JOURNAL OF CLINICAL MICROBIOLOGY, June 1991, p. 1095–1099
0095-1137/91/061095-05$02.00/0

Vol. 29, No. 6

New Aniline Blue Dye Medium for Rapid Identification and Isolation of *Candida albicans*

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Received 1 November 1990/Accepted 6 March 1991

Organic dyes have long been used in diagnostic microbiology to differentiate species by color reactions. We studied the ability of a new noninhibitory medium, YM agar containing 0.01% aniline blue WS dye, Colour Index 42780 (YMAB), to identify *Candida albicans* among 1,554 yeast specimens obtained from seven clinical laboratories. Appropriate American Type Culture Collection and other characterized strains served as controls. A total of 487 of the clinical strains were identified as *C. albicans*. The remainder were other *Candida* species and non-*Candida* yeasts. Clinical isolates and controls were grown on Sabouraud agar for 18 h at 30°C and then transferred to YMAB. Plates were incubated for 12 to 18 h at 30°C, and colonies were observed for yellow-green fluorescence under long-wave UV light (A_{365}). All control strains of *C. albicans* and *Candida stellatoidea* fluoresced, as did 480 of the 490 isolates designated as *C. albicans* (which included 3 strains of *C. stellatoidea*). Cells of *C. albicans* grown on YMAB produced germ tubes in serum. Only five of the other 1,062 non-*C. albicans* yeasts fluoresced. The sensitivity and specificity were 98.0 and 99.5%, respectively, with a predictive value of 99.1%. A fluorescent metabolite was found in cell wall particulate fractions of *C. albicans* sonic extracts grown on YMAB but not in non-*C. albicans* yeasts. This metabolite showed the same spectral curve as those of metabolites from whole cells in a recording spectrofluorometer when it was excited at 400 nm and scanned from 420 to 550 nm. Thus, growth on YMAB generates the production of a fluorescent moiety that can be used to specifically identify *C. albicans* within 12 to 18 h.

The more frequent use of broad-spectrum antimicrobial agents can be correlated with the increased presence of fungi among the general population. Fungi which may be present as normal flora, such as *Candida albicans*, may predominate in the ever increasing numbers of immunocompromised, immunoincompetent, and aging hosts in the population (3). Moreover, *C. albicans* is the yeast species most often found in early cases of AIDS, especially from epithelial tissue specimens. In addition, Krogh et al. (9) reported that *C. albicans* is one of the most common fungal isolates from leukoplakia lesions. For these reasons, the ability to rapidly isolate and identify fungi, especially *C. albicans*, becomes ever more important. It is safe to say that the use of various dyes and stains has continually helped revolutionize the manner in which microorganisms have been detected and identified. New uses for dyes are constantly being developed that involve both instrumented and noninstrumented methodologies (2, 8, 10, 12, 14–17, 20, 22). Several of these procedures have been specifically developed for yeast identification (12, 14, 16, 20, 22). The YM+aniline blue dye medium was initially developed by Fung and Liang (4) to rapidly isolate and identify *C. albicans* from food and environmental samples. In response to the need for rapid procedures mentioned above, this study was undertaken to assess the value of the YM+aniline blue WS dye agar in rapidly identifying *C. albicans*.

