Evaluation of a Caffeic Acid-Ferric Citrate Test for Rapid Identification of *Cryptococcus neoformans*

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Received for publication 30 June 1977

An evaluation of a rapid caffeic acid-ferric citrate paper disk test for the identification of *Cryptococcus neoformans*, using 474 isolates of yeasts and yeastlike organisms, showed that 96.6, 97.7, and 98.3% of 176 isolates of *C. neoformans* produced brown to dark-brown pigment on disks incubated for 6 h at room temperature, 30°C, and 37°C, respectively. All *C. neoformans* produced brown to dark-brown pigment within 24 h. However, nonspecific pigmentation was encountered at all temperatures of incubation with one isolate of *Trichosporon cutaneum* and, at room temperature only, with one isolate of *C. luteolus* after 6 h. Other genera of yeasts produced similar pigmentation after 24 h at all temperatures. The age of the cultures tested or the types of media used before testing did not significantly affect the ability of *C. neoformans* to produce pigmentation at 37°C. A positive test may prove useful for presumptive identification of *C. neoformans*, but a negative reaction should not be used to rule out an identification of this yeast. Since a number of false-negative and false-positive tests occur, it is necessary to confirm, by other biochemical tests, the identification of all organisms suspected of being *C. neoformans*, to reduce the serious risk of missing or misidentifying this important pathogen.

Because of the wide spectrum of cryptococcosis (11) and the opportunistic nature of such infection, particularly in immunosuppressed patients with cryptococcal meningitis (2, 3, 6, 12), rapid laboratory identification of the causative agent, *Cryptococcus neoformans*, is necessary so that therapy can be started without delay.

During the past decade it has been shown that the production of a brown pigment on media containing an extract of *Guizotia abyssinica* seeds (10, 16, 19) and the assimilation of creatinine (17) by *C. neoformans* are two of the characteristics that can be used in the selective isolation and identification of this organism (1, 5, 9, 15, 18). The 3,4-dihydroxycinnamic acid (or caffeic acid, an *o*-diphenol), when isolated from extracts of *G. abyssinica* seeds (10, 19), was found to produce a brown coloration of *C. neoformans* colonies in the presence of iron compounds such as ferric chloride or ferric citrate (10, 13).

Hopfer and Blank (7) incorporated caffeic acid and ferric citrate in culture media and were able to reduce the incubation time required for pigment production on the conventional *G. abyssinica* medium from 7 days to 3 to 4 days. Recently, Hopfer and Gröschel (8) developed a rapid 6-h pigmentation test in which organisms were inoculated onto caffeic acid-ferric citrate (CAFC)-impregnated paper disks, which were

then incubated at room temperature for pigment production. They studied 8 isolates of *Cryptococcus* species, 24 isolates of *C. neoformans*, and 40 isolates of yeasts. All cultures of *C. neoformans* produced a dark-brown pigment within 6 h at this temperature. The effect of higher temperatures of incubation on the rate and intensity of pigmentation was not studied. Shaw and Kapica (14) showed that phenoloxidase, the enzyme isolated from *C. neoformans* that is responsible for the oxidation of *o*-diphenols to melanin, was most active when incubated with L-3,4-dihydroxyphenylalanine (an *o*-diphenol) at 35 to 40°C.

The current study was designed to evaluate the CAFC test by using a larger number of addition, the effects of temperature of incubation, types of culture media used before testing, and age of cultures used for testing were investigated.

MATERIALS AND METHODS

Organisms. Four hundred seventy-four isolates of yeasts and yeastlike organisms from the Mayo Clinic mycology laboratory collection were used in this study (Table 1). The identification of each organism was confirmed by methods previously described (4).

Organisms were inoculated onto Sabouraud dextrose (Emmon's modification) agar (SAB) slants and incubated at 30°C for 48 h before testing for pigment

TABLE 1. *Organisms tested for brown pigment production on CAFC-impregnated paper disks*

