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Evaluation of a Simplified *Guizotia abyssinica* Seed Medium for Differentiation of *Cryptococcus neoformans*

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A simplified *Guizotia abyssinica* seed medium, eliminating glucose, creatinine, and phosphate, was evaluated for the isolation and presumptive identification of *Cryptococcus neoformans*. Of 80 isolates of *C. neoformans* tested, 69 (86%) developed the characteristic brown pigment within 12 h on this medium as against only 5 isolates (6%) on the complete medium. In primary cultures of experimentally seeded specimens of sputum, bronchial aspirate, soil, and pigeon excreta, *C. neoformans* was recognizable within 3 to 5 days on the modified medium in contrast to the 3 to 8 days required on the complete medium. The results demonstrated that the simplified *G. abyssinica* seed agar, with or without diphenyl, is superior to the complete medium for the rapid development of the brown pigment by *C. neoformans*.

Guizotia abyssinica seed agar, variously referred to in the literature as niger seed creatinine agar, thistle seed medium, bird seed agar, Staib medium, etc., is now recognized as a valuable tool for the selective isolation and presumptive identification of *Cryptococcus neoformans* (1-14). This communication deals with a reappraisal of the composition of this medium, leading to its simplification.

MATERIALS AND METHODS

Media. The basal *G. abyssinica* seed agar was prepared as described by Staib et al. (11) except that chloramphenicol (50 μg/ml) was substituted for penicillin and streptomycin sulfate. A 50-g amount of *G. abyssinica* seed (supplied by Manilal Lallubhai & Co., Bombay), pulverized in an electric mixer, was boiled for 25 to 30 min in 1,000 ml of distilled water, followed by filtration through gauze cloth and filter paper. To the filtrate were added glucose, 10 g; potassium dihydrogen orthophosphate, 1 g; creatinine, 1 g; and agar, 15 g, and the volume was made up to 1 liter with distilled water. The medium was sterilized by autoclaving at 110°C for 25 min. When the medium had cooled down to about 50°C, chloramphenicol (50 mg) and diphenyl (1 g) were added after dissolving these in 10 ml of absolute alcohol. A number of modified media were prepared from the basal *G. abyssinica* seed medium by depleting its constituents one by one. These were tested regarding suitability for growth and pigmentation of *C. neoformans*.

Strains. A total of 80 isolates of *C. neoformans* maintained in the Fungus Culture Collection of the Vallabhbhai Patel Chest Institute were used in the tests.

Test procedure. Plates of *G. abyssinica* seed agar and its modifications were point inoculated in duplicate by using a 2-mm-diameter loop directly from a 2- to 3-day-old growth of *C. neoformans* on Sabouraud agar (1% peptone-2% glucose-2% agar) slants at 25°C, taking care that each plate received a more or less uniform inoculum. The plates were incubated at 25°C and were observed for pigmentation and growth twice a day over a period of 2 weeks.

With a view to exploring the suitability of various *G. abyssinica* seed-based media for the primary isolation of *C. neoformans*, eight sputum specimens, a solitary bronchial aspirate, and six specimens each of soil and old mortar-like pigeon excreta were experimentally seeded with the fungus. Sputa and bronchial aspirates came from the inpatients of the Clinical Research Centre of the Vallabhbhai Patel Chest Institute, whereas the soil and old pigeon excreta were collected from the premises of the same institute and its neighborhood. Sputum specimens required homogenization by shaking with glass beads before they could be processed further. Culture Cr 402/55 of *C. neoformans*, isolated in this laboratory from the sputum of a patient, served as the test organism. The inoculum was prepared in sterile physiological saline from a 48-h-old culture on Sabouraud agar at 25°C, and its cell density was determined with a hemocytometer. The amount of inoculum added to the sputa and bronchial aspirates was so adjusted that a loopful (≈ 0.1 ml) would carry about 40 cells of *C. neoformans*. From each of the clinical specimens thus seeded, a loopful was cultured on plates of the various *G. abyssinica* seed-based media.

To process the soil samples, about 5 g of the material was added to 45 ml of sterilized physiological saline and kept at 37°C for 1 h with intermittent shaking. A 1-ml amount of the resulting suspension was transferred to 9 ml of sterile distilled water, followed by addition of chloramphenicol at a concentration of 50 μg/ml. Serial dilutions were made from this suspension after incubating it for 2 h at 37°C. A part of $1:10^5$

dilution was so mixed with a culture suspension that it contained approximately 100 *C. neoformans* cells per ml. Quantities (0.5 ml) of the supension thus prepared from each of the solid specimens were cultured on the test media. Specimens of pigeon excreta were also processed in the same way as soil except that both the $1:10^4$ and the $1:10^5$ dilutions, each containing nearly 200 cells per ml, were plated.

