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Caffeic Acid-Containing Medium for Identification of *Cryptococcus neoformans*

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A new growth medium containing caffeic acid and ferric citrate is described. The pigment produced on this medium is specific for the identification of *Cryptococcus neoformans* and differentiates it from other cryptococci. The medium is more easily compounded and requires less time for pigment formation than the conventional *Guizotia* extract media. The medium is stable in the dry form as well as in the prepared form.

Staib (4) observed that a hot water extract of *Guizotia abyssinica* seeds (nigerseed), when added to growth medium, caused a characteristic and specific brown pigmentation of *Cryptococcus neoformans* colonies. Strachan et al. (5) isolated 3,4-dihydroxy-transcinnamic acid (caffeic acid) from nigerseed. They showed that this compound, added to growth medium, induced a similar pigmentation of *C. neoformans*. It was therefore tempting to use this finding for the simplification and standardization of Staib's important technique.

MATERIALS AND METHODS

Source of yeasts tested. Of the 62 yeast strains tested, 47 were clinical isolates and 15 were stock cultures. The following species were tested (the number of stock strains is in parentheses): *Cryptococcus neoformans*, 24 (4) strains; *Cryptococcus albidus*, 5 (1) strains; *Cryptococcus laurentii*, 2 (1) strains; *Crytsococcus uniguttulatus*, 1 strain; *Candida albicans*, 7 (1) strains; *Candida tropicalis*, 5 (1) strains; *Candida guilliermondii*, 3 (2) strains; *Candida krusei*, 3 (1) strains; *Candida parapsilosis*, 3 (1) strains; *Candida pseudotropicalis*, 4 (1) strains; *Torulopsis glabrata*, 3 strains; and *Saccharomyces cerevisiae*, 2 (1) strains.

Growth of organisms. All organisms were maintained on Sabouraud dextrose agar slants. Inocula for pigmentation studies were prepared semiquantitatively by suspending 48-h-old cells in saline and applying equal amounts to the surface of the tested media with a cotton swab.

Media. The composition of medium I was arrived at after addition of a variety of compounds to a basal glucose-ammonium salts medium. Test compounds included peptone, yeast extract, Casamino Acids, Casitone, creatinine, inositol, ammonium chloride, ammonium sulfate, and dibasic potassium phosphate. The formulation of the medium appears in Results.

Medium II is the caffeic acid-containing agar described by Pulverer and Korth (1). It requires pH adjustment with NaOH and contains higher levels of glucose (10 g/liter), caffeic acid (0.3 g/liter), and ferric citrate (0.05 g/liter) than the medium we found to be optimal and specific for pigment production by *C. neoformans*.

Medium III is the *Guizotia abyssinica* extract medium described by Shields and Ajello (3).

RESULTS

Different concentrations of various compounds were investigated as possible sources for carbon and nitrogen together with a number of growth substances in order to arrive at a medium permitting fast and specific pigmentation. In addition, optimal conditions for incubation temperature with and without light were

TABLE 1. *Pigment formation by Cryptococcus neoformans (24 strains tested)*

Days after inoculation (25 C)	Medium		
	I[a] (dark-brown pigmentation)	II[b] (dark-brown pigmentation)	III[c] (gold-brown pigmentation)
2	9 (4)[d]	1 (4)	1 (8)
3	17 (7)	12 (3)	8 (11)
4	24	17 (2)	18 (5)
5	24	21 (3)	18 (6)
6	24	23 (1)	21 (3)
7	24	24	24

[a] Caffeic acid-ferric citrate medium of Hopfer and Blank.

[b] Caffeic acid-ferric citrate medium described by Pulverer and Korth (1).

[c] *Guizotia abyssinica* extract medium described by Shields and Ajello (3).

