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Six-Hour Pigmentation Test for the Identification of *Cryptococcus neoformans*

ROY L. HOPFER* AND DIETER GRÖSCHEL

The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

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Cryptococcus neoformans colonies can be identified within 6 h using paper disks containing caffeic acid and ferric citrate. Indentification is based on the development of a dark brown pigment. Saprophytic *Cryptococcus* species and common clinically isolated yeasts do not develop the brown color. The concentration of the reagents and the method of storage of the impregnated paper disks are critical for the rapid and specific development of the pigment.

In 1962, Staib (8) observed that colonies of *Cryptococcus neoformans* produced a characteristic brown pigment when grown on media containing an extract of *Guizotia abyssinica* seeds. Since then, several media containing similar extracts have been described (1, 3, 7). In addition, caffeic acid has been isolated from *G. abyssinica* seeds (10), and it, as well as a variety of related phenolic compounds, has been shown to cause similar pigmentation of *C. neoformans* colonies (2, 4, 5, 10). Most of these media did not significantly reduce the time necessary (4 to 6 days) for pigment formation by *C. neoformans* on *Guizotia* extract agar. Recently we described a caffeic acid-ferric citrate medium (CAFC) that allows pigment formation of *C. neoformans* within 3 to 4 days (2a).

Breyer et al. (2) reported that the *o*-diphenoloxidase activity of *C. neoformans* was probably responsible for the differential pigment formation. These investigators flooded Sabouraud-dextrose agar plates with catechol, a substrate of this enzyme, in an effort to produce a rapid diagnostic test for *C. neoformans*. Initial observations were very encouraging; however, subsequent testing with additional strains of *C. neoformans* showed the catechol-diphenoloxidase reaction to be unsuitable for identification of *C. neoformans* (9).

Shaw and Kapica (6) characterized the 3-4-dihydroxyphenylalanine-phenoloxidase enzyme activity of *C. neoformans*. They were able to show that the phenoloxidase activity was localized within the cell wall of the organism. Incubation of the organism for 8 h in a buffered 3-4-dihydroxyphenylalanine solution was shown to cause pigment formation.

Because caffeic acid has been isolated from *G. abyssinica* seeds (10) and has been shown to cause pigment production in *C. neoformans*, particularly in the presence of ferric citrate (5), we decided to incorporate these compounds in paper disks as substrates for the phenoloxidase enzyme activity in an effort to develop a rapid identification test for *C. neoformans*.

MATERIALS AND METHODS

The numbers and strains of yeasts tested are listed in Tables 1 and 2. The majority of the organisms were clinical isolates. The organisms were cultured on Sabouraud-dextrose agar slants 48 h before testing on caffeic acid-ferric citrate (CAFC)-impregnated disks.

Blank paper disks (BBL) were saturated with 0.5 M Sorensen buffer (pH 7.0) containing 0.2 mg of caffeic acid and 0.05 mg of ferric citrate per ml. For convenience, 1 ml of stock solution of caffeic acid (1 mg/ml of 0.5 M buffer) and 0.5 ml of a stock solution of ferric citrate (0.5 mg/ml of H_2O) were added to 3.5 ml of phosphate buffer.

The CAFC-impregnated disks were allowed to dry at 37 C overnight and were stored in dark containers at −20 C, 4 C, or room temperature. CAFC disks were moistened with sterile distilled water prior to testing.

Yeast cells were transferred from Sabouraud-dextrose agar slants to the CAFC disks with an inoculating loop. Control disks impregnated with buffer only were run in parallel. The quantity of yeast cells transferred is not critical; however, results are more easily discerned if liberal amounts are applied (see Fig. 1). Incubation of disks was carried out at room temperature.

RESULTS

All 24 strains of *C. neoformans* tested produced a dark brown pigment within 4 to 6 h. In most instances, development of pigment was discernible within 1 h of application of growth to the CAFC disks (Table 1). The intense pigmentation produced by four representative strains of *C. neoformans* can be seen in Fig. 1.

Eight strains of saprophytic *C* species were

TABLE 1. *Pigment production by 24 strains of C. neoformans on CAFC disks*

No. of strains	Response (h after transfer to CAFC disk)				
	1	2	3	4	6
5	1+[a]	2+[b]	3+[c]	3+	3+
2	1+	2+	2+	3+	3+
11	1+	1+	2+	3+	3+
3	1+	1+	2+	2+	3+
3	—[d]	1+	1+	2+	3+

[a] 1+, Pigmentation weak, but discernible.
[b] 2+, Brown pigment.
[c] 3+, Dark brown pigment.
[d] —, No detectable pigment produced.

