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Esculin-Based Medium for Isolation and Identification of *Cryptococcus neoformans*

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A simple medium was developed, using esculin as the substrate, for the isolation and identification of *Cryptococcus neoformans*. *C. neoformans* produced a brown-black pigment on the medium; all other yeasts produced no pigment or were light yellow. Esculin is β-glucose-6,7-dihydroxycoumarin. *C. neoformans* produced pigment because the 6,7-dihydroxycoumarin component of the esculin molecule was converted to a melanin-like pigment. We think the reaction was similar to the conversion of diphenols, aminophenols, and diaminobenzenes to melanin. Laboratory studies with isolates of *C. neoformans*, *C. albidus*, *C. luteolus*, and *C. terreus* and representatives of the genera *Candida*, *Torulopsis*, *Geotrichum*, and *Rhodotorula*, plus environmental field studies, demonstrated that over 95% of *C. neoformans* isolates were correctly identified, whereas all other fungi were excluded. Esculin agar was a sensitive, specific medium for the isolation and identification of *C. neoformans*. It was inexpensive and had a long storage life.

Esculin is 6-β-D-glucose-6,7-dihydroxycoumarin, a compound derived from the horse chestnut tree. It has a vitamin P activity. The molecule can be hydrolyzed by the β-glucosidase esculinase to yield two end products, glucose and esculetin. The hydrolysis of esculin can be determined by three methods: (i) detection of the black color resultant from the combination of esculetin with ferric ions, (ii) determination of the loss of fluorescence (366 nm) as esculin is hydrolyzed, and (iii) measurement of the decrease in pH of the medium if glucose is fermented and acid is produced (4, 5, 9).

The taxonomic use of the esculin hydrolysis reaction dates to H. Muelen's 1907 description of studies in which he used the test for the elucidation of enteric bacteria (6). The reaction was subsequently applied to the identification of the genus *Streptococcus* in the 1920s and to the family *Enterobacteriaceae* in the 1940s. Although not used widely, the reaction has recently been reexamined and its taxonomic usefulness has been refined (4, 5, 9). We could find no report in the literature in which the ability of a microorganism to produce pigment directly from esculin without hydrolysis was investigated.

Cryptococcus neoformans shares with mammalian cells the ability to convert 3,4-dihydroxyphenylalanine to melanin. This is an oxidative biochemical conversion requiring many individual steps in a series. Pigment production can be a useful aid for the isolation, identification, and classification of microorganisms. A useful method for the presumptive identification of *C. neoformans* was based on the observation that it was the only species of yeast able to convert 3,4-dihydroxyphenylalanine and other *o*- and *p*-diphenols to melanin. Pigment production from aminophenols and diaminobenzenes is thought to be closely related to melanin production. However, only two aminophenols (4-hydroxymethanilamine and 3-aminotyrosine) were highly specific substrates. Intracellular pigments were generally produced when the substrate was an *o*-diphenol, whereas soluble extracellular pigments were often the major pigment product when the hydroxyl arrangement was *para* (1-3). Ferric citrate can stimulate pigment production from *p*-diphenols (7, 8). A medium for the presumptive identification of *C. neoformans* by melanin produced from these substrates present in *Guizotia* seeds was described by Staib some years ago (11).

Because esculin bore a resemblance to diphenol, a study was undertaken to determine if this compound could prove to be a specific substrate for the identification of *C. neoformans*. This report describes the formulation and utility of an esculin-based medium for this purpose.

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MATERIALS AND METHODS

Fungal strains. The strains used in this study were all clinical isolates obtained both in the Montefiore Hospital and Medical Center (Bronx, N.Y.) and through the courtesy of mycologists throughout the United States. In addition, some more were obtained from the American Type Culture Collection (Rockville, Md.). Eight isolates that produced small amounts of melanin were obtained from Ira Salkin (New York State Department of Health, Albany, N.Y.) and Glenn D. Roberts (Mayo Clinic, Rochester, Minn.). All fungi were identified by conventional means in accordance with tests currently recommended (10).

The majority of the *C. neoformans* isolates were not serotyped. However, at least two isolates each of serotypes A, B, C, and D were tested (courtesy of I. Weitzman, New York City Department of Health, New York). In addition, one isolate of the perfect stage of *C. neoformans* (*Filobasidiella neoformans*) and one isolate of the perfect stage of *C. bacillispora* (*F. bacillispora*) were examined.

Esculin medium. To prepare the medium, all ingredients (except the antibiotics) were added to distilled water, heated to dissolve all constituents, and sterilized by autoclaving at 121°C for 15 min. After sterilization, the medium was allowed to cool to 55°C and then the antibiotics were added, with thorough mixing. Twenty milliliters of medium was poured into each 100-mm-diameter petri plate. Whether from environmental sampling area, patient, or pure culture, a sample of each specimen was streaked over the surface of the plate in the standard manner (10). Colonies that became black were presumptively identified as *C. neoformans* (Table 1).