[d] Numbers in parentheses indicate light pigmentation.

studied. The following medium was found to be satisfactory: glucose, 5 g; $(NH_4)_2SO_4$, 5 g; yeast extract, 2 g; K_2HPO_4, 0.8 g; $MgSO_4 \cdot 3H_2O$, 0.7 g; caffeic acid, 0.18 g; ferric citrate, 0.002 g (added as 4 ml of a stock solution); agar, 20 g; and distilled water to 1,000 ml. Final pH without adjustment is 6.5. For ferric citrate stock solution, 10 mg of ferric citrate in 20 ml of distilled water is dissolved in a boiling water bath. The medium is autoclaved for 12 min at 15 lb/in^2. Ten milliliters of medium is poured into petri dishes (60 by 15 mm). The medium may appear faintly blue upon cooling. The medium should be protected from direct light during prepara-

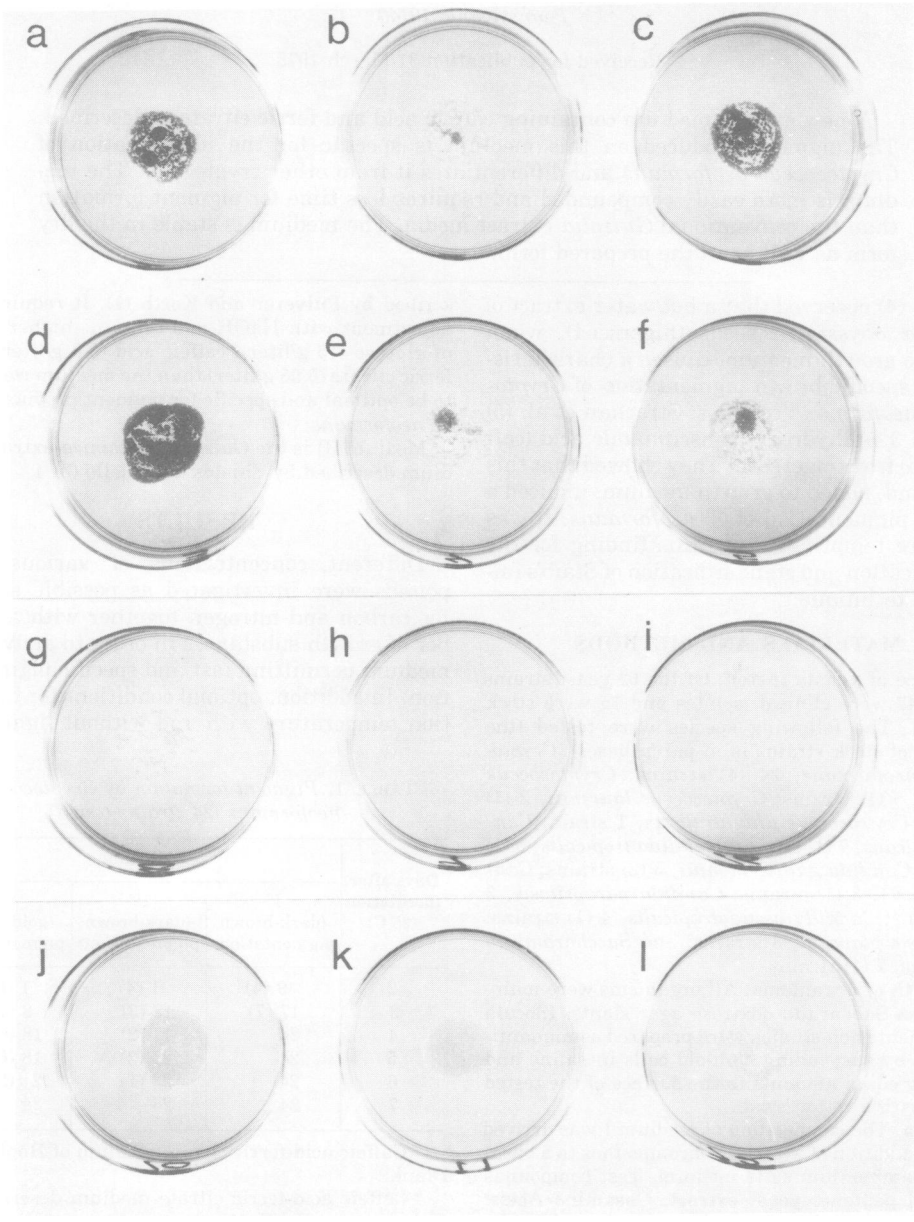

FIG. 1. *Yeasts on medium I after 2 days of incubation. (a) Cryptococcus neoformans 565-74; (b) C. neoformans S-30-74; (c) C. neoformans 1250-74; (d) C. neoformans 2149-74; (e) C. neoformans 490-74; (f) C. neoformans MDA-664; (g) C. laurentii (stock); (h) C. albidus 019; (i) C. uniguttulatus TSDH-3895; (j) Candida albicans MDA 701-74; (k) Candida tropicalis MDA 210-74; (l) Torulopsis glabrata MDA 468-74.*

tion and be stored at 4 C (wrapped in foil). Incubation temperature is 25 C in the dark. Addition of chloramphenicol (0.05 g/liter) does not alter pigment production. We found it more convenient to place one or two chloramphenicol (30 μg) antimicrobial susceptibility disks onto the surface of the medium after inoculation.