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MATERIALS AND METHODS

Strains. The following characterized strains served as controls for these experiments: *C. albicans* ATCC 10231, ATCC 26790, and ATCC 10261; *Candida stellatoidea* ATCC 36232 and ATCC 28836; *Candida lipolytica* ATCC 34088 and ATCC 20362; *Candida catenulata* NRRL Y-1508, *Candida famata* NRRL Y-1453, *Candida guilliermondii* NRRL Y-7572, *Candida krusei* ISU HB-2, *Candida parapsilosis* NRRL Y-2315 and ISU MM-4; *Candida pseudotropicalis* NRRL Y-1264, *Candida pulcherrima* NRRL HB-29, *Candida reukaufii* NRRL Y-6343, *Candida sake* NRRL Y-1622, *Candida tropicalis* NRRL Y-1552, *Candida utilis* NRRL Y-900, *Candida vini* NRRL Y-6658, *Candida rugosa* NRRL Y-1249, *Hansenula anomala* NRRL Y-366, *Saccharomyces cerevisiae* NRRL Y-12647, *Saccharomyces chevalieri* NRRL Y-12533, *Saccharomyces dairensis* NRRL Y-1353, *Saccharomyces delbrueckii* NRRL Y-1567, *Saccharomyces rosei* NRRL Y-866, *Saccharomyces rouxii* ISU FY-4, *Zygosaccharomyces rouxii* NRRL Y-2547, *Debaryomyces hansenii* NRRL Y-7426 and NRRL Y-1454, *Debaryomyces subglobosus* NRRL Y-6666, *Rhodotorula minuta* NRRL Y-1589, *Pichia fermentans* NRRL Y-1619, *Brettanomyces anomalus* NRRL Y-1415, *Trichosporon beigelii* NRRL Y-1490, *Trichosporon pullulans* NRRL Y-1522, and *Bullera alba* NRRL Y-6655. ATCC is the American Type Culture Collection, Rockville, Md.; NRRL is the Northern Regional Research Laboratory, Peoria, Ill.; and ISU is Iowa State University, Ames.

Yeast isolates were obtained from seven clinical microbiology laboratories in the Houston, Tex., area. The names assigned by these laboratories and the sites of their isolation were recorded and given a code number. This code number was used in all experiments to prevent prejudice in reading the results. Over 1,554 cultures were tested. If needed for

verification, identifications were performed by using the Silva-Hutner-Cooper schema based on auxanographic carbohydrate assimilation and germ tube formation (19). The names listed in Tables 1 and 2 are, for the most part, those names designated by the various laboratories. The majority of cultures were tested only once. Repeat cultures were picked as part of quality control procedures.

Media. Since cultures were received on a variety of media (including Vitek yeast identification cards), all strains were grown overnight on Sabouraud agar (Difco, Kalamazoo, Mich.) at 30°C. Overnight colonies were then transferred to polystyrene quad-plate petri dishes containing 0.01% aniline blue dye WS (Colour Index [C.I.] 42780) in YM agar (Difco). The aniline blue WS dyes (acid blue, methyl blue 93; from Aldrich Chemical Co., Milwaukee, Wis., or Sigma Chemical Co., St. Louis, Mo.) were both equivalent in producing fluorescence in *C. albicans*. The dye was added to the medium before autoclaving.

Fluorescence determinations. Plates were placed under long-wave UV light (A_{365}) in a Chromato-Vue model C5 (Ultra-Violet Products Inc., San Gabriel, Calif.). A 0.45-μm-pore-size Millipore filter was placed on a YM+dye plate and heavily inoculated with a 24-h culture of *C. albicans*. Duplicate plates were inoculated with either *C. krusei* or *C. tropicalis*. After a 24-h incubation at 30°C, the filters were removed. Both of the filters and the areas of the plates under the filters were observed for fluorescence. These experiments were repeated twice. The white filters had turned blue, indicating that the YM+dye medium had also diffused upward through the filter. In addition, growth was removed from 18-h dye plates and either smeared on 24-mm-pore-size Millipore filters or suspended in 1 ml of saline placed in a disk and inserted into a recording scanning Spectronic 88 spectrofluorometer (Bausch & Lomb, Rochester, N.Y.). Excitation was set at 400 nm, and the emission scanning range was 420 to 550 nm. Cells of *C. albicans* were washed four times by filtration, and the retentate was scanned as described above. Additionally, *C. albicans* cells were removed from an 18-h plate resuspended in 5 ml of saline and sonicated for 15 to 30 min (model W-10; Ultrasonics Heat Systems, Plainview, N.J.). The sonic extract was filtered through a 0.22-μm-pore-size Millipore filter. Both the filter (particulate fraction) and the filtrate were scanned as described above for fluorescence. The filtrate was also centrifuged for 60 min at 40,000 rpm in a Beckman L8-70M ultracentrifuge (Beckman Instruments, Fullerton, Calif.).

Formula. The following formulas were used to determine sensitivity, specificity, and predictive value: sensitivity, (true positives × 100)/(true positives + false negatives) = percent; specificity, true negatives/(true negatives + false negatives) = percent; predictive value, (true negatives × 100)/(true negatives + false negatives) = percent.

