. dubliniensis* and *C. albicans* has been developed, until now. Accurate species identification requires the use of genotype-based techniques that are not routinely available in most clinical microbiology diagnostic laboratories. The present study was designed to evaluate the efficiency of a new test (the immunochromatographic membrane [ICM] albi-dubli test; SR2B, Avrillé, France) to differentiate between *C. albicans* and *C. dubliniensis*. The organisms evaluated were strains whose identities had previously been confirmed by PCR tests and freshly isolated clinical strains and included 58 *C. albicans* isolates, 60 *C. dubliniensis* isolates, and 82 isolates belonging to other species of yeast. The ICM albi-dubli test is based on the principle of immunochromatographic analysis and involves the use of two distinct monoclonal antibodies that recognize two unrelated epitopes expressed by both species or specific to only one species. The assay requires no complex instrumentation for analysis and can be recommended for routine use in clinical microbiology laboratories. Results are obtained within 2 h and 30 min and are easy to interpret. This evaluation demonstrated the good performance of this immunochromatographic test for *C. albicans* and *C. dubliniensis* isolated on Sabouraud dextrose agar, CHOROMagar Candida, and CandidaSelect, with sensitivities and specificities ranging from 93.1 to 100%. These parameters decreased, however, to 91.4% when the test was performed with yeast isolated with Candida ID.

Over the past decade there has been a significant increase in the number of reports of systemic and mucosal infections caused by *Candida* species, and since the recognition that *Candida* species differ in the expression of putative virulence factors and susceptibilities to antifungal agents, greater emphasis has been placed on the identification of isolates to the species level.

Candida dubliniensis is a recently described pathogenic *Candida* species which was originally identified in cases of recurrent oral candidosis in human immunodeficiency virus (HIV)-infected and AIDS patients (34). Several studies have reported an overall prevalence of 26.4 to 34% in the oral cavities of HIV-positive patients and 3.5 to 13.9% among HIV-negative patients (9, 36). Moreover, it has been suggested that *C. dubliniensis* can be induced to develop resistance to fluconazole in vitro, a phenomenon which may have resulted in its emergence in the HIV-infected population (23, 24, 33). More recently, it has also been identified in a small number of cases of systemic infection. In retrospective analyses of stock collections, *C. dubliniensis* was found to account for at least 1.2 to 2% of the yeasts initially identified as *C. albicans* (14, 25). *C. dubliniensis* shares many phenotypic characteristics with *C. albicans*, including the ability to form germ tubes and chlamydospores, and is phylogenetically closely related to *C. albicans*. It belongs to *C. albicans* serotype A, and chlamydospores of *C. dubliniensis* are produced abundantly and often in clusters or contiguous pairs (10). These similarities have caused significant problems with the identification of *C. dubliniensis* by the average clinical mycology laboratory. Several phenotypic methods for the identification of *C. dubliniensis* and discrimination from *C. albicans* have been reported: (i) *C. dubliniensis* has been shown to produce a distinctive dark green color on CHROMagar Candida (30); however, this atypical color may not persist after serial passage of the organism and may be less useful for isolate identification (32, 36); (ii) following isolation, colonies of *C. dubliniensis* do not exhibit fluorescence on methyl blue-Sabouraud agar under Wood's light, but this lack of fluorescence may not be reproducible with isolates subjected to storage and repeated subculture (30); (iii) unlike *C. albicans*, *C. dubliniensis* does not grow at 45°C (27), but discrimination by thermotolerance was not confirmed (16); (iv) in contrast to *C. albicans*, *C. dubliniensis* is able to reduce 2,3,5-triphenyltetrazolium chloride (37); and (v) *C. dubliniensis* has been found to be β-glucosidase negative, a phenotypic characteristic that may, however, also occur in *C. albicans* (7, 25). More recently,

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Staib agar was shown to allow differentiation between these two species on the basis of abundant chlamydospores and the growth of rough colonies (3, 31). Unfortunately, none of these easy-to-perform methods appear to definitively identify all *C. dubliniensis* isolates. To attain this goal it is necessary to perform other, time-consuming tests such as carbohydrate assimilation assays, by which *C. dubliniensis* strains exhibit unique assimilation profiles (26). It has recently been shown that on Pal's agar, a medium prepared with sunflower seed extract, *C. dubliniensis* isolates yield rough colonies surrounded by a hyphal fringe, while *C. albicans* colonies are smooth without any evidence of hyphae (2). Modification of this medium to make it transparent has also allowed the direct observation of chlamydospore production through the petri dish (1).