Organism	No. of isolates tested
<i>Cryptococcus albidus/albidus</i>	26
<i>C. albidus/diffluens</i>	38
<i>C. gastricus</i>	1
<i>C. laurentii</i>	23
<i>C. luteolus</i>	10
<i>C. neoformans</i>	176
<i>C. terreus</i>	9
<i>C. uniguttulatus</i>	3
<i>Candida albicans</i>	24
<i>C. guilliermondii</i>	8
<i>C. krusei</i>	18
<i>C. parapsilosis</i>	17
<i>C. pseudotropicalis</i>	10
<i>C. stellatoidea</i>	6
<i>C. tropicalis</i>	19
<i>Geotrichum candidum</i>	2
<i>Rhodotorula</i> species	18
<i>Saccharomyces cerevisiae</i>	4
<i>Saccharomyces</i> species	16
<i>Torulopsis glabrata</i>	27
<i>Trichosporon cutaneum</i>	9
<i>Trichosporon</i> species	10

production on CAFC-impregnated disks. To study the effects of media and age of cultures on pigment production, 112 isolates of cryptococci, including 71 isolates of *C. neoformans*, were inoculated onto SAB, brain heart infusion agar that contained 10% sheep blood, 5 µg of gentamicin per ml, and 16 µg of chloramphenicol per ml (BHI-III), and inhibitory mold agar that contained 125 µg of chloramphenicol per ml (IMA). Cultures were incubated for 48 h, 96 h, and 1 week at 30°C. The media used in this study were those employed for the routine culturing of most clinical specimens submitted to the clinical mycology laboratory at the Mayo Clinic.

Preparation of CAFC disks. Sterile 0.5-inch (1.27-cm) blank paper disks (Difco) were saturated with a fresh mixture of 1 ml of a stock solution containing 1 mg of caffeic acid per ml (Nutritional Biochemicals Corp., Cleveland, Ohio) in 0.5 M Sørensen buffer (pH 7.0), 0.5 ml of an aqueous stock solution containing 0.5 mg of ferric citrate per ml (Fisher Scientific Co.), and 3.5 ml of Sørensen phosphate buffer. The buffer, stock solutions of caffeic acid, and ferric citrate were prepared according to methods described by Hopfer and Gröschel (8). Control disks were saturated with Sørensen buffer only. All disks were allowed to dry overnight in sterile petri dishes at 37°C and were stored in dark bottles at room temperature for no more than 2 months. They were moistened with 2 to 3 drops of sterile distilled water before testing.

Test procedures. A liberal amount of cells from each isolate was smeared onto CAFC and buffer disks with an inoculating loop. For each test, a moistened CAFC and buffer disk without an organism were included as uninoculated controls. The effect of temperature on pigment production was studied by incubating each test in a sterile petri dish at room temperature (≥22.5°C), 30°C, and 37°C. The effects of culture

media and age were evaluated only at 37°C. Petri dishes were incubated in stainless-steel cans to prevent excessive loss of moisture and to maintain complete darkness. Disks were read hourly for 6 h and finally at 24 h for the production of a brown pigment as compared with the buffer disk control, as described by Hopfer and Gröschel (8).

RESULTS

Most (96.6, 97.7, and 98.3%) of the 176 isolates of *C. neoformans* tested produced a brown to dark-brown (2+ to 3+) pigment on CAFC disks after 6 h of incubation at room temperature, 30°C, or 37°C, respectively, and all isolates showed pigmentation after 24 h of incubation, with most producing a dark-brown (3+) pigment (Table 2). Of the 110 isolates of saprobic cryptococci tested, only 1 isolate of *C. luteolus* produced a nonspecific brown coloration of the inoculum without noticeable diffusion into the CAFC disks after 6 h of incubation at room temperature. No nonspecific pigmentation of any other cryptococci was observed at higher temperatures of incubation within 6 h. Of the 188 isolates of organisms other than *Cryptococcus* species tested, only 1 isolate of *Trichosporon cutaneum* produced nonspecific pigmentation after 6 h of incubation at room temperature, 30°C, and 37°C.

Table 3 shows the number of yeastlike organisms that produced nonspecific pigmentation after 24 h of incubation at room temperature, 30°C, and 37°C. Most of the isolates produced a weak brown (1+) and a few produced a dark-brown (3+) coloration of smears at all three temperatures studied. A larger number of isolates produced nonspecific pigment at 30 and

TABLE 2. *Rate and intensity of pigment production by 176 isolates of C. neoformans on CAFC-impregnated paper disks*

Temp of incubation (°C)	Intensity of brown pigment produced ^a	No. of isolates producing pigmentation (%)	
		≤6 h ^b	24 h ^b
Room	1+	30 (17.0)	0 (0)
	2+	62 (35.2)	30 (17.0)
	3+	78 (44.3)	146 (82.9)
		Total 170 (96.6)	Total 176 (100)
30	1+	24 (13.6)	0 (0)
	2+	46 (26.1)	26 (14.8)
	3+	102 (57.9)	150 (85.2)
		Total 172 (97.7)	Total 176 (100)
37	1+	23 (13.1)	0 (0)
	2+	26 (14.8)	23 (13.1)
	3+	124 (70.5)	153 (86.9)
		Total 173 (98.3)	Total 176 (100)

^a 1+, Weak-brown pigment; 2+, brown pigment; 3+, dark-brown pigment.