RESULTS

The yeast strains received from the contributing laboratories were isolated from 25 anatomical sites. The eight most common sites were the genitourinary tract (n = 644), sputum (n = 268), blood (n = 119), wounds (n = 94), respiratory (n = 93), stool (n = 19), drains (n = 13), and cerebrospinal fluid (n = 6).

There were relatively few misidentifications by the participating clinical laboratories. All discrepancies were investigated for germ tube formation, and many were also identified biochemically in our laboratory. Otherwise, the laboratory identification was used as provided.

TABLE 1. Comparison of the fluorescence reactions of *Candida* species with germ tube formation

Original laboratory designation	No. of cultures tested	Fluorescence reaction[a]	Germ tube reaction[b]
C. albicans	477	+	+
C. stellatoidea	3	+	+
C. albicans	10	−	+
C. tropicalis	454	−	−
C. parapsilosis	148	−	−
C. parapsilosis	1	+	−
C. krusei	24	−	−
C. krusei	1	+	−
C. guilliermondii	17	−	−
C. pseudotropicalis	5	−	−
C. famata	8	−	−
C. lusitaniae	12	−	−
C. rugosa	2	−	−
C. lambica	1	−	−
C. zeylanoides	1	−	−
C. pulcherrima	1	+	−
C. lipolytica	2	−	−
C. catenulata	1	−	−
C. reukaufii	1	−	−
C. sake	1	−	−
C. utilis	1	−	−
C. vini	1	−	−

[a] +, fluorescence; −, no fluorescence.
[b] +, presence of a germ tube; −, absence of a germ tube.

Table 1 shows the fluorescence reactions of 1,172 organisms designated as *Candida* species from the contributing laboratories and control strains. Uninoculated YM+dye medium did not fluoresce. Three strains of *C. stellatoidea* and 477 strains of *C. albicans* fluoresced. Ten strains labeled as *C. albicans* did not fluoresce. These were germ tube-positive isolates and were subsequently biochemically identified as *C. albicans* in our laboratory. Eleven other strains that were submitted as strains of *C. albicans* were germ tube negative and were not identified as *C. albicans* biochemically. Three other germ tube-negative strains that were accurately identified as *C. parapsilosis*, *C. krusei*, and *C. pulcherrima* fluoresced. Nineteen cultures designated as *C. tropicalis* and *Torulopsis glabrata* also fluoresced. However, upon repeat testing, they were germ tube positive in our hands, and 10 of them were biochemically identified as *C. albicans* strains. The other nine were not further identified. In evaluating the aniline blue-derived fluorescence of 382 non-*Candida* yeasts, only two strains were positive (Table 2). These were subsequently shown by morphology and biochemical reactions to be two isolates of *Trichosporon beigelii*. On the basis of these data, the fluorescence test for *C. albicans* had a sensitivity of 98.0% and a specificity of 99.5%. The overall predictive value for the test was 99.1%.

The aniline dye was necessary for the reaction to occur, since no fluorescence was observed when the control strains were grown on the YM medium without the dye. A typical example is shown in Fig. 1. Temperatures between 25 and 30°C were necessary to maintain the specificity of the reaction. *C. parapsilosis* and several other species had a tendency to fluoresce at 35 to 37°C. Although fluorescence could be detected as early as 12 h, there was no significant difference for up to 48 h of incubation. However, longer times resulted in nonspecific fluorescence. *C. albicans* grown on YM+aniline blue dye medium retained its fluorescence for several months in the refrigerator, indicating that

TABLE 2. Production of fluorescence reactions of non-*Candida* yeasts on YM+aniline blue WS dye medium

Original laboratory designation	No. of cultures tested	Fluorescence reaction[a]
Torulopsis glabrata	309	−
Trichosporon beigelii	16	−
Trichosporon beigelii	2	+
Trichosporon pullulans	10	−
Saccharomyces cerevisiae	14	−
Saccharomyces dairensis	1	−
Saccharomyces chevalieri	1	−
Saccharomyces rouxii	1	−
Saccharomyces delbrueckii	1	−
Saccharomyces rosei	1	−
Zygosaccharomyces rouxii	1	−
Cryptococcus neoformans	10	−
Cryptococcus laurentii	1	−
Cryptococcus albidus	1	−
Rhodotorula glutinis	4	−
Rhodotorula minuta	1	−
Rhodotorula frutins	1	−
Pichia fermentans	1	−
Bullera alba	1	−
Brettanomyces anomalus	1	−
Debaryomyces hansenii	2	−
Debaryomyces subokobosus	1	−
Hansenula anomala	1	−

