JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1977, p. 387-391
Vol. 6, No. 4

Printed in U.S.A.

Diagnostic Medium Containing Inositol, Urea, and Caffeic Acid for Selective Growth of *Cryptococcus neoformans*

MARK E. HEALY,[1] CLAYTON L. DILLAVOU,[2]* AND GLENHALL E. TAYLOR[1]

Department of Biology, The University of San Francisco,[1] and Clinical Mycology Laboratory, Department of Pathology, Letterman Army Medical Center, The Presidio of San Francisco,[2] San Francisco, California 94129

Received for publication 11 April 1977

An agar medium containing inositol and urea as sole carbon and nitrogen sources, caffeic acid and ferric citrate as agents for the selective pigmentation of *Cryptococcus neoformans*, gentamicin as a broad-spectrum bacterial antibiotic, and yeast nitrogen base without amino acids and ammonium sulfate (Difco) was tested against 137 clinical isolates, 4 survey specimens, and 11 ATCC yeast and yeast-like strains. All 28 strains of *C. neoformans* showed heavy growth and dark brown pigmentation after 36 h. All other tested species of *Cryptococcus* showed heavy growth after 36 h but only light brown pigmentation after 48 h. No growth was observed in any tested strains of *Geotrichum, Pityrosporum, Rhodotorula, Saccharomyces,* and *Torulopsis.* Only the *Cryptococcus*-like *Candida humicola* grew of the 8 species and 62 strains of *Candida* tested. Six of 15 strains of *Trichosporon cutaneum* and 1 of 2 strains of *Trichosporon pullulans* showed moderate growth after 48 h. Very different colonial and microscopic morphology and/or the absence of brown pigmentation easily differentiated these strains of *T. cutaneum, T. pullulans,* and *C. humicola* from *C. neoformans.* The growth- and pigmentation-providing characteristics of the medium were unaffected by 2 h of exposure to 254 nm of ultraviolet light.

In 1948, Littman (19) introduced an Oxgall-containing medium for growth of pathogenic fungi that yielded three times more fungal species and colonies than did Sabouraud dextrose agar.

In 1963, Staib (28) reported that *Cryptococcus neoformans* was the only tested yeast species to develop dark-brown pigmentation when grown on an agar medium containing a hot water extract of common bird seed, *Guizotia abyssinica.* Staib's study included species of the genera *Candida, Cryptococcus, Lipomyces, Rhodotorula, Trichosporon,* and *Torulopsis.*

Shields and Ajello, in 1966, (26) also incorporated extract of *G. abyssinica* into a medium containing chloramphenicol, creatinine, and diphenyl for isolation of *C. neoformans.* However, in 1968 Botard and Kelley (6) noted that the efficiency of the Shields and Ajello medium was reduced by overgrowth of saprophytic fungi and went on to incorporate the *G. abyssinica* pigmentation reaction into Littman Oxgall agar, which reduced the growth of saprophytic fungi.

In 1971, Korth and Pulverer (18) described various substrates that produced the brown pigmentation of *C. neoformans.* These included a variety of related compounds, namely *o*- and *p*-diphenols and *o*-triphenols. The compounds tested included caffeic (3,4-dihydroxycinnamic) acid. Later that year they reported (23) that the presence of iron from ferric citrate had a positive influence upon growth and pigmentation of *C. neoformans.*

Strachan et al. (33) amplified the findings of Korth and Pulverer in 1971 by determining that hydroxyl groups in the 3- and/or 4-position of the phenyl ring were common to all compounds producing the specific dark pigmentation. They also reported that common organic solvents were ineffective in extracting the brown pigment from *C. neoformans,* and that caffeic acid isolated from hydrolyzed methanol extract of *G. abyssinica* seeds induced a similar brown pigmentation reaction when incorporated into a glucose-creatinine medium.

In 1972, Shaw and Kapica (25) reported that 3,4-dihydroxyphenylalanine phenoloxidase, found within the cell wall of *C. neoformans,* catalyzes the oxidation of *o*-diphenols to melanin. In the same year Breyer et al. (7) confirmed the findings of Shaw and Kapica and also developed a rapid diagnostic test for *C. neoformans* that consisted of flooding Sabouraud dextrose agar plates with catechol, a substrate of *o*-diphenyloxidase. However, subsequent testing by Staib

(31) in 1973 with added strains of *C. neoformans* showed the catechol test to be unsatisfactory.

