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New Culture Medium for the Presumptive Identification of *Candida albicans* and *Cryptococcus neoformans*

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A new medium composed of Tween 80, oxgall, caffeic acid, and Davis agar (TOC) that provides for the rapid presumptive identification of *Candida albicans* and *Cryptococcus neoformans* is described herein. *C. albicans* is differentiated from other yeasts by the sequential production of germ tubes and chlamydospores. In a comparison with cornmeal agar control plates, there was an increase of chlamydospore-forming strains of *C. albicans* (97.1% versus 87.2%) and a decrease in the time required for chlamydospore formation (24 h versus 48 h). *C. neoformans* produced a brown pigment on TOC, which is specific for its identification, thus differentiating it from the other yeasts. A comparison of 24-h pigment production by *C. neoformans* on TOC with that of birdseed agar showed a dark, coffee brown color in the former cultures and a light brown color in the latter. The change in pigmentation of *C. neoformans*, as well as morphological changes in *C. albicans*, can be induced within 3 to 12 h and in not more than 24 h on the TOC medium.

In the culturing of immunosuppressed patients, we have found *Candida albicans* to be the fungus most commonly encountered in clinical samples. This potential pathogen is presumptively identified by the formation of germ tubes in biological fluids (3, 19) and by the formation of chlamydospores on various vegetable-polysaccharide agars (17, 31). Recently, two solid media have been introduced for the sequential development of germ tubes and chlamydospores by *C. albicans* on a single agar plate (4, 15). The first medium is a combination of rice infusion, oxgall, and Tween 80, whereas the second medium consists of 0.1% glucose in 2% New Zealand agar. Both agars permit morphological identification of *C. albicans* within 24 to 48 h.

Another fungus of great clinical importance, due to the severity of infection in certain predisposed patients (2) as well as its predilection for the central nervous system, is the encapsulated yeast *Cryptococcus neoformans*. As in the identification of *C. albicans*, various media have been recently developed for the presumptive identification of *C. neoformans*. One medium consists of Sabouraud dextrose agar supplemented with antibiotics and trypan blue dye (28). A colony that grows at 37°C and has turned dark blue is presumed to be that of *C. neoformans*. The serious disadvantage of this agar is that *Trichosporon cutaneum* and *Rhodotorula* species, both common contaminants, also stain a dark blue. The inability to differentiate between these contaminants and *C. neoformans* by colony color would necessitate identification by more conventional methods, such as urease production, creatinine utilization, or the assimilation of certain sugars, particularly inositol (1).

A second medium relies upon an extract of *Guizotia abyssinica* seeds to provide a substrate for a phenol oxidase enzyme present within *C. neoformans* (24, 26, 27). The subsequent enzymatic reaction produces a pigment that impregnates the yeast cell wall, turning the colony brown. Other investigators have suggested that solid media made from extracts of potatoes, carrots, or chemicals can also act as substrates for the phenol oxidases. The *Guizotia* extract has also been combined with Littman oxgall agar to provide a medium that can suppress the microbial contamination found in some primary specimens, yet allowing growth and identification of *C. neoformans* (6). In the third medium, caffeic acid and ferric citrate serve as substrates for the phenol oxidase; again, a brown colony results from the activity of the enzyme (11). The phenol oxidase reaction in the caffeic acid-ferric citrate media, as well as in the other agars mentioned, is specific for *C. neoformans* and further identification is not necessary (23).

We felt that it would be possible to combine the advantages of the rice infusion, oxgall, and Tween 80 medium, or the more simple combination of 0.1% glucose in 2% New Zealand agar

along with caffeic acid, and develop a multipurpose medium for the presumptive identification of *C. albicans* and *C. neoformans*. This paper reports the successful development of a medium that will enable the clinical laboratory to identify *C. albicans* and *C. neoformans* on a single agar plate.