[a] +, fluorescence; −, no fluorescence. All of these cultures were germ tube negative.

the fluorescent moiety was stable. *C. albicans* did not fluoresce when it was incubated for up to 48 h in dye alone. When the dye was added to Sabouraud medium, *C. albicans* and several other yeasts fluoresced. YM medium appeared to restrict fluorescence to *C. albicans* with a specificity of 99.5%.

The emission spectrum of cells of *C. albicans* showed a maximum peak at 450 nm (Fig. 2). The emission spectra of *C. tropicalis*, *C. parapsilosis*, *T. glabrata*, and *S. cerevisiae* had no evidence of fluorescence, paralleling that of the uninoculated medium (Fig. 2). When cells of *C. albicans* were washed four times by filtration, the spectrum of the filtrate resembled that of the uninoculated medium, while the spectrum of the retentate resembled that of unwashed *C. albicans* (Fig. 2). When *C. albicans* and either *C. tropicalis* or *C. krusei* were inoculated onto a 0.45-μm-pore-size Millipore filter placed on YM+dye medium and incubated as usual, there was no fluorescence either on the undersides of the filters or on the plates under the filters. The *C. albicans* organisms on top of the filter fluoresced. When cell sonic extracts of *C. albicans* were observed (Fig. 3), the fluorescent moiety similarly appeared to be located in the particulate or cellular fraction (which appeared to be broken cell walls under light microscopy) rather than the supernatant (Fig. 3B). The fluorescence spectrum of the particulate fraction was similar to that of the whole cells (Fig. 2 and 4). Again, four repeated washings did not remove the fluores-