In 1975 Hopfer and Blank (13) developed a caffeic acid-containing medium for preliminary identification of *C. neoformans*. However, with the medium described by Hopfer and Blank, as well as those reported previously, successful isolation of *C. neoformans* from any clinical specimen containing bacteria or fungi other than *C. neoformans* is seriously threatened by overgrowth of these organisms.

In 1970 Lodder (22) described 39 genera of yeast-like fungi. Only one genera, *Cryptococcus*, showed inositol assimilation in all species and varieties. Of the 332 other species described, only 17 were reported to assimilate inositol as a sole carbon source.

In 1956 Seeliger (24) found that all 49 strains of *Cryptococcus* tested, including 37 strains of *C. neoformans*, were capable of hydrolyzing urea. Eight years later, Van Uden (35) tested 10 strains of *C. neoformans* and found that every strain used urea as a sole nitrogen source by means of hydrolysis.

In genera other than *Cryptococcus* and *Rhodotorula*, urea hydrolysis is species specific and seems dependent upon a number of factors, such as the concentration of urea and the presence of vitamins (1, 24, 35, 37).

Because *Cryptococcus* species are among the few yeast-like organisms able to utilize inositol as their sole carbon source and urea as their sole nitrogen source, and since *C. neoformans* is the only yeast species to develop intense dark-brown pigmentation on media containing caffeic acid and ferric citrate (13, 23), we have incorporated these four chemicals into a medium that facilitates selective growth of *Cryptococcus* species and rapid detection of *C. neoformans*.

MATERIALS AND METHODS

Source of organisms. The following facilities contributed to the 137 clinical isolates, 4 survey specimens, and 11 ATCC strains that were tested: (i) Medical Mycology Laboratory, University of California Medical Center at San Francisco, (ii) Microbial Diseases Laboratory, California State Department of Health at Berkeley, (iii) School of Public Health, University of California at Berkeley, (iv) Public Health Service Hospital at San Francisco, (v) Veterans Administration Hospital at San Francisco, (vi) San Francisco General Hospital, (vii) Naval Biosciences Laboratory at Oakland, and (viii) Letterman Army Medical Center at the Presidio of San Francisco. The authors confirmed the identity of all strains, prior to inoculation, by means of standard tests involving assimilation of carbohydrates and nitrate and production of urease.

Maintenance of cultures. The viability of all strains was proven, prior to testing, by their ability to grow on a medium containing potatoes and vegetable extracts (a medium we intend to report on in the near future).

Medium preparation. The medium contains: agar (Difco), 15 g; inositol, 10 g; urea, 5 g; yeast nitrogen base without amino acids and ammonium sulfate (Difco), 1.45 g; caffeic (3,4-dihydroxycinnamic) acid, 0.2 g; ferric citrate, 0.01 g; gentamicin, 40 mg; distilled water 1,000 ml.

Fifteen grams of agar (Difco) was first autoclaved with 900 ml of the distilled water at 15 lb/in^2 for 15 min. A stock solution of ferric citrate was then prepared by dissolving 1 g of ferric citrate in 100 ml of distilled water. A 1-ml portion of this solution was then added to a vessel containing 100 ml of distilled water, inositol, yeast nitrogen base without amino acids and ammonium sulfate (Difco), caffeic acid, and gentamicin in the quantities decribed above. The mixture was then heated and swirled until all of the contents dissolved. Next, 5 g of urea was added after the solution had cooled to a temperature between 80 and 90°C. After the urea was added, it was imperative that care be taken not to further heat the solution. Heating the solution to a temperature in excess of 95°C noticeably increased turbidity of the solution. This excessive heating may have resulted in decomposition of the urea, producing various nitrogenous compounds that could feasibly permit growth of some organisms unable to use urea as a nitrogen source. Next, the solution was filter sterilized, using a 0.45-μm membrane filter.