This medium (I) was compared with those media (II and III) described by Pulverer and Korth (1) and Shields and Ajello (3), respectively (Table 1). The medium of Pulverer and Korth contains caffeic acid; that of Shields and Ajello contains an extract of the seeds of *G. abyssinica* as pigment inducers. Our medium induced pigmentation earlier than did the two other media (Fig. 1–3). Four days after inoculation, all 24 strains of *C. neoformans* tested showed dark-brown pigmentation, whereas 7

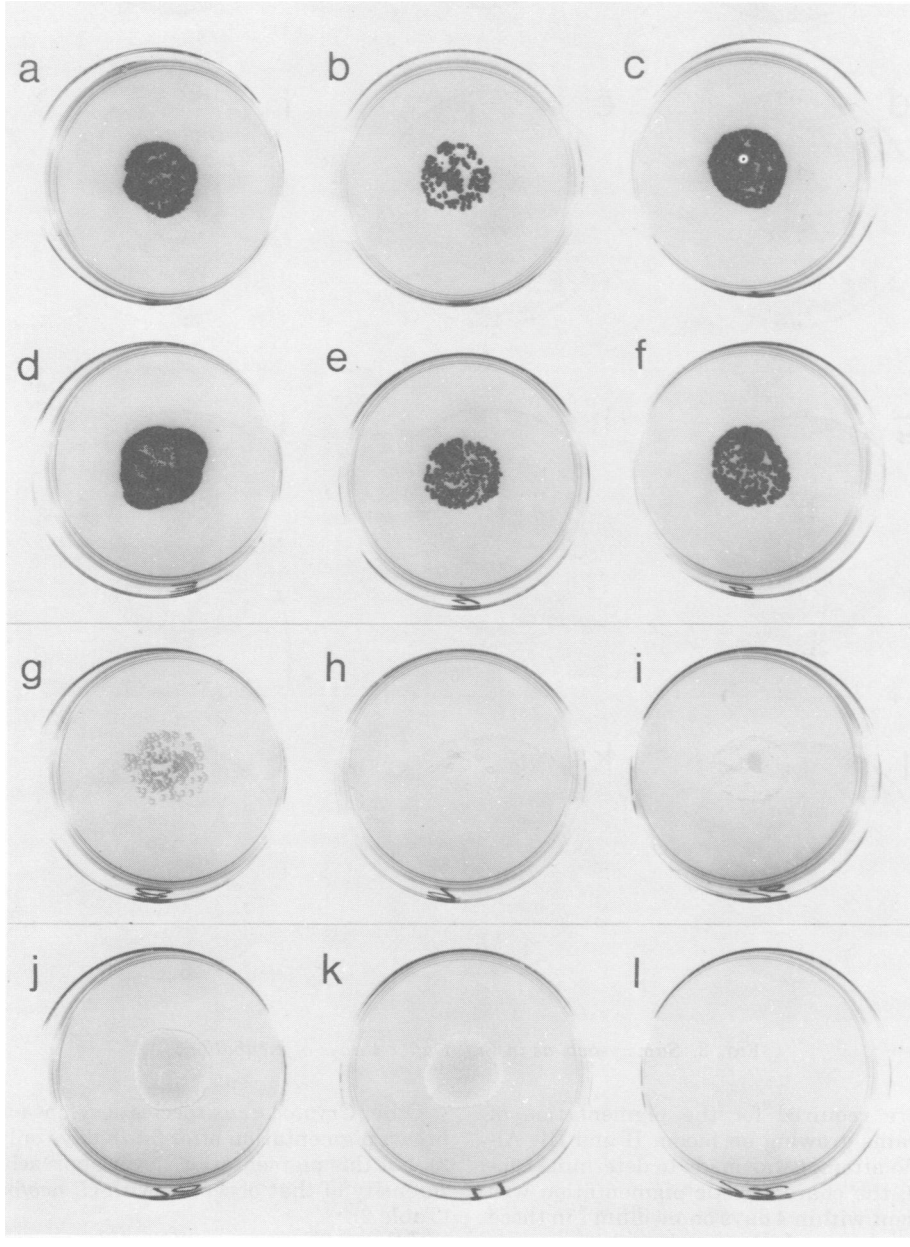

FIG. 2. *Same yeasts as in Fig. 1 after 3 days of incubation.*

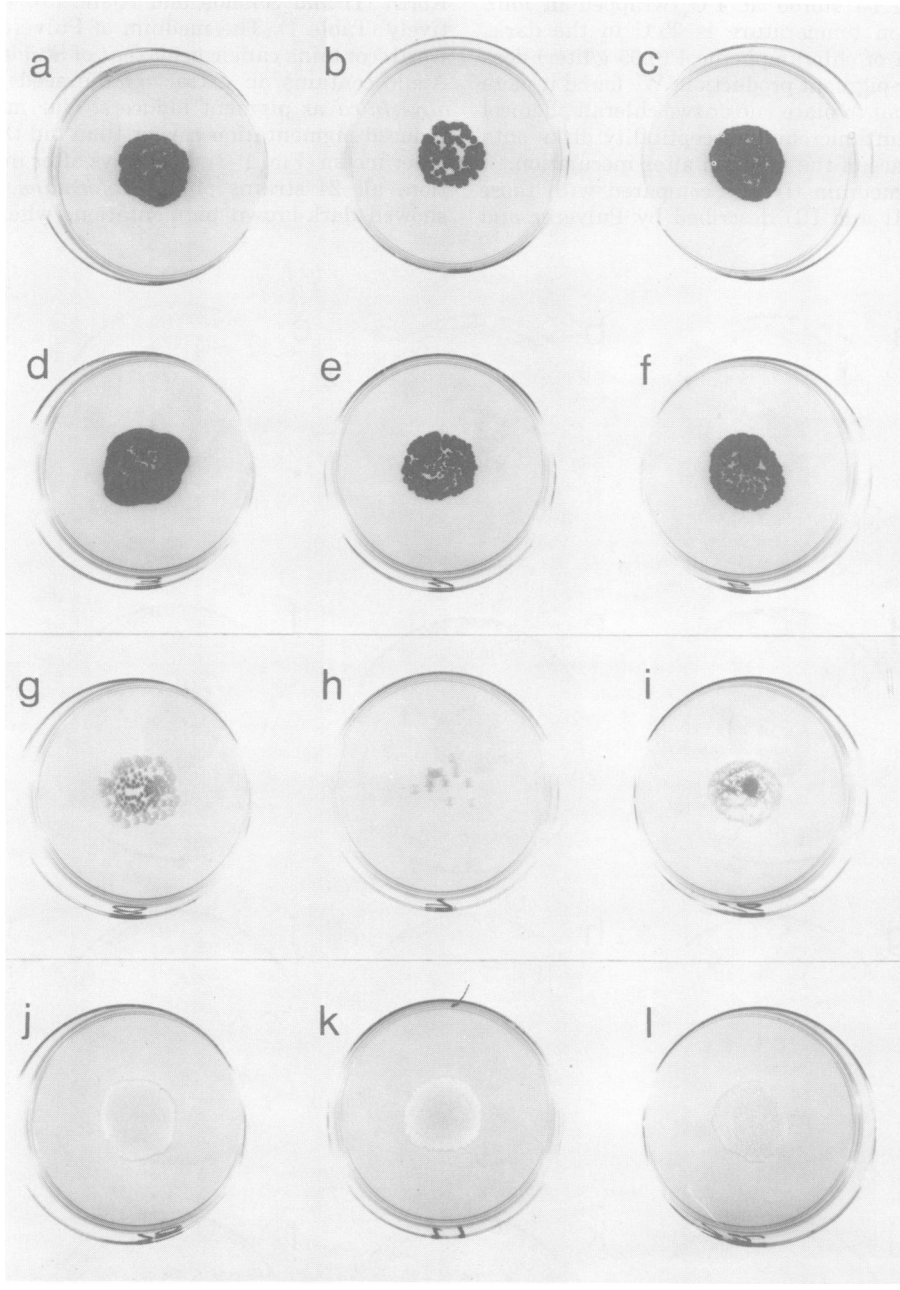

FIG. 3. *Same yeasts as in Fig. 1 after 4 days of incubation.*

days were required for the pigmentation of some strains growing on media II and III. Although no attempt was made to determine capsule size, the characteristic pigmentation was also present within 4 days on medium I in those strains producing thick capsules.