^b Hours after transfer to CAFC disks.

37°C than at room temperature.

One hundred twelve isolates of *Cryptococcus* species, including 71 isolates of *C. neoformans*, were further tested for their ability to produce a brown pigment at 37°C on CAFC disks with the use of cultures that had been incubated for 48 h, 96 h, and 1 week on SAB, BHI-III, and IMA slants. Table 4 shows that most of the *C. neoformans* isolates produced pigment within 6 h regardless of the age of the culture or the type of medium used. However, one 96-h-old isolate and four 1-week-old isolates of *C. neoformans*, cultured on IMA, produced only weak brown pigmentation (1+) within 24 h of incubation and were therefore not identified as possible *C. neoformans*. Furthermore, one 1-week-old *C. neoformans* isolate, cultured on IMA, failed to produce any pigment even after 24 h of incubation.

Table 4 also shows that the frequency of non-specific pigmentation among saprobic cryptococci was lower when disks were incubated for 6 h than when they were incubated for 24 h. This observation was consistent with the previous finding (Table 3).

DISCUSSION

The unavailability of commercially prepared Niger seed agar has limited its usefulness as a pigmentation test for the identification of *C. neoformans* in clinical laboratories. The development of a more rapid pigmentation test using readily available, defined reagents would thus be a valuable adjunct for the identification of this important pathogen. The CAFC disk test (8) seems to offer all of these advantages. Data from our evaluation of this test, however, showed certain minor discrepancies between our

TABLE 3. Nonspecific pigmentation exhibited by yeastlike organisms other than *C. neoformans* within 24 h

Organism	No. of isolates tested	No. of isolates having nonspecific pigmentation ^a					
		Room temp		30°C		37°C	
		1+	>1+	1+	>1+	1+	>1+
<i>Cryptococcus albidus/albidus</i>	26	2	0	2	0	7	2
<i>C. albidus/diffluens</i>	38	0	0	2	0	4	2
<i>C. gastricus</i>	1	0	0	0	0	0	1
<i>C. laurentii</i>	23	4	0	6	1	8	5
<i>C. luteolus</i>	10	4	0	5	1	5	5
<i>C. terreus</i>	9	5	0	5	0	6	2
<i>C. uniguttulatus</i>	3	1	0	2	0	2	0
<i>Candida</i> species ^b	83	5	0	9	0	14	2
<i>Torulopsis glabrata</i>	27	3	0	3	0	3	0
<i>Trichosporon cutaneum</i>	9	1	2	2	3	3	3
<i>Trichosporon</i> species	10	3	0	3	0	6	0
Total (%)	239	28 (11.7)	2 (0.8)	39 (16.3)	5 (2.1)	58 (24.3)	22 (9.2)

^a 1+, Weak-brown nondiffusible pigment; >1+, moderate to dark-brown nondiffusible pigment.

^b Includes all species of *Candida* tested except *C. tropicalis*.

TABLE 4. Effect of culture age and type of medium used before testing for pigment production on CAFC disks at 37°C with 112 isolates of cryptococci

Age	Medium ^a	No. of <i>C. neoformans</i> producing pigment within 6 and 24 h of incubation out of a total of 71 used ^b (%)	No. of cryptococci other than <i>C. neoformans</i> showing nonspecific pigmentation within 6 and 24 h of incubation out of a total of 41 used (%)	
			6 h	24 h
48 h	SAB	70 (99)	3 (7)	18 (44)
	BHI-III	71 (100)	7 (17)	17 (41)
	IMA	71 (100)	6 (15)	18 (44)
96 h	SAB	71 (100)	1 (2)	8 (20)
	BHI-III	71 (100)	5 (12)	15 (37)
	IMA	71 (100)	2 (5)	9 (22)
1 week	SAB	70 (99)	0 (0)	3 (7)
	BHI-III	70 (99)	13 (32)	16 (39)
	IMA	69 (97)	2 (5)	8 (20)

^a See text for abbreviations of media.