After the agar-containing solution was removed from the autoclave, and it had cooled to between 80 and 90°C, the two solutions were combined, then swirled gently and thoroughly. The medium was poured into petri dishes with dimensions of 100 by 15 mm.

Method of inoculation. A comparable portion of viable inoculum from each of the original plates (see above) was suspended in equal volumes of sterile water by means of a cotton swab. After vortex mixing, equal amounts of liquid were transferred from these water suspensions to the caffeic acid-containing medium by means of an additional cotton swab. This inoculum was distributed over the entire surface of the medium. Incubation termperature was 29°C in a forced-air incubator. Incubation temperatures above 30°C are not recommended due to inability of certain species of *Cryptococcus* to grow at such temperatures.

RESULTS

All 28 strains of *C. neoformans* showed heavy growth and dark-brown pigmentation after 36 h. All other species of *Cryptococcus* showed heavy growth after 36 h but only light brown pigmentation after 48 h. Examination of all 152 cultures at 72 and 96 h showed no visible changes in growth or pigmentation, as compared with reports taken at 48 h (Table 1). This medium proved to be highly selective against genera other than *Cryptococcus*. No growth was observed in any tested strains of *Geotrichum, Pityrosporum, Rhodotorula, Saccharomyces,* and *Torulopsis*. Of the 8 species and 62 strains of

TABLE 1. *Growth and pigmentation of tested strains*

Organism	No. of strains tested	Culture incubation (h)					
		24		36		48	
		Growth	Extent of brown pigmentation	Growth	Extent of brown pigmentation	Growth	Extent of brown pigmentation
Cryptococcus neoformans	28	Moderate	Medium	Heavy	Dark	Heavy	Dark
C. albidus	5	Moderate	–	Heavy	–	Heavy	Light
C. albidus var. *diffluens*	2	Moderate	–	Heavy	–	Heavy	Light
C. laurentii	3	Moderate	–	Heavy	–	Heavy	Light
C. terreus	3	Moderate	–	Heavy	–	Heavy	Light
Candida albicans	31	–	–	–	–	–	–
C. guilliermondii	4	–	–	–	–	–	–
C. humicola	1	Moderate	–	Heavy	–	Heavy	–
C. krusei	5	–	–	–	–	–	–
C. parapsilosis	8	–	–	–	–	–	–
C. pseudotropicalis	5	–	–	–	–	–	–
C. stellatoidea	3	–	–	–	–	–	–
C. tropicalis	5	–	–	–	–	–	–
Trichosporon cutaneum	6	Light	–	Light	–	Moderate	–
T. cutaneum	9	–	–	–	–	–	–
T. pullulans	1	Light	–	Light	–	Moderate	–
T. pullulans	1	–	–	–	–	–	–
T. penicillatum	1	–	–	–	–	–	–
Geotrichum candidum	4	–	–	–	–	–	–
Pityrosporum pachydermatis	1	–	–	–	–	–	–
P. ovale	1	–	–	–	–	–	–
Torulopsis candida	2	–	–	–	–	–	–
T. glabrata	8	–	–	–	–	–	–
Rhodotorula glutinis var. *glutinis*	2	–	–	–	–	–	–
R. rubra	9	–	–	–	–	–	–
Saccharomyces cerevisiae	4	–	–	–	–	–	–

Candida tested, only the *Cryptococcus*-like *Candida humicola* exhibited growth. Cultures of *C. humicola* remained unpigmented throughout the duration of each test. Six of 15 strains of *Trichosporon cutaneum* and 1 of 2 strains of *Trichosporon pullulans* showed moderate growth after 48 h. These strains of *Trichosporon* were easily differentiated from *C. neoformans* by their lack of brown pigmentation as well as their characteristic arthrospores and pseudohyphae. *C. neoformans* produced uniformly dark-brown colonies with moderately mucoid consistency, the unicellular nature of which could be demonstrated microscopically.

