JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2006, p. 138–142 Vol. 44, No. 1
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.1.138–142.2006
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Development and Evaluation of a Rapid Latex Agglutination Test Using a Monoclonal Antibody To Identify *Candida dubliniensis* Colonies

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Received 20 July 2005/Returned for modification 12 September 2005/Accepted 12 October 2005

Cell components of the dimorphic pathogenic fungus *Candida dubliniensis* were used to prepare monoclonal antibodies (MAbs). One MAb, designated 12F7-F2, was shown by indirect immunofluorescence to be specific for a surface antigen of *Candida dubliniensis* yeast cells. No reactivity was observed with other fungal genera or with other *Candida* species, including *Candida albicans*, that share many phenotypic features with *C. dubliniensis*. The use of different chemical and physical treatments for cell component extraction suggested that the specific epitope probably resides on a protein moiety absent from *C. albicans*. However, we failed to identify the target protein by Western blotting, owing to its sensitivity to heat and sodium dodecyl sulfate. MAb 12F7-F2 was further used to develop a commercial latex agglutination test to identify *C. dubliniensis* colonies (Bichrodubli Fumouze test; Fumouze Diagnostics). The test was validated on yeast strains previously identified by PCR and on fresh clinical isolates; these included 46 *C. dubliniensis* isolates, 45 *C. albicans* isolates, and other yeast species. The test had 100% sensitivity and specificity for *C. dubliniensis* isolated on Sabouraud dextrose, CHROMagar Candida, and CandiSelect media and 97.8% sensitivity for *C. dubliniensis* grown on Candida ID medium. The test is rapid (5 min) and easy to use and may be recommended for routine use in clinical microbiology laboratories and for epidemiological investigations.

Over the past decade there has been a significant increase in the number of reports of systemic and mucosal *Candida* infections. The potential clinical importance of species-level identification has been recognized since the discovery that *Candida* species differ in expression of putative virulence factors and in antifungal susceptibility (7, 22, 23).

Candida dubliniensis is a newly described pathogenic *Candida* species originally isolated from patients with human immunodeficiency virus infection and recurrent oral candidosis. *C. dubliniensis* is now reported to account for between 3.5% and 34% of all *Candida* infections (9, 29).

In-depth epidemiological investigations are required to determine the precise clinical significance and incidence of *C. dubliniensis* infection and the reasons for its recent emergence. However, the development of rapid and simple means of *C. dubliniensis* identification has been hampered by the very close phenotypic and genotypic relationships between *C. dubliniensis* and *C. albicans*, the latter remaining the most common cause of candidosis (22). At present, the most accurate means of differentiating between isolates of these two species is based on molecular biology-based techniques (5, 8, 10, 12, 13, 14, 15, 16, 17, 21, 30). Several phenotype-based methods for identifying *C. dubliniensis* and discriminating it from *C. albicans* have been reported. (i) *C. dubliniensis* has been shown to produce a distinctive dark green color on CHROMagar Candida medium, and (ii) colonies of *C. dubliniensis* do not fluoresce on methyl blue-Sabouraud agar under Wood's light (27); however, these two methods are not reproducible after subculture and storage (31). (iii) Unlike *Candida albicans*, *C. dubliniensis* does not grow at 45°C (25), but this discrimination based on thermotolerance was not confirmed (17). (iv) *C. dubliniensis*, unlike *C. albicans*, is able to reduce 2,3,5-triphenyltetrazolium chloride (32). (v) Fourier transform infrared spectroscopy analysis of all cell components present in a cell can phenotypically discriminate these two species (31). More recently, it was reported that the two species could be distinguished by culture on Staib agar, Pal's agar, modified Pal's agar, and casein agar, on which only *C. dubliniensis* produces abundant chlamydospores and rough colonies (1, 2, 3, 24, 28). We also described an immunochromatographic assay differentiating between *C. albicans* and *C. dubliniensis*, but *C. dubliniensis* was identified only by default, owing to the specificity of the monoclonal antibodies (MAbs) (19). The aim of the present study was to produce a monoclonal antibody specific for *C. dubliniensis* cells and to investigate its potential use in a rapid latex agglutination test for identifying *C. dubliniensis* colonies.