MATERIALS AND METHODS

Strains. All yeast isolates were obtained from the following sources: (i) the Tulane University Mycology Collection, (ii) clinical isolates from the Granville C. Morton Cancer and Research Hospital, and (iii) the Mycology Laboratory of Baylor University Medical Center at Dallas. All isolates were identified by the currently accepted identification criteria for yeasts (16, 25).

Media. Cornmeal agar (CMA, Difco) was made up according to the manufacturer's directions and supplemented with 0.1% Tween 80. Birdseed agar was prepared by a modification of the procedure recommended by Silva-Hutner and Cooper (25). Briefly, 50 g of birdseed (*G. abyssinica*) was suspended in 100 ml of distilled water and pulverized in a blender (Waring, commercial) for 5 min; it was then brought to 1,000 ml with distilled water. After the disrupted seeds were boiled in the distilled water for 30 min, the extract was filtered through a 9.0-cm filter (Whatman GF1a). To the filtrate was added the following: 1.0 g of dextrose, 1.0 g of KH_2PO_4, 1.0 g of creatinine, 20 g of agar, and 0.1 g of chloramphenicol. The test medium (TOC), consisting of Tween 80, oxgall, caffeic acid, and Davis agar, was prepared as follows: to 1 liter of distilled water was added 10 g of oxgall, 20 g of agar, 10 ml of a 10% solution of Tween 80, and 0.3 g of caffeic acid. The mixture was stirred, heated to boiling to allow the components to go into solution, and sterilized at 121°C with 15 lb/in^2 of pressure for 15 min. After sterilization and slight cooling, 30 ml of medium was poured into each petri dish (100 by 15 mm). After solidification of the medium, the plates were inverted and allowed to dry overnight at room temperature.

Procedures. Stock yeast strains were subcultured onto Sabouraud dextrose agar and allowed to grow for 72 h. After this preliminary growth period, a means of simulating growth on a primary plate, yeasts were subcultured by the Dalmau technique (29) onto either TOC or cornmeal plates. To obtain a heavy inoculum, a sterile swab was used to make a sweep of the colonies on the Sabouraud dextrose agar plates. Four specimens were inoculated onto each plate and incubated at 37°C for 3 h, after which the inoculated areas were examined for germ tube formation. After examination for germ tubes, we allowed the plates to stand at room temperature, with periodic observations over the next 72 h for chlamydospore production. Chlamydospore production was determined by scanning the inoculated areas of the plates with the low-power objective of a microscope for areas rich in chlamydospores.

Cryptococcus species were tested for pigment production by applying a heavy inoculum, as above, to both TOC and birdseed plates. Each plate was inoculated with eight isolates of *Cryptococcus* and incubated at room temperature for 72 h. Strains were checked periodically during this incubation period for brown pigment production.

To evaluate the TOC medium as a possible primary plating medium in the culturing of blood, the reconstruction procedure of Dorn et al. (7, 8) was used. An overnight broth culture of *C. albicans* or *C. neoformans* was diluted to give a range of 1,000 to 3,000 colony-forming units per ml. Portions of each inoculated dilution (0.1 ml) were added to 7 ml of 21-day-old human donor blood in citrate-phosphate-dextrose, obtained from the Wadley Central Blood Bank, Dallas, Tex. The blood and yeast suspension was shaken vigorously, introduced into an evacuated blood culture tube, processed as previously described (7, 8), and inoculated onto each of five TOC plates. All plates were incubated for 72 h and periodically examined for yeast growth, morphology, and colonial pigmentation.

RESULTS

Table 1 records the formation of germ tubes and chlamydospores by 214 isolates of *C. albicans*, *C. tropicalis*, and *C. stellatoidea*. Ninety-six percent of all *C. albicans* strains and 80% of all *C. stellatoidea* strains tested formed germ tubes within 3 h when incubated at 37°C. We found that many strains of both positive species varied in their ability to form germ tubes, supporting the results of other investigators (4). In some cases, the majority of cells in the inoculum formed germ tubes, whereas in other

TABLE 1. *Comparative production of germ tubes and chlamydospores in Dalmau slide cultures on cornmeal or TOC plates*