Medium coloration alone was not important in revealing the growth of *C. neoformans*. All strains of *C. neoformans* showed dark-brown pigmentation after 36 h. In these cases, the medium itself often manifested a light-to-medium brown color approximately 12 h after the colonies became pigmented. However, we also observed that heavy inocula of *Rhodotorula* species frequently induced light-brown medium coloration without any growth whatsoever. As such, the isolation of *C. neoformans* cannot be inferred from medium coloration in the absence of dark-brown colonial pigmentation. This phenomenon may be due to the fact that the enzymes responsible for brown pigmentation are found in both *Rhodotorula* and *Cryptococcus*, two genera which exhibit many taxonomic similarities. Lighter inoculum did not produce this effect.

To determine the effects of light exposure upon the growth- and pigmentation-providing characteristics of the medium, plates were exposed, at a distance of 56 cm, to a 91-cm bulb emitting 254 nm of ultraviolet light for a period of 2 h. Results, in terms of both growth and pigmentation, after time intervals of 24, 36, 48, 72, and 96 h, were identical to those obtained with medium that had been protected from light during both storage and incubation.

DISCUSSION

The selectiveness of this diagnostic medium should increase the incidence of isolating *Cryptococcus* species as well as the probability of rapidly detecting *C. neoformans* from most clinical samples, in particular, specimens from cutaneous lesions and the sputum, which usually produce cultures grossly contaminated with bacterial and fungal growth. Despite the optimistic results we have observed when using this exper-

imental medium with clinical isolates, survey specimens, ATCC strains, and a limited number of clinical specimens, it is strongly recommended that this new medium always be used in conjunction with a standard isolation medium until further clinical trials provide additional evidence of its capacity to selectively isolate all strains of *Cryptococcus* species and to rapidly pigment all strains of *C. neoformans*. In addition, we recommend that any yeast-like organism isolated on this medium always be identified by standard auxanographic procedures.

Based on data (22) describing the ability of yeast species to hydrolyze urea and to assimilate inositol, some or all of the strains of yeast species listed below can be expected to produce growth on this medium. These organisms include a total of only 17 of the 332 non-*Cryptococcus* species described by Lodder (22). The remaining 315 species are deficient in one or both of these prerequisites: *Cryptococcus* species (all), *Bullera alba, Candida blantii, C. ciferrii, C. curiosa, C. curvata, C. humicola, C. marina, Endomycopsis fibuligera, Leucosporidum capsuligenum, L. poridum frigidum, L. gelidum, L. nivalis, L. stoklsii, Lipomyces starkeyi, Trichosporon cutaneum, T. pullulans,* and *Wingea robertsii*.

The probability of isolating the organisms listed above is extremely low; however, each should be assumed to be capable of growing on this medium by virtue of their urea- and inositol-utilizing characteristics. In those cases where the ability to hydrolyze urea was either described as +/− or was unreported (22), we have assumed that the organism does have the ability to hydrolyze urea. Conclusive data concerning the assimilation of inositol was available in the case of all strains and species described (22). Of the *Candida* species listed (above), only *C. curvata* has been isolated from the 4,800 yeast strains that we recovered from the 12,000 specimens received by this laboratory within the last 4 years. Considering the comparative specificity of caffeic acid and ferric citrate as *C. neoformans* pigmentation agents (13, 23), it is not likely that any one of these organisms will exhibit brown pigmentation on this medium, when and if it is clinically isolated.

In numerous cases, we found that stock cultures and clinical isolates of *Cryptococcus* species, which had been stored for from 2 to 5 years, had lost their ability to assimilate inositol or to hydrolyze urea. As such, in the case of strains which have undergone a lengthy period of storage, it is imperative that the ability to assimilate inositol and to hydrolyze urea be determined just prior to the time of inoculation.

Our observation that *C. neoformans* produces moderately mucoid growth on this new medium is in contrast to the dry-looking colonies that Staib et al. (29) reported when using a different medium, which also contained urea as the sole nitrogen source.

Gentamicin was chosen as the antibiotic because of its broad antibacterial spectrum (10) and because its actions are not fungicidal (3).

It is evident that the exogenous hydrolysis of urea by the action of urease establishes strongly alkaline conditions within the medium. These strongly alkaline conditions seem to favor the dark-brown pigmentation of *C. neoformans* (32). Due to this effect, during the preparation of the medium, an adjustment of the pH is unnecessary.