Esculin medium was stable for approximately 4 to 6 months in the refrigerator (4 to 8°C), whereas 3,4-dihydroxyphenylalanine medium (1) will begin to autoxidize after 3 weeks at 4°C. Both 3,4-dihydroxyphenylalanine and esculin media were stable for at least 1 year when stored at 4°C under anaerobic conditions.

TABLE 1. *Esculin medium*[a]

Ingredient	Source[b]	Amt/liter of distilled water
Agar, purified	Difco Laboratories (Detroit, Mich.)	15 g
Dextrose	Fisher Scientific Co. (Pittsburgh, Pa.)	5 g
Peptone	Difco	10 g
Esculin	ICN Pharmaceuticals, Inc. (Cleveland, Ohio)	0.5 g
Yeast extract	Difco	1.0 g
Gentamicin sulfate[c]	Schering Corp. (Bloomfield, N.J.)	25 mg
Chloramphenicol[c]	Parke, Davis & Co., (Detroit, Mich.)	10 mg

[a] All ingredients are dissolved in 1 liter of distilled water. The final pH is 7.1. The medium (without antibiotics) is sterilized by autoclaving at 121°C for 15 min.

[b] Other sources of equivalent material may be suitable.

[c] Antibiotics are added by sterile filtration after autoclaving.

Esculetin medium. To determine if the entire esculin molecule or one part, esculetin (6,7-dihydroxycoumarin), was necessary for pigment production, a medium was formulated as described for esculin medium except that esculetin was substituted for esculin.

Esculin hydrolysis. To determine esculin hydrolysis (not pigment production from esculin), 1 or 2 drops of 0.1% ferric ammonium citrate was dropped on a colony. The development of a brown to black color was taken as positive; no change in colony color was recorded as negative.

Defined medium. To determine if a constituent other than esculin was needed for pigment production, a minimal medium consisting of 15 g of purified agar, 4 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of glutamine, 1 g of asparagine, 1 g of glycine, and 0.5 g of esculin per liter was made as described for the parent esculin-based medium.

RESULTS

Table 2 shows the abilities of different species of cryptococci to produce pigment on esculin medium. Only *C. neoformans* produced a brown-black pigment; representative isolates of *C. laurentii*, *C. albidus*, and *C. terreus* produced no pigment (Fig. 1). If ferric ammonium citrate (0.1%) was incorporated in the medium or if several drops of 0.1% ferric ammonium citrate solution was added to the surface of the colonies, esculin hydrolysis could be determined by the black color produced. Many species of *Cryptococcus* hydrolyzed esculin; pigment production from esculin was not related to the brown-black color produced by the reaction of ferric ions with esculin, although the colors produced by both mechanisms were similar. Occasionally, isolates of non-*C. neoformans* species of *Cryptococcus* produced an extremely pale yellow pigment we designated "torpid." This weak pigment was clearly distinguishable from the dark brown to black color produced by *C. neoformans*, and, because it was nearer to nonpigmented colonies, it was considered negative. The one *C. neoformans* isolate torpid on esculin agar produced pigment from 3,4-dihydroxyphenylalanine.

Forty-two isolates of *C. neoformans* were studied (Table 2). Forty produced pigment, one produced no pigment, and one was torpid. The one negative strain was obtained from I. Salkin (New York State Department of Health) and failed to produce pigment from any diphenol, aminophenol, or diaminophenol substrate or from *Guizotia* seed agar. Thirty-six of the forty isolates of *C. neoformans* were positive within 48 h; the other four isolates were positive within 72 h. *C. neoformans* produced pigment at both 37 and 22°C. The isolates of *C. laurentii*, *C.*

TABLE 2. *Pigment production by yeast on esculin medium*

Yeast	No. tested	No. positive	No. negative	No. torpid[a]	% Positive
Cryptococcus neoformans	42	40	1[b]	1[b]	95.3
C. laurentii	6	0	6	0	0.0
C. albidus	8	0	7	1	0.0
C. terreus	7	0	6	1	0.0
C. luteolus (ATCC 10671)	1	0	0	0	0.0
C. gastricus (ATCC 24225)	1	0	0	0	0.0
C. uniguttulatus (ATCC 24227)	1	0	0	0	0.0
Candida albicans	105	0	105	0	0.0
C. krusei	12	0	12	0	0.0
C. tropicalis	30	0	30	0	0.0
C. parapsilosis	8	0	8	0	0.0
C. guilliermondii	1	0	1	0	0.0
C. pseudotropicalis	1	0	1	0	0.0
Torulopsis glabrata	29	0	29	0	0.0
Geotrichum	1	0	1	0	0.0
Rhodotorula	2	0	2	0	0.0

[a] Torpid is defined as a weak yellow pigment that is distinct from the brown-black melanin color and should be considered negative.

[b] Does not produce pigment on *Guizotia* seed agar.