^b Data are the same for 6 and 24 h of incubation.

results and those of Hopfer and Gröschel (8).

Whereas those authors were able to demonstrate pigmentation of 3+ intensity after 6 h at room temperature in all of 24 strains of *C. neoformans* tested, only 78 of 176 isolates of *C. neoformans* in our study produced pigmentation of similar intensity after 6 h at the same temperature. Sixty-two isolates produced a brown pigment (2+), and the 30 remaining isolates produced a weak-brown pigment (1+). Six isolates were not pigmented until after 24 h, and these represented false-negative tests. This apparent difference in sensitivity could be due to a number of factors, including (i) the number of organisms used for testing, (ii) the inherent differences in phenoloxidase levels in different isolates of *C. neoformans*, (iii) the purity of the caffeic acid and ferric citrate used, (iv) variability in the manner and concentration in which the aforementioned reagents were prepared, and (v) the conditions under which the CAFC disks were incubated. In our study, stock solutions of the above reagents were freshly prepared in concentrations recommended by Hopfer and Gröschel (8).

All disks were incubated in petri dishes placed in stainless-steel cans to prevent excessive loss of moisture and to maintain complete darkness during incubation. This was done to avoid nonspecific pigmentation or loss of activity due to the instability of the CAFC mixture used in the disks. As emphasized by Hopfer and Gröschel (8), the CAFC reagents are very unstable when exposed to light. However, these authors demonstrated no evidence of deterioration of the prepared disks for up to 6 months when they were stored in the dark at -20°C , 4°C , or room temperature. The caffeic acid stock solution, based on experience, exhibits a light purplish-blue color when freshly prepared but degenerates to a dark-brown solution even in the absence of light. CAFC-impregnated disks, however, showed some loss of sensitivity upon testing after storage of 4 to 6 weeks in dark bottles at room temperature.

Nonspecific weak-brown pigmentation was observed in one isolate of *C. luteolus* and one of *T. cutaneum* after 6 h of incubation at room temperature (Table 3). Since *T. cutaneum* is morphologically distinct from *Cryptococcus* species, it is unlikely to be confused for the latter and would probably not be tested in routine practice, nor would *C. luteolus*, for it is rarely encountered in clinical laboratories. Nevertheless, some isolates of *C. neoformans* exhibited weak-brown pigmentation (1+) or a green coloration after 6 h of incubation at room temperature. The possibility of misidentifying these organisms as saprobic cryptococci renders the

rapid CAFC test somewhat less specific than expected.

Although all of the 176 isolates of *C. neoformans* produced a brown to dark-brown pigment after 24 h of incubation at room temperature (Table 2), the incidence and intensity of nonspecific weak-brown pigmentation in saprobic cryptococci and other yeasts also increased during this extended time (Table 3). These organisms could routinely be misidentified as isolates of *C. neoformans*, especially if one overlooks the non-diffusive nature of the nonspecific brown pigment.

It was observed that an increase in incubation temperature did not reduce the time for all 176 isolates of *C. neoformans* to produce brown pigmentation of varying degrees on CAFC disks. However, the number of isolates producing dark-brown (3+) pigment was increased, particularly at 37°C , beginning 2 h after inoculation onto the disks (Table 2). Nonetheless, a number of isolates still exhibited weak-brown pigmentation (1+) after 6 h and were therefore unaffected by the increase in temperature. The increase in temperature still did not increase the sensitivity to 100% within 6 h. Four isolates of *C. neoformans* did not produce pigmentation at 30°C , and three failed to pigment at 37°C . This may well suggest that certain isolates of this pathogen do have inherently low levels of phenoloxidase and are incapable of producing a darker pigment in a shorter period. Of the isolates tested, all produced a brown to dark-brown pigment after 24 h (Table 2), but incubation at this temperature resulted in an increased number of false positives.

Nonspecific pigmentation at higher temperatures after 6 h of incubation occurred with only one isolate of *T. cutaneum*. However, the large number of saprobic cryptococci and other yeast-like organisms that produced a nonspecific pigment (weak brown, 1+) after 24 h at 37°C (Table 3) makes the interpretation of pigmentation formed at 30 and 37°C after 24 h difficult.

The age of the cultures or the types of media used did not significantly affect the ability of *C. neoformans* to produce a brown pigment. However, all cultures of cryptococci exhibited a brown coloration when grown on IMA and BHI-III agar. Even with the help of buffer control disks, there was some difficulty in interpreting results of pigmentation. Therefore, SAB agar seems to be the ideal medium for culturing organisms before testing.