We have isolated *C. neoformans* from a subcutaneous abcess on the buttocks of a patient having a diagnosis of sarcoid. The patient had been on high levels of steroids for several months. Before being submitted to this laboratory, the isolate was first recovered from a routine bacteriological culture of pus from the described abcess. It was next cultured on routine mycological media and showed the morphological characteristics of *Cryptococcus* species. The isolate was subcultured to our experimental medium. Within 24 h, the growth and characteristic dark-brown pigmentation of *C. neoformans* became evident. Biochemical identification, involving tests for the assimilation of carbohydrates and nitrate and the production of urease, next confirmed the isolate as *C. neoformans*.

The cultural diagnosis of pulmonary cryptococcosis is complicated by the fact that patients frequently cannot produce the quantities of sputum required for a conclusive laboratory diagnosis. Despite previous diagnostic methods not allowing for consistently reliable isolation of cryptococci from heavily contaminated samples, it is probable that, in some cases, small numbers of cryptococci originating from bronchial secretions do pass through the gastrointestinal tract and enter feces in viable form. Noting that this new medium will not support the growth of most bacterial and fungal species that exist within fecal samples, and that some such samples probably contain small numbers of viable cryptococci, we propose regular implementation of this new medium, in conjunction with standard isolation media, for diagnosis of pulmonary cryptococcosis by means of growth obtained from fecal samples in addition to that obtained from sputum.

LITERATURE CITED

1. **Abadie, F.** 1967. L'uréase chez les levures. Ann. Inst. Pasteur (Paris) **113**:791–813.
2. **Ajello, L.** 1958. Occurrence of *Cryptococcus neoformans*