Organism	No. of isolates	Medium	Germ tube formation		Chlamydospore production					
					24 h		48 h		72 h	
			No.	%	No.	%	No.	%	No.	%
Candida albicans	138	CMA	0	0.0	25	18.1	84	60.9	112	81.2
		TOC	132	95.7	119	86.2	133	96.3	134	97.1
C. stellatoidea	30	CMA	0	0.0	0	0.0	0	0.0	0	0.0
		TOC	24	80.0	24	80.0	24	80.0	24	80.0
C. tropicalis	46	CMA	0	0.0	0	0.0	0	0.0	0	0.0
		TOC	0	0.0	0	0.0	0	0.0	0	0.0

strains (particularly those of *C. stellatoidea*), a very small percentage of cells formed germ tubes. Thus, the variance of germ tube formation necessitated scanning the entire inoculated area under the cover slip before judging a particular *Candida* species to be germ tube negative.

Chlamydospores were produced by 97.2% of the *C. albicans* isolates and by 80% of the *C. stellatoidea* isolates on the TOC medium. In addition to achieving a greater percentage of chlamydospore-forming strains, chlamydospores appeared earlier in TOC cultures when compared with the CMA control plates (Table 1). For example, within 24 h, 86.2% of the *C. albicans* strains had formed chlamydospores on TOC as opposed to only 18.1% on CMA. Chlamydospores were present in 80% of the *C. stellatoidea* strains after 24 h of incubation on TOC, but the same strains failed to form chlamydospores on CMA. It should also be noted that *C. tropicalis* did not form germ tubes or chlamydospores on either TOC or CMA. *C. albicans* and *C. stellatoidea* formed a few chlamydospores within 14 h of growth, and they had produced numerous clusters by 18 to 24 h. However, on companion CMA plates, chlamydospore production began only after a minimum of 24 h of incubation.

Germ tubes formed by either *C. albicans* or *C. stellatoidea* on TOC medium are identical in morphology, making differentiation between the two yeasts on this basis impossible. It should also be emphasized that the germ tube morphology on TOC medium is compatible with its classical description in biological fluids (3, 13), since the tube lacks any constriction at its point of origin (Fig. 1). In addition, the morphology of chlamydospores formed on TOC by *C. albicans* and *C. stellatoidea* also remains unaltered from that reported for the vegetable-polysaccharide agars (Fig. 2) (17). Studies of clinical specimens of yeasts contaminated with bacteria demonstrated that pure cultures were not necessary for the desired changes in morphology to occur.

In a survey of yeasts other than *C. albicans* and *C. stellatoidea* but commonly encountered in the laboratory, neither germ tubes nor chlamydospores were produced on TOC (Table 2). The characteristic pigmentation and morphology of other yeasts remained unaltered on TOC, as indicated by the following illustrations: (i) *Rhodotorula* species were red yeasts with limited pseudohyphae, (ii) *Torulopsis glabrata* were white yeasts, and (iii) *Trichosporon* species were basically cream-colored and produced hyphae and arthrospores. An exception was *C. krusei*, which, of all *Candida* species tested, was the only one failing to produce pseudohyphae on the TOC medium.

The TOC medium was also evaluated as a differential medium for separating *C. neoformans* from the other cryptococci by the phenol oxidase reaction. Only strains of *C. neoformans* were positive for the brown pigment, and positivity was attained within 24 h (Table 3). In fact, the majority of *C. neoformans* strains showed the color change within 6 to 12 h (Fig. 3). A comparison of 24-h pigment production by *C. neoformans* on TOC with that of birdseed agar showed the pigment to be dark, coffee brown in the former cultures and only light brown in the latter. As mentioned above, the characteristic pigmentation of other yeasts, such as the white color of *T. glabrata* and red color of the *Rhodotorula* species, was not altered by growth on the TOC medium.