MATERIALS AND METHODS

Strains, media, and growth conditions. *Candida dubliniensis* strain ATCC MYA-646 was used throughout this work, and *Candida albicans* strain ATCC 66396 (serotype A) was used for hybridoma screening.

Other strains of *C. albicans* and *C. dubliniensis* were obtained from the Dublin Dental School and Hospital Yeast Collection, Dublin, Ireland, and the Bilbao Facultad de Medicina Collection, Bilbao, Spain. All had been identified by a number of techniques, including PCR based on the intron sequence of the *ACT1* gene or immunofluorescence with a specific monoclonal antibody (20). Fresh

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clinical isolates of *C. albicans* and *C. dubliniensis* were also investigated, and their identities were confirmed by growth on modified Pal's agar (1, 19).

Clinical isolates of other *Candida* spp., *Saccharomyces cerevisiae*, and *Cryptococcus neoformans*, identified by using the ID 32C system (bioMérieux SA, Marcy l'Étoile, France), were obtained from the Mycology Laboratory of Angers Medical School, Angers, France.

Cells were first subcultured twice on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 24 h at 37°C. All species studied grew as blastospores on this medium.

Germ tubes were prepared by incubating *C. albicans* and *C. dubliniensis* yeast cells for 3 h at 37°C in medium 199 (pH 6.7; Gibco Laboratories, Grand Island, N.Y.) and were recovered by centrifugation.

To evaluate the agglutination test, 36 strains and 9 clinical isolates of *C. albicans* and 34 strains and 12 clinical isolates of *C. dubliniensis* were grown for 48 h at 37°C on commercial solid media, such as Sabouraud glucose agar with chloramphenicol and gentamicin, CHROMagar Candida (Becton Dickinson Microbiology Systems, Sparks, Md.), Candida ID (bioMérieux SA, Marcy l'Etoile, France), and Candiselect (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Three *C. albicans* serotype B isolates were included in the study.

Preparation of MAbs. (i) Immunogen preparation. *C. dubliniensis* ATCC MYA-646 was used to prepare the immunogen after growth on SDA slants for 26 h at 37°C. Cell components were extracted by Zymolyase 20T (*Arthrobacter luteus*; Seikagaku, Kogyo Co., Tokyo, Japan) as previously described by Marot-Leblond et al. (20). Solubilized antigenic components were recovered by centrifugation at 10,000 × *g* for 10 min and were stored at −20°C.

C. dubliniensis and *C. albicans* yeast cell extracts (45 mg) were fractionated by hydrophobic interaction chromatography (HIC) on a phenyl-Sepharose 6 Fast Flow (low-substitution) column (Pharmacia Biotech, Uppsala, Sweden). Elutions were carried out with a stepwise decrease in the concentration of ammonium sulfate (20). Each fraction was dialyzed against distilled water, freeze-dried, and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and gels were stained for proteins by using Coomassie brilliant blue R250 (18). As very similar protein band patterns were obtained in contiguous tracks, elution fractions were gathered, such as fractions containing 1.9 and 1.8 M, 1.7 and 1.6 M, etc., ammonium sulfate; 0.1 M ammonium sulfate to 50 mM phosphate; and 20% ethanol-distilled water.

(ii) Immunization. Adult 8-week-old female BALB/c mice (Iffa-Credo, L'Arbresle, France) received eight subcutaneous injections (100 μl each) of dialyzed freeze-dried pooled chromatographic fractions at 1-week intervals. The fractions were emulsified in complete Freund's adjuvant (Sigma, St. Louis, Mo.) for the first injection and in incomplete Freund's adjuvant (Sigma) for the subsequent injections. Mice were bled from the tail vein, and blood samples were tested for specific antibodies by immunofluorescent assay (IFA) with *C. dubliniensis* yeast cells. All animal protocols were conducted according to the recommendations of the Institutional Animal Care and Use Committee and performed by appropriately qualified personnel.