in soils. Am. J. Hyg. **67**:72-77.
3. **American Medical Association Department of Drugs.** 1973. AMA Drug Evaluations, 2nd Edition. Publishing Sciences Group, Acton, Mass.
4. **Bennett, J. E.** 1972. Cryptococcosis, p. 945-952. *In* P. D. Hoeprich (ed.), Infectious diseases. Harper and Row, New York.
5. **Blum, W.** 1968. Cryptococcosis: a laboratory diagnosis. Am. J. Med. Technol. **34**:409-410.
6. **Botard, R. W., and D. C. Kelley.** 1968. Modified Littman oxgall agar to isolate *Cryptococcus neoformans*. Appl. Microbiol. **16**:689-690.
7. **Breyer, D., F. Staib, M. Senska, and A. Blisse.** 1972. Suitability of O-diphenoloxidase demonstration by means of catechol for the differentiation of *Cryptococcus neoformans*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **222**:540-543.
8. **Chaskes, S., and R. L. Tyndall.** 1975. Pigment production by *Cryptococcus neoformans* from para- and orthodiphenols: effect of the nitrogen source. J. Clin. Microbiol. **1**:509-514.
9. **Dolan, C. T., and M. R. Woodward.** 1971. Identification of *Cryptococcus* species in the diagnostic laboratory. Am. J. Clin. Pathol. **55**:591-595.
10. **Finland, M.** 1969. Gentamicin: antibacterial activity, clinical pharmacology and clinical applications. Med. Times **97**:161-174.
11. **Gervasi, J. P., and N. G. Miller.** 1975. Recovery of *Cryptococcus neoformans* from sputum using techniques for the isolation of fungi from sputum. Am. J. Clin. Pathol. **63**:916-920.
12. **Haley, L. D.** 1971. Identification of yeasts in clinical microbiological laboratories. Am. J. Med. Technol. **37**:125-131.
13. **Hopfer, R. L., and F. Blank.** 1975. Caffeic acid-containing medium for identification of *Cryptococcus neoformans*. J. Clin. Microbiol. **2**:115-120.
14. **Hopfer, R. L., and D. Groschel.** 1975. Six-hour pigmentation test for the identification of *Cryptococcus neoformans*. J. Clin. Microbiol. **2**:96-98.
15. **Jennings, A., J. E. Bennett, and V. Young.** 1967. Identification of *Cryptococcus neoformans* in a routine clinical laboratory. Mycopathol. Mycol. Appl. **35**:256-264.
16. **Kapica, L., and C. E. Shaw.** 1969. Improvement in laboratory diagnosis of pulmonary cryptococcosis. Can. Med. Assoc. J. **101**:582-585.
17. **Kaufman, L., and S. Blumer.** 1969. Value and interpretation of serological tests for the diagnosis of cryptococcosis. Appl. Microbiol. **16**:1907-1912.
18. **Korth, H., and G. Pulverer.** 1971. Pigment formation for differentiating *Cryptococcus neoformans* from *Candida albicans*. Appl. Microbiol. **21**:541-542.
19. **Littman, M. L.** 1948. Growth of pathogenic fungi on a new culture medium. Am. J. Clin. Pathol. **18**:409-420.
20. **Littman, M. L., and J. E. Walker.** 1968. Cryptococcosis: current status. Am. J. Med. **45**:922-932.
21. **Littman, M. L., and L. E. Zimmerman.** 1956. Cryptococcosis. Grune & Stratton, New York.
22. **Lodder, J. (ed.).** 1970. The yeasts. North-Holland Publishing Co., Amsterdam.
23. **Pulverer, G., and H. Korth.** 1971. *Cryptococcus neoformans*: Pigment bildung aus verschiedenen Polyphenolen. Med. Microbiol. Immunol. **175**:46-51.
24. **Seeliger, H. P. R.** 1956. Use of a urease test for the screening and identification of cryptococci. J. Bacteriol. **72**:127-131.
25. **Shaw, C. E., and L. Kapica.** 1972. Production of diagnostic pigment by phenoloxidase activity of *Cryptococcus neoformans*. Appl. Microbiol. **24**:824-830.
26. **Shields, A. B., and L. Ajello.** 1966. Medium for selective isolation of *Cryptococcus neoformans*. Science **151**:208-209.
27. **Staib, F.** 1962. *Cryptococcus neoformans* and *Guizotia abyssinica* (syn. *G. oleifera* D. C.) (Farbreaktion fur *C. neoformans*). Z. Hyg. **148**:466-475.
28. **Staib, F.** 1963. New concepts in the occurrence and identification of *Cryptococcus neoformans*. Mycopathol. Mycol. Appl. **19**:143-145.
29. **Staib, F., S. K. Mishra, Th. Abel, and A. Blisse.** 1976. Growth of *Cryptococcus neoformans* on uric acid agar. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **236**:374-385.
30. **Staib, F., H. S. Randhawa, G. Grosse, and A. Blisse.** 1973. Cryptococcosis: a contribution to the identification of *Cryptococcus neoformans* isolated from clinical specimens. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **225**:211-222.
31. **Staib, F., and M. Senska.** 1973. Experiences with the *o*-diphenol-oxidase reaction with pyrocatechol for the differentiation of *Cryptococcus neoformans*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **223**:419-420.
32. **Staib, F., and M. Senska.** 1973. Brown pigmentation (brown colour effect, BCE) of *Cryptococcus neoformans* on *Guizotia abyssinica*-creatinine-agar as dependent on the initial pH value. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **225**:113-124.
33. **Strachan, A. A., R. J. Yu, and F. Blank.** 1971. Pigment production of *Cryptococcus neoformans* grown with extracts of *Guizotia abyssinica*. Appl. Microbiol. **22**:478-479.
34. **Tynes, B., K. N. Mason, A. E. Jennings, and J. E. Bennett.** 1968. Variant forms of pulmonary cryptococcosis. Ann. Intern. Med. **69**:1117-1125.
35. **Van Uden, N., M. Vidal-Leiria, and H. R. Buckley.** 1964. Utilization of nitrogen compounds by *Cryptococcus neoformans* and other yeasts. Ann. Soc. Belge Med. Trop. **44**:619-640.
36. **Vickers, R. M., J. J. McElligott, J. D. Rihs, and B. Postic.** 1974. Medium containing trypan blue and antibiotics for the detection of *Cryptococcus neoformans* in clinical samples. Appl. Microbiol. **27**:38-42.
37. **Wickerham, L. J.** 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. J. Bacteriol. **52**:293-301.