The use of TOC as a primary plating medium for the isolation of yeasts from blood cultures had limited success. *C. neoformans* grew as isolated brown colonies, and *C. albicans* formed pseudohyphae and chlamydospores. These structures could be visualized by making a lacto-phenol cotton blue wet mount of an isolated colony, or by scanning the edge of an isolated colony on a plate with a microscope (×100). Although *Candida* morphology and *C. neoformans* pigmentation remained unchanged when TOC was used as a primary plate, the time taken for visible growth to occur (3 to 5 days) limits its usefulness in this capacity at present.

DISCUSSION

The increased incidence of oportunistic fungal infections in patients undergoing treatment with corticosteroids (21, 25), surgical manipulations (22), catheterization (30), immunosuppression (10, 18), or in the advanced state of malignancy (5, 9, 14) has made it necessary for many clinics and hospitals to improve their fungal-identification techniques (1, 16). Most small clinics and laboratories, however, have not taken the time for proper fungal identification, for reasons of work overload and lack of technical expertise (1).

Although many techniques and media have been developed to streamline the identification of bacteria from clinical samples, particularly the *Enterobacteriaceae*, few techniques have been developed to provide the same service in identifying fungi. The fungal-identification techniques and media that have been developed to date usually are dedicated techniques; i.e., they serve to identify one organism or group of organisms at a time. One such medium is the combination of Littman oxgall agar and *Guizo-*