Other *Cryptococcus* species developed a light-brown pigmentation after 3 to 4 days only (Fig. 3), but this pigmentation never approached the intensity of that observed with *C. neoformans* (Table 2).

Twenty-five strains of *Candida*, representing

six species, three strains of *T. glabrata*, and two *S. cerevisiae* strains were cultured on media I and II (Table 3). These yeasts did not produce brown pigmentation (Fig. 3). However, *Candida albicans*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis* tended to turn our medium slightly pink-tan. In addition, the colonies of these species became weakly pigmented (pink-tan); however, the intensity of the colony pigmentation was only slightly more intense than that of the surrounding medium. Medium II became bluish by the growth of *Candida albicans*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis*. These colonies turned gray-blue. One strain of *C. krusei* developed a relatively dark blue-brown pigment on medium II after 4 days of growth.

DISCUSSION

The medium we developed for the selective pigmentation of *C. neoformans* has several advantages. The substitution of the extract of *Guizotia* seeds by a commercially available compound, caffeic acid, simplifies the preparation of the medium, and the unvaried composition of different batches secures reproducibility of results. The major advantage of the medium is the reduction in incubation time (24 to 48 h) necessary for pigment formation. Other cryptococci become pigmented only 3 to 4 days after inoculation, but they are not so intensely colored and can therefore be distinguished from *C. neoformans*.

Pigment formation is delayed during luxuriant growth. This observation may explain why colonies growing in petri dishes show better and earlier pigmentation than those on the lower portion of agar slants in test tubes.

The light pink-tan color developed on our medium by several *Candida* strains is most likely a result of nonspecific accumulation of metabolic products from the medium. This pigmentation will not interfere with the identification of *C. neoformans*. In mixed cultures of *Candida albicans* or other *Candida* species, the dark-brown pigment of *Cryptococcus neoformans* develops more rapidly (2). Our preliminary studies suggest that this enhancement is not simply caused by a change in pH. We are attempting to determine the factors responsible for this phenomenon.

We have isolated *C. neoformans* from a variety of clinical specimens including spinal fluid, lung biopsy tissue, sputum, and autopsy tissue. In addition, we have added light and heavy suspensions of *C. neoformans* to heavily contaminated sputa and to stool specimens. In no instance were antimicrobial agents necessary for the identification of *C. neoformans;* however, chloramphenicol added either directly to the medium (0.05 g/liter) or as an antimicrobial susceptibility disk (30 μg) prevented growth of many of the contaminating bacterial organisms. It is our opinion that routine addition of antimicrobial agents is not necessary for clinical specimens.

TABLE 2. *Pigment formation by other cryptococci (eight strains tested)*

Days after inoculation (25 C)	Medium		
	I[a] (light-brown pigmentation)	II[b] (light-brown pigmentation)	III[c] (light-brown pigmentation)
2	0	0	0
3	0 (5)[d]	0 (4)	0
4	6 (2)	2 (5)	0
5	8	7	0
6	8	7 (1)	0
7	8	8	0

[a,b,c] See Table 1.

[d] Numbers in parentheses indicate very light pigmentation.

TABLE 3. *Pigmentation of Candida, Torulopsis, and Saccharomyces after 6 days (25 C)*

Organism	No. of strains tested	Intensity of pigmentation	Medium	
			I	II
Candida albicans	7	Light	7	7
C. guilliermondii	3	Light	3	3
C. krusei	3	Dark	0	1
C. parapsilosis	3	Light	3	3
C. pseudotropicalis	4	Light	0	0
C. tropicalis	5	Light	5	5
Torulopsis glabrata	3	Light	0	0
Saccharomyces cerevisiae	2	Light	0	0

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