(iii) Production and screening of hybridomas. Cell fusion and hybrid selection were performed as described by Dippold et al. (11) with minor modifications (20). Ten days after cell fusion, aliquots of medium from wells with growing hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) and IFA for antibodies directed to *C. dubliniensis* components and cell surface antigens, respectively. Hybrids recognizing epitopes expressed solely on the *C. dubliniensis* yeast cell surface were immediately subcloned and stored in liquid nitrogen. MAbs were obtained from confluent hybridoma cultures. Isotypes were determined with a homemade ELISA using class-specific antibodies.

IFA using *Candida* yeast cells and ELISA using *C. dubliniensis* and *C. albicans* Zymolyase extracts or Zymolyase solution were performed as previously described by Marot-Leblond et al. (20).

Biochemical characterization of antigen 12F7-F2. The heat and chemical stabilities of the *C. dubliniensis* epitopes recognized by MAb 12F7-F2 were examined by dot blotting. The *C. dubliniensis* components released by Zymolyase were boiled for 2 min or diluted 1:2 with 2% SDS (wt/vol). Ten microliters was dropped onto polyvinylidene difluoride sheets and allowed to dry. The filter was blocked with 10% (wt/vol) nonfat dry milk in phosphate-buffered saline at 4°C overnight and probed with hybridoma culture supernatant and goat anti-mouse immunoglobulin G1 coupled to horseradish peroxidase (Caltag, Burlingame, Calif.). Bound antibodies were revealed by submersing the sheets in 0.1 M Tris buffer (pH 7.6) containing 0.5 mg · ml^{-1} 3-3′diaminobenzidine (Sigma) and 0.1% (vol/vol) hydrogen peroxide. The color reaction was arrested by rinsing in 5% acetic acid (vol/vol).

In other experiments, *C. dubliniensis* and *C. albicans* cells (10^9) grown on SDA for 48 h at 37°C were incubated for 30 min at 37°C with 1 ml of 50 mM EDTA, pH 7.5; EDTA–2-mercaptoethanol (50 mM and 0.35 M, respectively; pH 9); or Zymolyase (2 mg/ml) in the presence of phenylmethylsulfonyl fluoride. Cell components released by these treatments were tested by ELISA.

MAb 12F7-F2 purification. MAb 12F7-F2 was purified from confluent hybridoma culture supernatants using affinity chromatography on HiTrap Protein G HP columns (5 ml) (Amersham Biosciences Europe, Orsay, France) by the company SR2B (Avrillé, France) and was then used to develop a latex agglutination test. The resulting kit, named Bichro-dubli Fumouze, is distributed by Fumouze Diagnostics, Asnières, France.

Latex agglutination slide test. The Bichro-dubli Fumouze test consists of blue latex particles coated with MAb 12F7-F2, which reacts specifically with an antigen located on the surface of *C. dubliniensis* yeast cells. The latex particles are in suspension in a dissociating pink dye, giving a purple mixture. We used the test as recommended by the manufacturer. Briefly, two or three isolated colonies of each isolate, grown for 48 h at 37°C on agar media, were emulsified in 20 μl of latex suspension on the card provided. After 3 min of manual rotation or mechanical agitation, the card was examined for agglutination. A positive reaction corresponds to large blue agglutinates on a pink background. The absence of agglutinates or the presence of white aggregates without a pink background corresponds to a negative reaction. All yeasts were tested in a blinded fashion, and the results were compiled when the study had been completed.

Data analysis. The test was evaluated in terms of the numbers of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). Sensitivity was calculated as TP/(TP + FN) and specificity as TN/(TN + FP). Sensitivity and specificity of the test performed on yeast grown on different media were compared by using the χ^2 test.

RESULTS

Zymolyase extract composition. The arrays of molecular species obtained by Zymolyase digestion of *C. dubliniensis* and *C. albicans* yeasts were compared by means of 5 to 15% linear gradient SDS-PAGE. Both cell extracts yielded a large number of molecular components, ranging from 14 kDa to 94 kDa. Most components had mobilities which were the same for both extracts, but some significant qualitative and quantitative differences in protein composition were noted. For example, the major component of approximately 43 kDa in *C. albicans* had greater electrophoretic mobility than its counterpart in *C. dubliniensis*. Three components of 18, 24, and 67 kDa present in *C. dubliniensis* extract were not seen with the *C. albicans* extract, which also contained some specific (glyco)proteins (Fig. 1A).