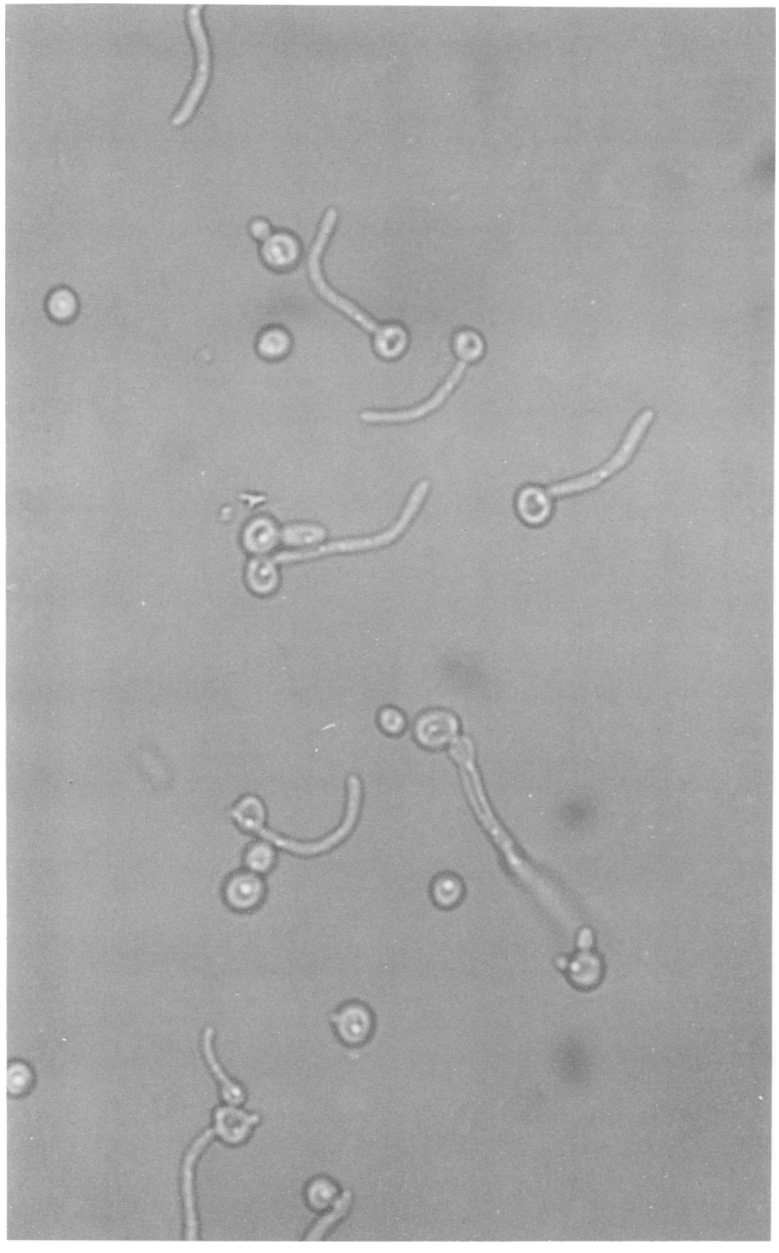

FIG. 1. *Typical germ tube formation by C. albicans after 3 h of incubation at 37°C on TOC solid medium.*

tia extract to form an agar that will suppress both saprophytic fungal and bacterial growth and permit the identification of only one organism, *C. neoformans* (6).

The Pagano-Levin base agar (20), used for the primary isolation of *Candida* species, is an example of a medium for group identification. *Candida* species are differentiated by their ability to reduce 2, 3, 5-triphenyl tetrazolium and produce a white to red colony, depending upon the species of *Candida*. Since some gradations of colonial color between genera and even species within genera do occur on this medium, there would be some ambiguity in determining the identity of a colony on the plate. Therefore, a conventional schema of differentiation would be required to completely identify the isolated colonies.

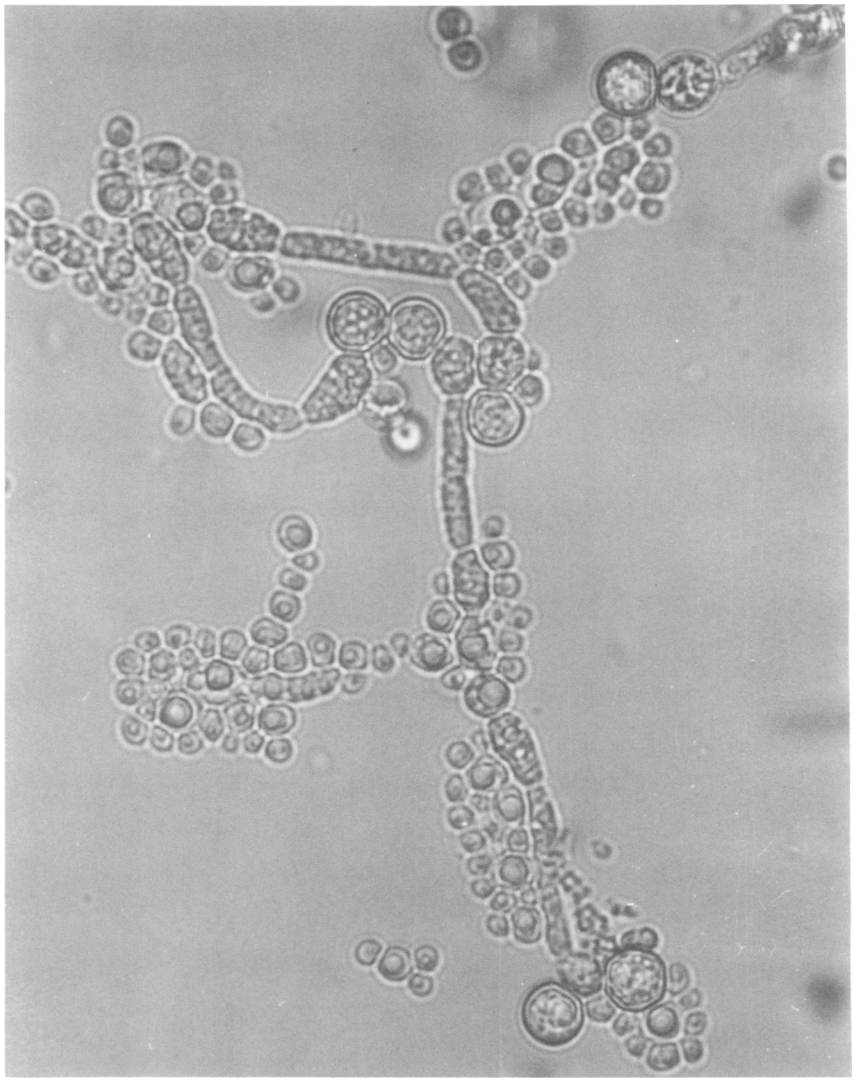

FIG. 2. *Characteristic pseudomycelium with chlamydospores formed by C. albicans after 3 h of incubation at 37°C and 18 h at room temperature on the TOC medium.*