We believe that the rapid CAFC disk test may not be specific and sensitive enough to be used for the definitive identification of *C. neoformans* without confirmation with other tests, including pigment formation on Niger seed agar,

nitrate reduction, urease production, and carbohydrate assimilation tests. However, since only a few yeastlike organisms exhibited nonspecific pigmentation within 6 h of incubation at room temperature, 30°C, or 37°C, the test could be used as a rapid screening tool in small laboratories where Niger seed agar plates are not readily available. Moreover, a few false-negative tests were observed, and one must not eliminate the possibility of *C. neoformans* when the CAFC test results are negative. Both caffeic acid and ferric citrate may readily be purchased commercially, and this availability along with the simplicity of the test may prove to be an important consideration for the clinical microbiology laboratory.

LITERATURE CITED

1. Botard, R. W., and D. C. Kelley. 1968. Modified Littman oxgall agar to isolate *Cryptococcus neoformans*. Appl. Microbiol. 16:689-690.
2. Collins, J. V., D. Tong, R. G. Bucknall, and A. P. Warin. 1972. Cryptococcal meningitis as a complication of systemic lupus erythematosus treated with systemic corticosteroids. Postgrad. Med. J. 48:52-55.
3. Diamond, R. D., and J. E. Bennett. 1974. Prognostic factors in cryptococcal meningitis: a study in 111 cases. Ann. Intern. Med. 80:176-181.
4. Dolan, C. T., and G. D. Roberts. 1974. Identification procedures: yeasts and yeastlike organisms, p. 153-201. In J. A. Washington (ed.), Laboratory procedures in clinical microbiology. Little, Brown and Co., Boston.
5. Dolan, C. T., and M. R. Woodward. 1971. Identification of *Cryptococcus* species in the diagnostic laboratory. Am. J. Clin. Pathol. 55:591-595.
6. Freed, E. R., R. J. Duma, H. J. Shadomy, and J. P. Utz. 1971. Meningoencephalitis due to hyphae-forming *Cryptococcus neoformans*. Am. J. Clin. Pathol. 55:30-33.
7. Hopfer, R. L., and F. Blank. 1975. Caffeic acid-containing medium for identification of *Cryptococcus neoformans*. J. Clin. Microbiol. 2:115-120.
8. Hopfer, R. L., and D. Gröschel. 1975. Six-hour pigmentation test for the identification of *Cryptococcus neoformans*. J. Clin. Microbiol. 2:96-98.
9. Jennings, A., J. E. Bennett, and V. Young. 1968. Identification of *Cryptococcus* in a routine clinical laboratory. Mycopathol. Mycol. Appl. 35:256-264.
10. Korth, H., and G. Pulverer. 1971. Pigment formation for differentiating *Cryptococcus neoformans* from *Candida albicans*. Appl. Microbiol. 21:541-542.
11. Lewis, J. L., and S. Rabinovich. 1972. The wide spectrum of cryptococcal infections. Am. J. Med. 53:315-322.
12. Massachusetts General Hospital. 1976. Case records of the Massachusetts General Hospital (case 27-1976). N. Engl. J. Med. 295:34-42.
13. Pulverer, G., and H. Korth. 1971. *Cryptococcus neoformans*: Pigmentbildung aus verschiedenen Polyphenolen. Med. Microbiol. Immunol. 157:46-51.
14. Shaw, C. E., and L. Kapica. 1972. Production of diagnostic pigment by phenoloxidase activity of *Cryptococcus neoformans*. Appl. Microbiol. 24:824-830.
15. Shields, A. B., and L. Ajello. 1966. Medium for selective isolation of *Cryptococcus neoformans*. Science 151:208-209.
16. Staib, F. 1962. *Cryptococcus neoformans* und *Guizotia abyssinica* (syn. *G. oleifera* D. C.) (Farbreaktion für *C. neoformans*). Z. Hyg. 148:466-475.
17. Staib, F. 1963. New concepts in the occurrence and identification of *Cryptococcus neoformans*. Mycopathol. Mycol. Appl. 19:143-145.
18. Staib, F., H. S. Randhawa, G. Grosse, and A. Blisse. 1973. Cryptococcose. Zur Identifizierung von *Cryptococcus neoformans* aus klinischem Untersuchungsmaterial. Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 225:211-222.
19. 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.