HIC fractionation. In order to separate and identify C. *dubliniensis*-specific cell surface antigens, *C. dubliniensis* and *C. albicans* crude extracts were fractionated by HIC. No significant difference was observed between the two chromatographic profiles (data not shown). However, SDS-PAGE with Coomassie blue staining revealed some qualitative and quantitative differences in the bands within homologous fractions from these two extracts (Fig. 1B, lanes 1 to 6). Fractions, pooled as indicated above, were used for mouse immunization.

Mouse immune serum reactivity by IFA. Reactivity of mouse antiserum boosted with pooled 1.7 to 1.6 M *C. dubliniensis* HIC fractions was further investigated. By IFA, the fluorescence was homogeneous on the cell wall surface. Some cells were not labeled, while about one-third of cells were intensely labeled. This heterogeneous reactivity may reflect heterogeneous expression or accessibility of the relevant epitopes among yeast cells (Fig. 2A and B).

MAb isolation. Hybrids resulting from the fusion of X63/Ag8.653 myeloma cells and lymphocytes from a BALB/c mouse that had been immunized with the pooled 1.7 to 1.6 M HIC fractions resulted in 4,000 hybridomas. Of these, 120 produced antibodies that recognized a *C. dubliniensis* cell component by ELISA or IFA. One cell line (12F7-F2) reacted

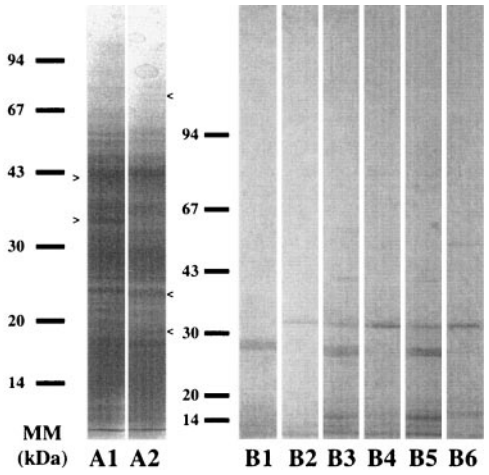

FIG. 1. Coomassie blue staining of 5 to 15% SDS-PAGE gels loaded with crude Zymolyase extracts (lanes A1 and A2) and 1.7 M (lanes B1 and B2), 1.6 M (lanes B3 and B4), and 1.5 M (lanes B5 and B6) HIC fractions of *C. albicans* (lanes A1, B1, B3, and B5) and *C. dubliniensis* (lanes A2, B2, B4, and B6) yeast cells grown at 22°C for 48 h on SDA. The molecular masses of standard proteins are listed on the left of the gels. Relevant components are indicated by arrowheads.

exclusively with the *C. dubliniensis* cell surface and cell extracts by IFA and ELISA, respectively. Hybridoma 12F7-F2 secreted an immunoglobulin G1 and was selected for further studies.

Cell surface antigen expression on *C. dubliniensis* MYA-646 and *C. albicans* 66396. In a given microscopic field, the IFA labeling intensity varied among *C. dubliniensis* yeast cells: some were negative and some were intensely stained (Fig. 2C and D). On germ tubes and pseudohyphae induced at 37°C, antigen expression was limited to the parent portion of the structure, and there was no antigen expression on the apical portion of the elongated structure (Fig. 2E and F). MAb 12F7-F2 showed no fluorescence by IFA with yeast cells and germ tubes of *C. albicans* strain 66396 (data not shown).