The TOC medium, however, should greatly aid the clinical laboratory in simplifying fungal identification, since presumptive identification of *C. albicans* and *C. neoformans* is possible within 3 to 12 h and in not more than 24 h. A disadvantage arises when the medium is used to differentiate *C. albicans* from germ tube- and chlamydospore-positive isolates of *C. stellatoidea,* as morphologically, the germ tubes and chlamydospores are identical. However, *C. albicans* is easily separated from *C. stellatoidea,* since it is the more common clinical isolate, and it assimilates sucrose (16, 25). Another disadvantage of the TOC medium is that *Candida krusei,* of all species of filamenting yeasts screened, will not form hyphae. However, as this organism is a rare clinical isolate, neither this nor the above disadvantage outweighs the medium's usefulness in prompt identification of the more common pathogens, *C. albicans* and *C. neoformans*.

Although the use of caffeic acid-ferric citrate-impregnated disks (12) for the identification of *C. neoformans* is a slightly faster procedure than using the TOC medium, this method has disadvantages. The apparent saving of identifi-

TABLE 2. *Appearance of selected fungi on TOC medium*

Organism	No. of strains	Morphology[a]	Pigmentation[b]	Time taken for characteristic growth (h)
Candida albicans	138	f + c	w	32
C. stellatoidea	30	f + c	w	31
C. tropicalis	46	f	w	25
C. parapsilosis	12	psh	w	38
C. krusei	10	y	w	NO[c]
C. guillermondii	12	y + psh	w	37
C. utilis	2	psh	w	24
C. aaseri	1	psh	c	24
C. rugosa	1	psh	w	24
C. macedoniensis	1	psh	w	38
C. pseudotropicalis	10	psh	w	43
Geotrichium candidum	1	f + a	w	48
Trichosporon beigelii	1	f,y,a	w	48
T. cutaneum	5	f,y,a	w	35
T. pullulans	3	f,y,a	w	52
Saccharomyces cerevisiae	5	y	w	24
S. rouxii	2	y	w	24
S. champagnii	11	y	w	24
Hansenula wingii	1	y	w	54
Aureobasidium pullulans	3	f + y	bl	38

[a] f, Filaments; c, chlamydospores; y, yeast; psh, pseudohyphae; a, arthrospores.
[b] w, White; bl, black; c, cream yellow.
[c] NO, Characteristic growth not obtained.

TABLE 3. *Appearance of some Cryptococcus species and other yeasts on TOC medium*

Organism	No. of strains	Medium	Pigment production	Morphology[a]	Time to characteristic growth (h)
C. neoformans	30	BS[b]	Light brown	y	24
		TOC	Dark brown	y	24
C. laurentii	15	BS	Cream yellow	y	24
		TOC	Cream yellow	y	24
C. albidus var. *diffluens*	3	BS	Cream yellow	y	24
		TOC	Cream yellow	y	24
C. innocuous	2	BS	Cream yellow	y	24
		TOC	Cream yellow	y	24
C. uniguttulatus	1	BS	Cream yellow	y	24
		TOC	Cream yellow	y	24
C. terreus	1	BS	White	y	24
		TOC	White	y	24
C. laurentii var. *magnus*	1	BS	Pink	y	24
		TOC	Pink	y	24
Torulopsis glabrata	64	BS	White	y	28
		TOC	White	y	28
T. torresii	1	BS	White	y	24
		TOC	White	y	24
Rhodotorula rubra	2	BS	Red	y	24
		TOC	Red	y	24
R. glutinis	2	BS	Red	psh + y	26
		TOC	Red	psh + y	26
R. graminis	2	BS	Red	psh + y	25
		TOC	Red	psh + y	25

[a] psh, Pseudohyphae; y, yeast.
[b] BS, Birdseed.