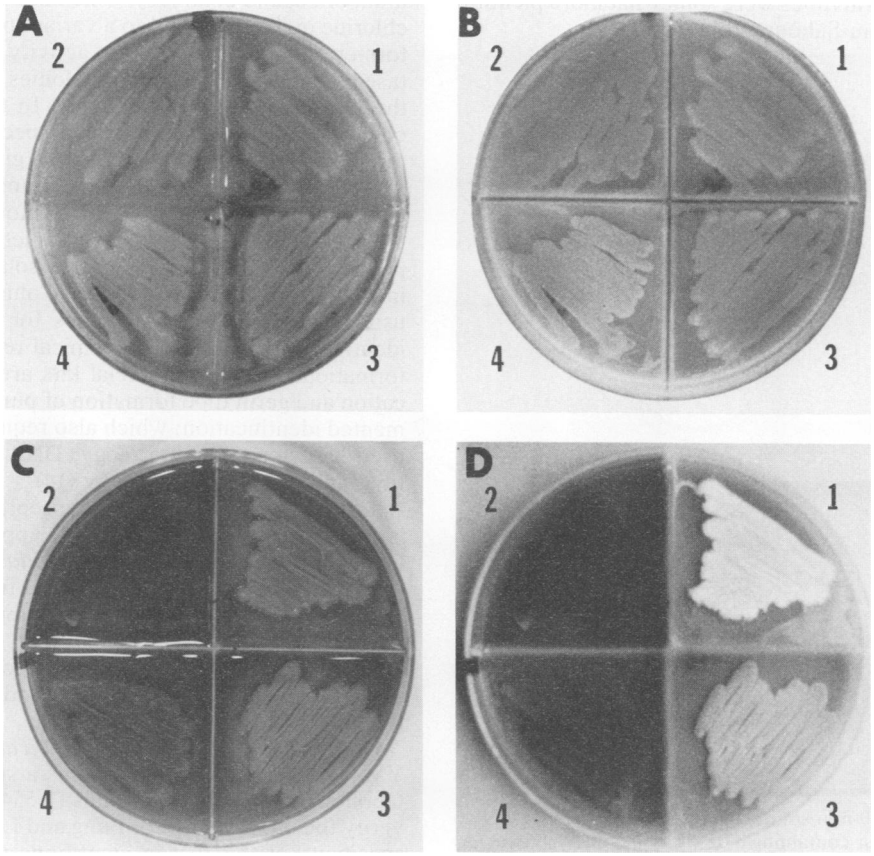

FIG. 1. Effect of 0.01% aniline blue WS dye on the fluorescence of *C. albicans* (quad 1), *T. glabrata* (quad 2), *C. albicans* (quad 3), and *C. tropicalis* (quad 4) with or without exposure to UV light (A_{365}). (A) YM agar with no dye under white light; (B) the same plate as in panel A under UV; (C) YM agar containing aniline blue WS dye under white light; (D) the same plate as in panel C under UV light showing fluorescence of only the two *C. albicans* strains.

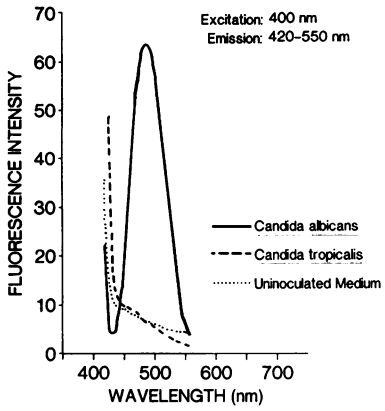

FIG. 2. Emission spectra of uninoculated YE agar with 0.01% aniline blue WS dye and spectra after inoculation of *C. albicans* and *C. tropicalis*. Excitation at 400 nm was followed by recording the fluorescence emission from 420 to 550 nm.

cence from the particulate fraction, and these washings (supernatant) did not fluoresce (Fig. 3 and 4).

Germ tube formation from yeasts grown on YM+aniline blue dye medium and Sabouraud medium were compared for *C. albicans* ATCC 10231, a freshly isolated *C. albicans* strain, and *C. tropicalis* (negative control). Both *C. albicans* cultures produced germ tubes on both media, while *C. tropicalis* did not. Germ tubes were somewhat more plentiful in cells harvested from Sabouraud medium.

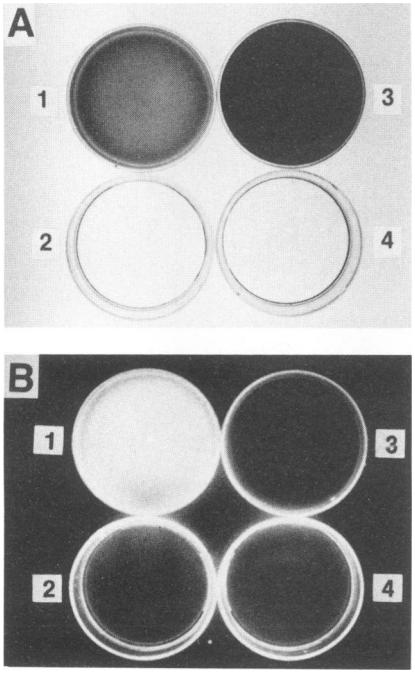

FIG. 3. Effect of a 30-min sonication on the fluorescence of two yeasts grown on YM agar containing 0.01% aniline blue WS dye. (A) exposure to white light; (B) exposure of the same plates to UV light (A_{365}); 1, cellular or particulate fraction of *Candida albicans*; 2, supernatant or soluble fraction of *C. albicans*; 3, cellular or particulate fraction of *T. glabrata*; 4, supernatant or soluble fraction of *T. glabrata*.

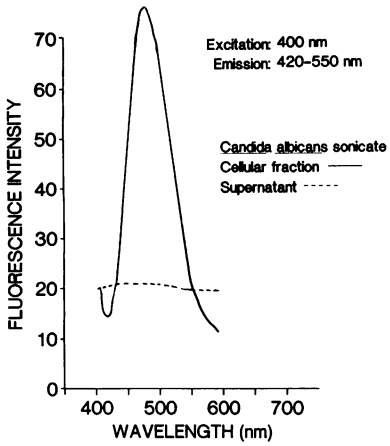

FIG. 4. Emission spectra of the cellular (particulate) and supernatant (soluble) fractions of sonicated cells of *C. albicans* grown on YM agar containing 0.01% aniline blue dye WS. Excitation at 400 nm was followed by recording of the fluorescence emission from 420 to 550 nm.