TABLE 1. MAb 12F7-F2 immunofluorescence reactivities of *C. dubliniensis* and related yeast strains grown on SDA for 24 h at 37°C

Organism	No. of strains tested	Reactivity[a]
Candida dubliniensis	22	+ (22)
Candida albicans	37	− (37)
Candida tropicalis	10	− (10)
Candida glabrata	7	− (7)
Candida krusei	10	− (10)
Candida kefyr	4	− (4)
Candida parapsilosis	5	− (5)
Candida guilliermondii	3	− (3)
Cryptococcus neoformans	2	− (2)
Saccharomyces cerevisiae	2	− (2)

[a] Numbers in parentheses indicate the numbers of strains with the indicated reactivity. −, no fluorescence; +, positive fluorescent labeling.

Cell surface antigen expression by other isolates and species. The antigen recognized by MAb 12F7-F2 was not detectable in the *Cryptococcus* and *Saccharomyces* genera. In the genus *Candida*, no binding was noticed for species of yeast other than *C. dubliniensis* (Table 1). All 22 *C. dubliniensis* strains tested were positive by IFA, despite slight differences in labeling intensity. These results suggest that MAb 12F7-F2 recognizes an epitope specific to the *C. dubliniensis* yeast cell surface.

Characterization of the antigenic determinant. Yeast cells of *C. dubliniensis* MYA-646 and *C. albicans* 66396 grown on SDA (48 h at 37°C) were subjected to several treatments, and the resulting extracted cell components were tested for their antigenicity by ELISA. Serial dilutions of the extracts were tested to avoid ambiguous weak signals due to steric hindrance occurring with highly concentrated solutions. MAb 12F7-F2

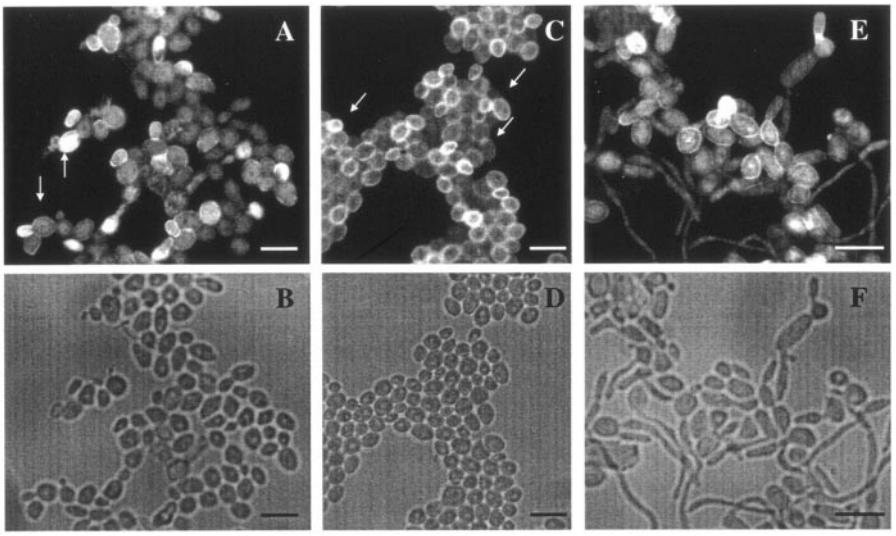

FIG. 2. Immunofluorescence (A, C, and E) and phase-contrast (B, D, and F) photomicrographs of the same microscopic fields of *C. dubliniensis* strain ATCC MYA-646 stained with immune serum from a mouse immunized with the HIC 1.7 to 1.6 M ammonium sulfate fraction (A and B) or stained with MAb 12F7-F2 (C and E), after growth on SDA for 24 h at 22°C (A to D) or in medium 199 for 3 h at 37°C (E and F). Note the heterogeneous fluorescent labeling of yeast cells (arrows) and the lack of germ tube labeling. Bars, 10 μm.