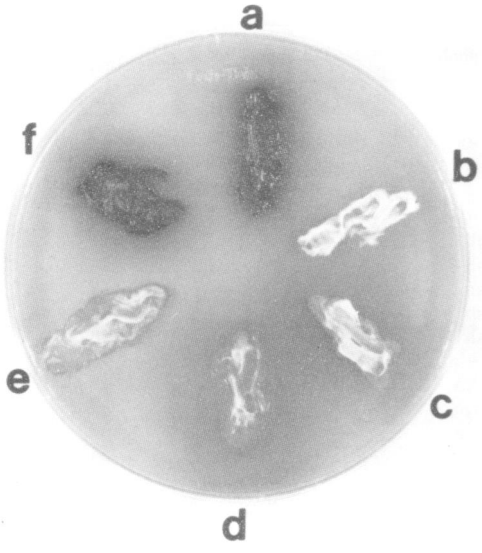

FIG. 3. *Production of brown pigment by C. neoformans (a, f) after 12 h of incubation at room temperature on TOC medium. Other species of Cryptococcus that failed to produce pigment during incubation over a 72-h period: (b) C. albidus; (c) C. laurentii; (d) C. diffluens; (e) C. laurentii.*

cation time must be weighed against two important factors: (i) the caffeic acid-ferric citrate-impregnated disks must be prepared and stored in the dark due to the instability of the components in light, and (ii) the concentrations of caffeic acid and ferric citrate are critical. The reduction of either component will increase the incubation time for brown pigment production, whereas an increase of either component will lead to nonspecific pigmentation of many other yeasts.

The TOC medium is as easily prepared as cornmeal agar. In contrast to other media, labile components (ferric citrate or birdseed extract) are not used, thus eliminating the disadvantage of careful storage. The optimum shelf life of TOC plates has not been determined; however, we have routinely used month-old TOC plates and obtained excellent results. Although we store our TOC medium in plate form, we foresee no problem with placing 30-ml portions of the medium in tubes and then melting and pouring the medium on plates as needed.

In using TOC as a primary plate for blood cultures, we found that both *C. albicans* and *C. neoformans*, although differentiable upon the medium, grew quite slowly. These results suggest that the TOC medium functions best as a differential medium. To demonstrate this concept, we tested a yeast isolate from a recent blood culture (processed by the centrifugation technique [7, 8]). A single yeast colony that appeared on a primary plate was streaked on a TOC plate and presumptively identified as *C. neoformans* within 12 h. Identification was confirmed by the presence of the enzyme urease, growth at 37°C, and a typical *C. neoformans* carbohydrate assimilation pattern (16). The presence of a 1:2,048 antibody titer to cryptococcal antigen (IBL, latex agglutination) in the acute serum of the patient, which declined after 14 days of antifungal therapy to 1:256, further supported TOC presumptive identification of *C. neoformans*.

In summary, we feel that the TOC medium offers several advantages to the clinical laboratory: (i) the medium is made from commercially available ingredients, facilitating preparation and assuring reproducibility of results from each lot of media, (ii) no special procedures for preparation and storage are necessary, since the medium can be either prepared as plates or placed in tubes and melted and poured as needed, and (iii) the interpretation of results is not ambiguous and does not require an inordinate amount of training. *C. neoformans* is the only yeast which acquires a brown pigment during growth on the plate. Morphological identification of *C. albicans* is quickly accomplished, since both germ tubes and chlamydospores are distinct and numerous. Furthermore, since morphology is not affected by the growth of bacteria upon the plate, the medium may be used with a contaminated isolate. Thus, the TOC medium provides a rapid and precise identification of the genera of filamenting yeasts and the specific identification of *C. albicans* and *C. neoformans*.

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