DISCUSSION

There have been several media proposed for the initial differential isolation of *Candida* species from clinical specimens. The two most commonly used are the medium of Nickerson, (12) which contains a polysulfite, and the medium of Pagano et al. (15), which has a triphenyl tetrazolium chloride indicator added to a variant of Sabouraud agar. The former medium indicates the activity of disulfhydryl reductase by the formation of dark colonies and was developed for the identification of *C. albicans*. In the latter, *C. albicans* colonies were colorless and other species varied from pink to red. However, both of these media give many false-positive and false-negative results (18, 21) and should be used with caution (13). Hunter et al. (7) used morphotype markers, and their test took over 10 days to complete. Clinical laboratories plate clinical specimens for yeast isolation on various agars, including blood, Sabouraud, and Columbia agars. Then, they usually streak selected colonies for isolation followed by identification by using biochemical reactions and germ tube formation. Many commercial kits are available for identification and germ tube formation of pure cultures (14). Instrumented identification, which also requires pure cultures, has used light scattering, serology, DNA restriction fragmentation, and gas chromatography (14).

The number of individuals susceptible to yeast infections is increasing, and *C. albicans* appears to be the most invasive and virulent of the *Candida* species as well as the one that is most commonly isolated from clinical specimens (7). Thus, there is a need for the rapid isolation and identification of this yeast. Toward that end, we explored the ability of this simple, relatively inexpensive YM+dye agar to serve both as an isolation medium and as a direct indicator of the presence of *C. albicans*.

Our data show that *C. albicans*, when grown on YM+aniline blue medium, forms a specific fluorescent metabolite. The number of strains (1,554) tested were ample to verify the initial finding of Fung and Liang (4) concerning the specificity of this test in identifying *C. albicans*. We found relatively few false-positive and false-negative results, 5 and 10, respectively. This is excellent when compared with the reported efficacy of the other primary isolation media (those of Nickerson [12] and Pagano et al. [15]) (13, 18, 21).

This study was undertaken to verify the specificity of the test and concentrated on the use of purified clinical isolates. However, preliminary studies of over 100 patients in which oral lavages were used on individuals undergoing cancer chemotherapy (6) and healthy oral clinic patients (5) have indicated the medium's efficacy as a primary isolation medium.

The fact that *C. albicans*, when grown on the YM+dye medium, forms germ tubes in serum also enhances its usefulness. Thus, positive identification of *C. albicans* from clinical specimens can be made (including germ tube formation data) within 18 to 24 h after receipt of the specimen in the laboratory. The exact reaction between the aniline dye and the cell wall of *C. albicans* which produces the fluorescent metabolite is not known. The sparse literature available on aniline blue dyes suggests that there is a possible reaction with specific cell wall polysaccharides (1, 11). One of these references indicates the presence of yeast-like glycan in *Pneumocystis carinii* which might be a potential binding site (11). This will be the subject of future investigations.

In summary, in this report we provided convincing evidence that a YM+aniline blue WS dye medium can be used to rapidly identify *C. albicans* from clinical specimens with high accuracy and predictability. The medium is simple to prepare and easy to use and only requires a long-wave UV (A_{365}) light for the detection of fluorescence.

ACKNOWLEDGMENTS

We acknowledge the following microbiology laboratories for the generous contributions of cultures and clinical isolates for this study: St. Luke's Episcopal Hospital, Methodist Hospital, Ben Taub General Hospital, Hermann Hospital, Texas Children's Hospital, Medical Clinic of Houston, and City of Houston Department of Health and Human Services. We also thank James Vogel for helping with the spectral curves, L. Brown for providing technical assistance, and J. Campbell for photographic help. We are also indebted to Thomas Wickersham of Aldrich Chemical Co., Inc., who verified that our dye samples were indeed aniline blue WS (acid blue 93, methyl blue, C.I. 42780) by high-performance liquid chromatography.

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