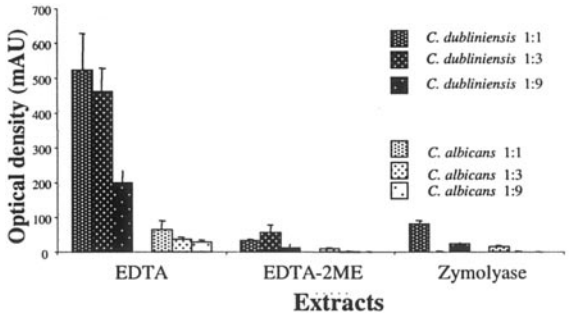

FIG. 3. MAb 12F7-F2 reactivity of chemical and enzymatic extracts of *C. dubliniensis* MYA-646 and *C. albicans* 66396 grown for 48 h at 37°C. Results are the means of triplicate determinations ± standard deviations for two independent experiments. 2ME, 2-mercaptoethanol.

did not significantly react with *C. albicans* extracts, even at high concentrations. EDTA treatment was the best extraction method for the *C. dubliniensis* antigen recognized by MAb 12F7-F2; Zymolyase treatment was less efficient (Fig. 3).

The sensitivity of the target antigen to heat and SDS was examined by dot blotting. *C. dubliniensis* Zymolyase extracts lost all their reactivity when boiled for 2 min or diluted with SDS, preventing molecular mass determination of the target antigen by SDS-PAGE and immunoblotting.

Latex agglutination test. A latex agglutination test using MAb 12F7-F2 for rapid identification of *C. dubliniensis* colonies, the Bichro-dubli Fumouze test (Fumouze Diagnostics, Asnières, France), was tested against 45 *C. albicans* and 46 *C. dubliniensis* strains and against other genera and species. The influence of the growth medium was studied with four isolation media. Table 2 summarizes the performance of the *C. dubliniensis* identification test according to the isolation medium. *C. albicans* and *C. dubliniensis* strains were tested after subculture on all the media, whereas the other species were tested only after isolation on nonchromogenic media. No false-positive results were obtained with the 45 *C. albicans* strains. Likewise, all non-*C. dubliniensis* yeasts were negative, irrespective of the growth medium. The specificity of the test was thus 100%, regardless of the growth medium. Only 1 of the 46 *C. dubliniensis* strains was negative after growth on Candida ID medium, giving a sensitivity of 97.8%. Sensitivity was 100% for *C. dubliniensis* grown on Sabouraud glucose agar with chloramphenicol and gentamicin, CandiSelect, and CHROMagar Candida (Table 2).

TABLE 2. Results of the Bichro-dubli Fumouze agglutination test and sensitivity and specificity of the test following isolation on standard media

Organism	No. of strains and isolates tested[b]	Reactivity[a] on[c]:			
		SDA-GC	CH	CS	CID
C. dubliniensis	46[d]	+ (46/46)	+ (46/46)	+ (46/46)	+ (45/46)
C. albicans	45[e]	− (45/45)	− (45/45)	− (45/45)	− (45/45)
C. glabrata	10	− (10/10)	ND[f]	ND	ND
C. guilliermondii	8	− (8/8)	ND	ND	ND
C. kefyr	6	− (6/6)	ND	ND	ND
C. krusei	8	− (8/8)	ND	ND	ND
C. parapsilosis	8	− (8/8)	ND	ND	ND
C. tropicalis	8	− (8/8)	ND	ND	ND
C. famata	7	− (7/7)	ND	ND	ND
C. lusitaniae	6	− (6/6)	ND	ND	ND
S. cerevisiae	8	− (8/8)	ND	ND	ND

[a] Values in parentheses are numbers of strains and isolates displaying the indicated reactivity. Sensitivity and specificity for SDA-GC, CH, and CS were 100%; for CID, sensitivity was 97.8% and specificity was 100%.

[b] The number of yeast strains varies, depending on the relevance of each isolation medium.

[c] SDA-GC, SDA with gentamicin and chloramphenicol; CH, CHROMagar; CS, CandiSelect; CID, Candida ID.

[d] 34 strains and 12 isolates.

[e] 36 strains and 9 isolates.

[f] ND, not determined.