At present, the most accurate means of differentiating between *C. dubliniensis* and *C. albicans* requires the use of molecular biology-based techniques, such as DNA fingerprinting analysis with repetitive sequence-containing DNA probes, randomly amplified polymorphic DNA analysis, PCR analysis, or pulsed-field gel electrophoresis (5, 8, 11, 12, 15, 16, 21, 22, 35). Although they are very effective, these techniques are expensive, time-consuming, and not readily applicable to the identification of large numbers of isolates, nor can they be conducted routinely in most standard mycology laboratories at present.

To our knowledge, few monoclonal antibodies (MAbs), polyclonal antibodies, or single-chain variable fragments have been demonstrated to differentiate yeast cells at the species level. Marot-Leblond et al. (19, 20) isolated an MAb (MAb 3D9) that specifically allows the identification of the *C. albicans* mycelial phase and, more recently, a second MAb (MAb 16B1-F10) that allows the discrimination between *C. albicans* and *C. dubliniensis* hyphae by immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay. Bikandi et al. (4) developed an immune serum that allows differentiation of *C. dubliniensis* and *C. albicans* blastospores; however, use of this immune serum is restricted to IFA because of cross-reactivity with a variety of intracellular antigens from other yeast species. More recently, Bliss et al. (6), using a combinatorial phage display library of human heavy and light chain variable regions, prepared two single-chain variable fragments that allowed the specific identification of *C. albicans* and *C. dubliniensis* germ tubes by IFA. However, they did not consider the possibility that the epitopes that were recognized might also be expressed within the cells of other *Candida* species or yeast genera (6). To date, two MAbs have led to the development of effective immunological tests for the identification of colonies of *C. albicans* and *C. krusei* (13, 18, 28, 29).

In the present study, we have investigated the potential use of an immunochromatographic assay as a basis for the identification of *C. albicans* and *C. dubliniensis*. This technique is specific, rapid, easy to perform, and applicable to large numbers of isolates and should enhance the rapid and accurate identification of *C. dubliniensis* strains in the future.

MATERIALS AND METHODS

Organisms and culture conditions. Strains of *C. albicans* (n = 47) and *C. dubliniensis* (n = 43) were obtained from the Dublin Dental School and Hospital Yeast Collection, Dublin, Ireland, and from the Bilbao Facultad de Medicina Collection. All have been identified by a number of techniques, including a PCR test based on the intron sequence of the *ACT1* gene or immunofluorescence with a specific MAb (19). The clinical isolates of *Candida* spp., and non-*Candida* yeast species included for comparison were obtained from the Mycological Laboratory of the Medical School Angers. These isolates were identified by using the ID 32C system (bioMérieux SA, Marcy l'Etoile, France). They consisted of 12 *C. tropicalis*, 14 *C. krusei*, 15 *C. glabrata*, 10 *C. parapsilosis*, 8 *C. guilliermondii*, 11 *C. kefyr*, 1 *Rhodotorula mucilaginosa*, and 11 *Cryptococcus neoformans* isolates. Freshly isolated clinical strains of *C. albicans* (n = 11) and *C. dubliniensis* (n = 17) were also investigated, and their identities were confirmed with modified Pal's agar (1). All yeasts were stored on cryobeads (Hardy Diagnostics, Santa Maria, Calif.) prior to use.

Cells were first subcultured twice onto Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) at 37°C for 48 h. Colonies from the second subculture were used for testing. Colonies were transferred in parallel onto SDA, CHROMagar Candida (Becton Dickinson Microbiology Systems, Sparks, Md.), CandiSelect (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France), or Candida ID (bioMérieux SA) and were then incubated for 48 h at 37°C on these media.

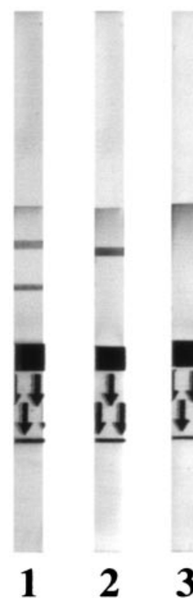

FIG. 1. Different features of the results obtained with the ICM (albi-dubli) test kit. Lane 1, *C. albicans*; lane 2, *C. dubliniensis*; lane 3, other yeast species.

ICM test. The immunochromatographic membrane (ICM) test (the ICM albi-dubli test) is a rapid immunochromatographic test for the identification of *C. albicans* and *C. dubliniensis* that uses a sandwich assay system with two MAbs (MAb LIB 3H8 and MAb 16B1-F10), each of which binds to a repetitive epitope of two distinct antigens. MAb LIB 3H8 reacts with the protein moiety of a glycoprotein of >200 kDa specifically expressed in the *C. albicans* and *C. dubliniensis* cell wall (18). In contrast, MAb 16B1-F10 reacts with a glycoprotein component expressed uniquely on the surfaces of *C. albicans* hyphae (19).