TABLE 2. *List of yeasts failing to produce pigment on CAFC disks after 24 h of incubation*

Organism	No. of strains tested
Cryptococcus albidus	5
Cryptococcus laurentii	2
Cryptococcus uniguttulatus	1
Candida albicans	10
Candida guilliermondii	3
Candida krusei	5
Candida parapsilosis	5
Candida pseudotropicalis	2
Candida tropicalis	10
Torulopsis glabrata	3
Saccharomyces cerevisiae	2

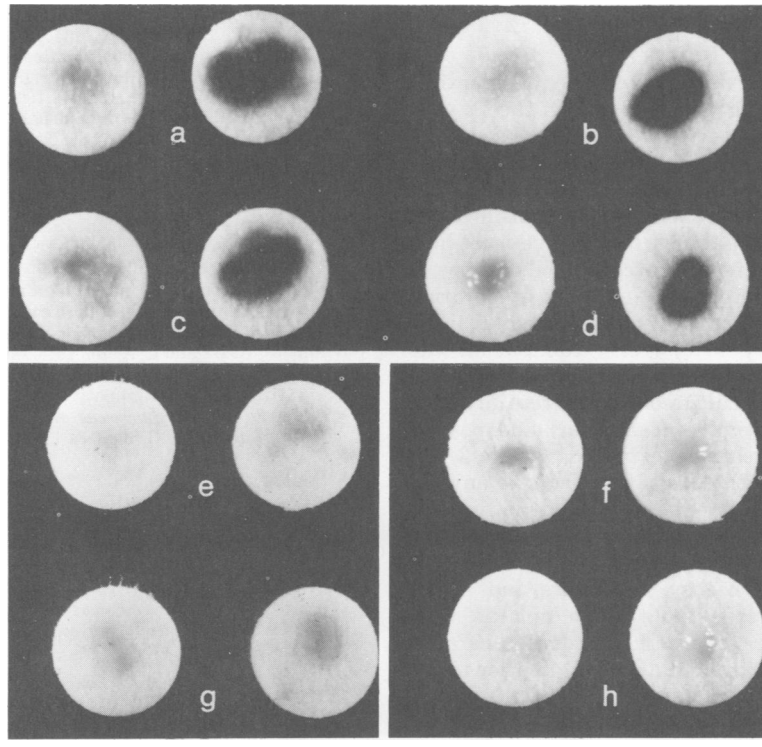

FIG. 1. *In each set of disks the control disk (buffer only) is on the left and the CAFC-containing disk is on the right. (a) C. neoformans MDA-664; (b) C. neoformans MDA-807; (c) C. neoformans (stock); (d) C. neoformans TSDH-3102; (e) C. laurentii (stock); (f) C. albidus 019; (g) C. albicans MDA-701-74; and (h) C. tropicalis MDA-210-74.*

also tested. Several of these strains are light tan-yellow when grown on Sabouraud-dextrose agar slants. Therefore, it is always necessary to include buffer-containing control disks. These saprophytic species do not develop any increased pigmentation on CAFC disks after 6 h (Fig. 1) and even 24 h (Table 2) of incubation.

Forty strains of other commonly isolated yeasts, including six species of *Candida*, *Torulopsis glabrata*, and *Saccharomyces cerevisiae*, were tested on CAFC disks. These yeasts failed to develop any pigmentation within 24 h (Table 2).

The stability of the CAFC-impregnated disks was studied using two representative strains of *C. neoformans*, one *Cryptococcus albidus* strain, one *Cryptococcus laurentii* strain, two *Candida albicans* strains, and one strain of

Candida tropicalis. CAFC disks stored in dark containers at −20 C, 4 C, and room temperature were removed and tested after 1 week, 2 weeks, 1 month, 2 months, 4 months, and 6 months of storage. No evidence or deterioration of chemical breakdown of CAFC components was noted. It should be pointed out that the CAFC solution used to impregnate the paper disks is quite unstable when exposed to light.

The concentrations of components are critical. Reduction in concentration of either caffeic acid or ferric citrate requires longer incubation periods for production of the dark pigment. Likewise, increased concentrations of either component (particularly ferric citrate) causes nonspecific pigmentation of saprophytic *Cryptococcus* species and several *Candida* species.

DISCUSSION

Cryptococcus neoformans can be identified within 6 h using CAFC disks. Of the 72 yeast isolates tested, only *C. neoformans* produced a dark pigment within 24 h. Because the color reaction appears to be complete within 6 h, we suggest using a 6-h time limit for routine use.

The CAFC rapid disk test will be most helpful in the diagnostic mycology laboratory. In the absence of a positive India ink preparation, *C. neoformans* is often not suspected until several laboratory tests (germ tube, chlamydospore, urease) have been performed. The isolates are then cultured on differential media (CAFC or *guizotia* extract agar) and positive identification can be expected within 3 to 6 days. Through the use of CAFC disks, any suspected colonies can be identified within 6 h, thereby decreasing both time and materials necessary for identification.

The disks are easy to prepare and, in our laboratory, were stable for 6 months when stored in dark containers at room temperature (22 C). The disks should be wetted prior to use.

It was noted during our investigation that continued wetting of the CAFC disks results in some of the saprophytic *Cryptococcus* species becoming lightly pigmented after incubation times of more than 24 h. It is possible that these cryptococci have phenoloxidase in very small quantity, but the pigment formation could also result from a nonenzymatic chemical reaction. This phenomenon is being investigated.

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