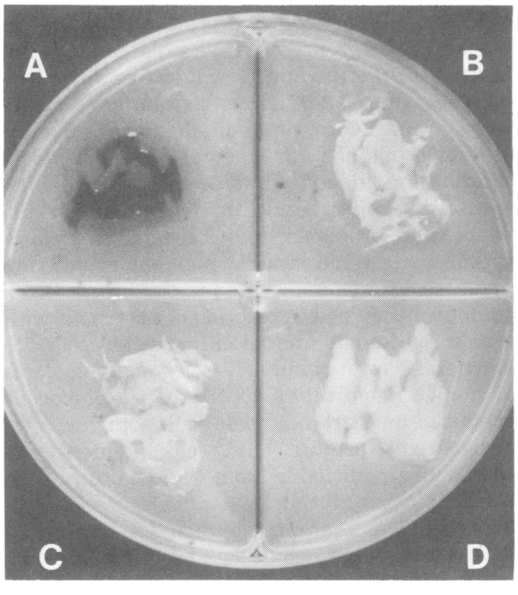

FIG. 1. *Appearance of four Crytococcus species on general esculin medium. (A) C. neoformans; (B) C. albidus; (C) C. terreus; (D) C. laurentii.*

albidus, and *C. terreus* available to us for this study produced either no pigment or a weak yellow (torpid) color.

Isolates of *Candida*, *Torulopsis*, *Geotrichum*, and *Rhodotorula* species tested did not produce pigment on esculin medium (Table 2).

Pigment production from esculetin (6,7-dihydroxycoumarin) was investigated with randomly selected isolates of *Cryptococcus* (Table 3). Approximately one-half of our isolates were tested on esculetin base, because esculetin was extraordinarily expensive and available in small quantities. The results obtained were not statistically different from those we found in experiments with the whole esculin molecule as the substrate (Table 2). Whether hydrolysis was required for pigment production or whether the yeast could utilize the intact molecule was not determined by this experiment.

TABLE 3. *Pigment production by Cryptococcus from esculetin*

Species	No. tested	No. positive[a]	No. negative[b]	No. torpid
C. neoformans	20	18	2	0
C. laurentii	4	0	4	0
C. albidus	4	0	4	0
C. terreus	4	0	3	1

[a] All produced pigment from esculin.

[b] All did not produce pigment from esculin.

All isolates of *C. neoformans* and all isolates of other species of *Cryptococcus* tested in this study (Table 2) showed similar reactivities on minimal salts medium to those shown on the general esculin medium. However, the yeasts did not grow as well on this medium as on the general medium.

Over 100 environmental samples from around the Montefiore Hospital and Medical Center, including pigeon nesting areas, were sampled and plated on both esculin and *Guizotia* seed agars. No *C. neoformans* was isolated from either medium; no other yeast produced a posi-

tive on either of these media.

To determine if pigment production on esculin agar might be useful for the rapid identification of *C. neoformans* isolated on other media, colonies of *C. neoformans* were first grown on Sabouraud dextrose agar and blood agar and then transferred to esculin agar. The results obtained were the same as those presented in Table 2.

DISCUSSION

It is well established that isolates of *C. neoformans* can produce melanin-like pigment from diphenol, aminophenol, and diaminophenol substrates. Esculetin, which is (together with glucose) one of the two products of the hydrolysis of esculin, contains two hydroxyl groups juxtaposed in the 6 and 7 positions on one of its two six-member carbon rings. *C. neoformans* can produce melanin-like pigment from both esculin and esculetin. It appears that the 6,7-dihydroxycoumarin structure is similar enough to diphenols to serve as an intermediate in the melanin biosynthetic pathway. Accordingly, an esculin-based medium proved to give results that were as specific as those obtained with media which incorporate diphenols, aminophenols, or diaminophenols for the identification of *C. neoformans*.

However, esculin hydrolysis was not a useful tool for the identification of *C. neoformans*. Many *Cryptococcus* species hydrolyze esculin, and there was variable hydrolysis amongst the *Candida* species. It should be emphasized that esculin hydrolysis, which was useful for the taxonomic description of bacteria (4, 5), was a different reaction than pigment production directly from esculin without hydrolysis by *Cryptococcus* as described here. There was no iron in the medium; the brown-black color produced was melanin, not the complex formed by esculetin and ferric ions.

Pigment production from esculin, with the strains tested in this study, was specific for the identification of *C. neoformans*. Of the forty-two isolates investigated, forty (95.3%) produced pigment. There were no false-positive tests with the species studied. As with any single test, pigment production should be considered presumptive evidence that the isolate is *C. neoformans*, and accessory biochemical and serological tests should be performed to establish the identity of a yeast with certainty. The medium was simpler to prepare and was less costly than *Guizotia* seed agar. Esculin agar should prove to be serviceable for both the epidemiological study of *C. neoformans* and for its identification from clinical material.

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