The apparatus comprises a dispstick, a test tube, medium for antigen induction (minimal essential medium and calf serum bovine), and an extraction solution (glucanase) for cell component solubilization. The dipstick for the ICM test is composed of a comb-shaped device that contains a sample pad, a reagent pad, a membrane, and an absorbent pad. Each antibody is immobilized on the membrane as the capture reagent (test lines). The same antibodies, but conjugated with colloidal gold particles, are contained in the absorbent area and are used for antigen capture and detection in the sandwich-type assay.

In the presence of relevant soluble antigens, the antibody-colloidal gold conjugates bind to specific antigen in the sample. The complexes migrate upward along the membrane by capillary action and then bind to the MAbs immobilized on the solid phase in the test zone, producing red bands. Finally, the liquid continues to migrate along the membrane. In the presence of *C. albicans* two lines appear, and in the presence of *C. dubliniensis* only one line appears. For other yeast species, no line appears in the positive reaction zone (Fig. 1).

Dipstick assays. (i) Germ tube induction. Samples were prepared according to the instructions of the manufacturer (SR2B). Briefly, two to four colonies were

TABLE 1. Sensitivity and specificity of the ICM (albi-dubli) test kit

Organism[a] and growth medium[b]	% Sensitivity	% Specificity
C. albicans (58)		
SDA	93.1	100
CH	96.6	100
CS	93.1	100
CID	91.4	100
C. dubliniensis (60)		
SDA	98.3	97.9
CH	98.3	99.3
CS	98.3	97.9
CID	98.3	97.2
Other yeasts (82)		
SDA	100	98.3
CH	100	98.3
CS	100	98.3
CID	100	98.3

[a] Clinical isolates and reference strains. Numbers in parentheses indicate the number of strains tested.
[b] CH, CHROMagar Candida; CS, CandiSelect; CID, Candida ID.

picked from the growth medium and transferred to the test tube included in the kit (which contained 1 ml of medium) to yield a suspension equal to a no. 3 McFarland turbidity standard. Cells were allowed to express specific antigens by incubation for 2 h at 37°C without any shaking. For some experiments, yeast suspensions with turbidity greater than a no. 3 McFarland standard were used.

(ii) Antigen extraction and dipstick assay. Following the incubation period, the medium was discarded, and the remaining cells coated onto the plastic were scraped and resuspended in 100 μl of dissociating agent added to the tube. Antigen extraction was performed by incubation of the tube for 15 min at 37°C without shaking.

After this time, membranes were inserted into the test tube and allowed to stand at room temperature for 5 min and then removed. Assay results were read after 5 min.

All strains were tested in a blinded fashion, and results were compiled at the end of the study.

Data analysis. The variables measured were the number of samples with true-positive results (TP), the number of samples with true-negative results (TN), the number of samples with false-positive results (FP), and the number of samples with false-negative results (FN). Sensitivity was calculated as TP/(TP + FN), and specificity was calculated as TN/(TN + FP).

RESULTS

The ICM albi-dubli test was first evaluated with yeast cells isolated on SDA. Among the 58 *C. albicans* strains tested, 4 were misidentified: 3 were misidentified as *C. dubliniensis* strains and 1 was misidentified as a non-*C. albicans* and non-*C. dubliniensis* strain. Two misidentifications were obtained among the 60 *C. dubliniensis* strains and isolates tested, and no false-positive results were noticed for the 82 other yeasts analyzed. These values indicated sensitivities and specificities of 93.1 and 100%, respectively, for *C. albicans* and 98.3 and 97.9%, respectively, for *C. dubliniensis*.

The influence of the growth medium on the expression of the epitopes reacting by the ICM test was also studied. Table 1 summarizes the performance characteristics of the ICM test for the identification of *C. albicans* and *C. dubliniensis* according to the growth medium used for isolation. Differences in the performance of the ICM test were observed depending on the isolation medium used, with more accurate results being obtained with SDA, CHROMagar Candida, and CandiSelect, whereas more strains were misidentified when growth was on Candida ID. Misidentifications occurred mostly with *C. albicans* isolates, which were wrongly identified as *C. dubliniensis*. For the other species tested, no difference in the performance of the ICM test according to the source of the isolates was observed, since identification as non-*C. albicans* and non-*C. dubliniensis* isolates was correct irrespective of the growth medium used. A unique *C. dubliniensis* strain was incorrectly identified as non-*C. albicans* and non-*C. dubliniensis* after subculture on all media.