RESULTS

Observations on the time taken by *C. neoformans* to develop the brown pigment on the various *G. abyssinica* seed-based agar media are presented in Table 1. The minimal *G. abyssinica* seed medium (N agar) yielded the most rapid results regarding pigmentation. Of the 80 cultures of *C. neoformans* tested, 69 (86%) exhibited the brown pigment within 12 h on this medium, as against only 5 (6%) on the complete *G. abyssinica* seed medium (NPGCD agar) that served as the control. The minimal medium that contained diphenyl (ND agar) also yielded excellent results, with 64 (80%) isolates showing pigmentation in 12 h. It was further noted that the exclusion from the complete medium of glucose, and possibly of creatinine to a lesser extent, promoted early pigmentation of *C. neoformans*, whereas phosphate exerted no effect. On the other hand, pigmentation appeared to be retarded when diphenyl was removed from the glucose-containing media, i.e., NPGCD agar and NPGD agar. Finally, it may be mentioned that the growth of *C. neoformans* on the minimal *G. abyssinica* seed agar was more or less equal to that on the control medium (NPGCD agar). However, growth was slightly reduced when the minimal medium was supplemented with diphenyl.

Table 2 provides data on the suitability of the

TABLE 1. *Efficacy of some G. abyssinica seed-based media[a] for the presumptive identification of 80 reference cultures of C. neoformans cultivated at 25°C*

Pigmentation time (h)	No. of isolates showing brown pigment on:									
	Control[b] (NPGCD agar)	Modified *G. abyssinica* seed-based agar media								
		NPCD	NPGD	NPD	ND	NPGC	NPC	NPG	NP	N
12	5	58	4	63	64	1	59	1	69	69
24	5	14	17	10	11	0	17	6	8	10
36	5	8	10	7	5	1	3	3	3	1
48	22		29			2	0	7		
60	13		10			4	0	8		
72	9		7			3	0	9		
84	10		2			5	0	8		
96	3		0			8	1	6		
108	1		1			5		2		
120	4					11		16		
132	0					1		1		
144	1					14		10		
168	1					3		2		
180	0					6		0		
192	1					5		1		
204–336						11				

[a] All the test media contained chloramphenicol at a concentration of 50 μg/ml.
[b] Denotes the complete medium comprising an aqueous extract of *G. abyssinica* seeds (N), potassium dihydrogen orthophosphate (P), creatinine (C), glucose (G) and diphenyl (D).

TABLE 2. *Suitability of the minimal G. abyssinica seed agar supplemented with diphenyl (ND agar) for isolation of C. neoformans from experimentally inoculated clinical materials and natural substrates[a]*

Substrates	No. of samples tested	Inoculum (no. of cells/plate)	Avg colony count/plate on:		Avg pigmentation time (days) on:	
			Minimal medium (ND agar)[b]	Control (NPGCD agar)	Minimal medium (ND agar)	Control (NPGCD agar)
Soil	6	50	45	43	3.8	5.5
Pigeon excreta	6	100	88	86	4.0	6.0
Clinical material[c]	9	40	36	37	3.8	5.8

[a] Cultures were incubated at 25°C and observed up to 8 days.
[b] See Table 1, footnote *b* for abbreviations of agars.
[c] Includes eight samples of sputum and one bronchial aspirate.

minimal *G. abyssinica* seed agar supplemented with diphenyl (ND agar) for the primary isolation of *C. neoformans* from clinical specimens and natural substrates that had been experimentally seeded with the fungus. *C. neoformans* was recovered with equal frequency from all of the 21 test specimens on both the ND agar and the complete medium. However, ND agar was preferable to the complete medium (NPGCD agar) in that brown pigmentation was evident within 3 to 5 days as against 3 to 8 days on the latter medium.

DISCUSSION

The results demonstrate that the minimal *G. abyssinica* seed agar, apart from supporting adequate growth, allows the early development of brown pigmentation in *C. neoformans*. Thus, 86% of the *C. neoformans* cultures developed the brown pigment within 12 h on the minimal medium, whereas the complete medium (NPGCD agar) yielded pigmentation in only 6% of the isolates in the same time period. There seems to be little justification, therefore, in following the current practice of incorporating glucose, creatinine, and phosphate (4–6, 8, 10–14) in the *G. abyssinica*-based selective medium. In fact, colonies of *C. neoformans* exhibited delayed pigmentation on the complete medium. Our preliminary observations suggest that glucose is the principal factor responsible for the delayed pigmentation of *C. neoformans* on the complete *G. abyssinica* seed medium, but the underlying mechanism is not understood. Further investigations are in progress to clarify this question.

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