DISCUSSION

C. dubliniensis infection is a growing cause for concern, especially in patients with human immunodeficiency virus infection/AIDS. Currently, accurate differentiation between *C. albicans* and *C. dubliniensis* requires the use of molecular technologies in reference laboratories or Fourier transform infrared spectroscopy (31). To our knowledge, only one MAb and a single-chain variable fragment have been shown to discriminate these two closely related yeast species on the basis of a peculiar epitope on *C. albicans* cells (6, 20). Here, we developed and tested a rapid latex agglutination method based on a MAb recognizing an epitope specific to *C. dubliniensis*. Bikandi et al., using adsorbed antiserum, described a cell surface molecule of 33 kDa capable of distinguishing between *C. albicans* and *C. dubliniensis* (4). The immunization strategy that we chose for developing a *C. dubliniensis*-specific monoclonal antibody consisted in fractionating by HIC a *C. dubliniensis* yeast cell extract. A mouse immunized with 1.7 to 1.6 M HIC fractions yielded very intense IFA labeling of *C. dubliniensis* yeast cells. Following hybridization and a two-step screening, hybridoma 12F7-F2 secreting a MAb specific for the *C. dubliniensis* cell surface was isolated. All other yeast species and genera tested against MAb 12F7-F2, including *C. albicans*, gave negative results by IFA. Furthermore, *C. albicans* labeling was always negative whatever the extraction procedure. Several lines of evidence suggest that the antigen recognized by MAb 12F7-F2 is a protein and/or is conformational. Indeed, reactivity was entirely lost when Zymolyase extracts were heated or incubated with SDS and also when 2-mercaptoethanol was present. Unfortunately, this meant that the molecular mass of the target antigen could not be established by SDS-PAGE and immunoblotting. Comparative ELISA studies of *C. dubliniensis* yeast cell extracts indicated that EDTA treatment was the most efficient and/or least caustic procedure for antigen 12F7-F2 extraction.

As the epitope is located solely at the *C. dubliniensis* cell surface, no extraction step is necessary for the test procedure based on MAb 12F7-F2. We chose to develop a latex agglutination format that could be directly applied to fresh colonies. A similar rapid colored-latex test (Bichrolatex Albicans; Fumouze Diagnostics) is already commercially available for rapid identification of *C. albicans* colonies after a 5-minute antigen extraction step (26). However, we have found that this test cross-reacts with *C. dubliniensis* colonies. The specific test for *C. dubliniensis* described here (Bichro-dubli Fumouze) is also based on colored latex beads; this avoids the need to check for autoagglutination. We evaluated the Bichro-dubli Fumouze test on colonies grown on four standard media for 48 h at 37°C

and obtained perfect specificity and 97.8 to 100% sensitivity; however, these two values are not significantly different. Two or three colonies were used for the test, as recommended by the manufacturer, but we also obtained excellent results with only one-half of a colony grown on SDA medium and with single colonies grown on CHROMagar. At least two colonies had to be used with the other media. The use of 10 colonies did not lead to a zone phenomenon. The following approach for *C. dubliniensis* colony identification can be suggested. As *C. dubliniensis* is less prevalent than *C. albicans*, we recommend testing first for the *C. albicans*-*C. dubliniensis* group by isolation on chromogenic media. Specifically colored colonies can then be tested with the rapid Bichro-dubli Fumouze test for *C. dubliniensis*. A negative result identifies the yeast as *C. albicans* and a positive result as *C. dubliniensis*. If yeast colonies are isolated on nonchromogenic media, the *C. albicans*-*C. dubliniensis* group can be identified with standard techniques, such as the germ tube test (2 to 4 h) or the more convenient rapid latex agglutination test Bichrolatex Albicans, followed by the Bichro-dubli Fumouze test to discriminate between the two species.

In conclusion, we describe the first MAb specific for *C. dubliniensis*. The Bichro-dubli Fumouze test based on this MAb is sensitive, specific, rapid (5 min), and simple. The results are easy to read, and the test does not require sophisticated equipment or experienced technicians. Moreover, given the high analytical sensitivity of the test, the prevalence and the real proportion of *C. dubliniensis* yeast cells in mixed-yeast populations can be evaluated by applying the test to several individual colonies grown on the same primary plates. Work is under way to identify the *C. dubliniensis*-specific target antigen.

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