The same *C. albicans* yeast strains were generally misidentified by the test regardless of the growth medium used; thus, among the 58 *C. albicans* strains tested, 5 yielded false-negative results. One strain was misidentified as a non-*C. albicans* and non-*C. dubliniensis* isolate, whatever medium was used, although it was clearly identified as *C. albicans* with the ID 32C carbohydrate assimilation system. The other four *C. albicans* strains were incorrectly identified as *C. dubliniensis*. One strain that was misidentified when it was isolated on SDA was also misidentified when it was grown on Candida ID and Candi Select, while another strain was also misidentified following culture on these two chromogenic media. The two remaining misidentifications were observed for two independent strains grown on SDA and Candida ID, respectively. In summary, the best results were obtained when the test was performed with *C. albicans* strains grown on CHROMAgar Candida (sensitivity, 96.6%), while the worst results were obtained when the *C. albicans* strains were grown on Candida ID (sensitivity, 91.4%); however, these values are not significantly different.

The *C. albicans* strains with false-negative results were further investigated. Microscopic examination of cells was performed after antigen induction in the test tube and before the culture medium was discarded. From the observations it was concluded that the *C. albicans* isolates ($n = 4$) that were misidentified as *C. dubliniensis* could be characterized by a lack or a low rate of germination. Increasing the density of the yeast cell inoculum to greater than a no. 3 McFarland standard before germ tube induction did not significantly improve the performance of the test for these strains.

DISCUSSION

Accurate analysis of the epidemiology of infections caused by *C. dubliniensis* has been hampered by the lack of effective methods to identify this species in clinical samples. In addition, although *C. dubliniensis* and *C. albicans* isolates are both susceptible to azoles, fluconazole resistance has been observed in clinical isolates of *C. dubliniensis* from AIDS patients who have had prior exposure to fluconazole. Moreover, stable fluconazole resistance can readily be induced in *C. dubliniensis* isolates following direct exposure to the antifungal in vitro (23, 24). Consequently, these findings may have implications for antifungal therapy and indicate an important reason for distinction between these two species.

Identification of yeast species usually requires prior isolation on a primary isolation medium, which takes 24 to 48 h. When a nonchromogenic medium is used, recognition of *C. albicans* can be obtained on the basis of a single characteristic (e.g., rapid production of germ tubes in serum or chlamydosporulation). The production of germ tubes and hyphae can be in-

duced by changes in a variety of environmental factors, including ambient pH, nutritional status, and temperature. Germ tube formation is also influenced by the inoculum size, with densities of 10^5 to 10^7 cells/ml having been found to be the most conducive to germ tube induction (17). Some other *Candida* spp., e.g., *C. tropicalis*, can also generate germ tubes or pseudohyphae which can be confused with the true germ tubes produced by *C. albicans*. Moreover, this strategy ignores *C. dubliniensis*, since this species produces germ tubes that cannot be differentiated from those of *C. albicans*. However, in a previous study (19) it was noted that some *C. dubliniensis* strains did not produce true germ tubes but were shown to exhibit elongated pseudohyphae produced by polarized cell division of the yeast cells and characterized by constrictions. Solely on the basis of the germ tube test, these strains would have been considered non-*C. albicans* and non-*C. dubliniensis* and would have been further investigated by physiologic tests, such as those used by commercial yeast identification systems. Today, many laboratories use commercial systems for *Candida* species identification. However, these tests are expensive and laborious, and the samples must be incubated for greater than 48 h to obtain accurate results. Comparing such systems, Pincus et al. (26) showed that, according to the kit, doubtful results or misidentifications were obtained for *C. dubliniensis* (30% for the ID 32C system and 19% after 48 h for the API 20C AUX system). In their study, most isolates identified as *C. dubliniensis* strains were concluded to be *C. albicans*. The results obtained with the Vitek 2 ID YST system showed a very high correlation with those obtained by molecular methods, yielding mistaken identities for only 2% of isolates. Chromogenic media allow the simultaneous isolation and identification of *C. albicans* and *C. dubliniensis.* A dark green color on CHROMAgar Candida has been described as a potential phenotypic marker for C. *dubliniensis* in primary cultures (30). However, not all proven *C. dubliniensis* isolates yield the dark green color in primary cultures (36), thus possibly leading to the potential underestimation of the prevalence of *C. dubliniensis*.

The reasons for the variation in the results obtained with different isolation media are not clear. However, the differences observed may be the result of differential rates of germ tube induction or antigen expression in each circumstance. According to our observations, it might be useful to examine each sample microscopically before antigen extraction and the dipstick assay are performed. In our study, if the results for *C. albicans* or *C. dubliniensis* strains that did not form germ tubes were discarded, the sensitivity of the test for *C. albicans* identification increased from the range of 91.4 to 93.1% to 98.2%, although no improvement was seen in the case of *C. dubliniensis*.

The ICM albi-dubli test is a new rapid test that allows the accurate discrimination between *C. albicans* and *C. dubliniensis*. The results obtained by this assay have a high specificity and a high sensitivity. The assay is easy to perform, and it offers early results compared to the time that the results of molecular approaches are obtained and can easily be scaled up for use with a high volume throughput.

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