Rapid Presumptive Identification of *Cryptococcus neoformans*

HAROLD G. MUCHMORE,* FRANCES G. FELTON, AND E. NAN SCOTT

Infectious Disease Section, Veterans Administration Hospital, and Departments of Medicine and Microbiology and Immunology, University of Oklahoma, College of Medicine, Oklahoma City, Oklahoma 73104

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Carbohydrate-containing extracts were prepared from mature yeast colonies grown on Sabouraud dextrose agar by mixing a 0.001-ml loopful of yeast cells for 30 s in phenolized saline and removing the cells by centrifugation. Extracts were prepared from 54 *Cryptococcus neoformans* isolates, 29 isolates of other *Cryptococcus* species, 16 isolates of *Candida* species, 2 *Rhodotorula*, 2 *Torulopsis*, and 1 *Saccharomyces* species. Initially the carbohydrate content of each extract was estimated (Molisch method) and adjusted to 1, 5, and 10 μ g/ml. Twofold dilutions of each extract were tested for reactivity with the cryptococcal latex agglutination reagent of Bloomfield et al. (N. Bloomfield, M. A. Gordon, and D. F. Elmendorf, Jr., Proc. Soc. Exp. Biol. Med. 114:64-67, 1963). All 54 *C. neoformans* extracts gave strong agglutinations (3+ to 4+) in dilutions of 1:4 or greater. None of the other yeasts produced any agglutination, except for 1 of 15 *C. laurentii* isolates, which showed a 1+ reaction that disappeared at a dilution of 1:4 and above. Subsequent testing established that a single extract made from 0.001 ml of yeast cells in 6 ml of phenolized saline contained less than 5 μ g of carbohydrate per ml, was suitable for a single rapid screening dilution, and eliminated any cross-reaction from the *C. laurentii* isolates. In our hands this method has provided a reliable differentiation of *C. neoformans* from other unknown yeast colonies in less than 20 min exclusive of a Molisch determination.

Upon primary isolation, *Cryptococcus neoformans* isolates grow as buttery or mucoid colonies, resembling many other species of yeast. The definitive identification of an unknown yeast as *C. neoformans* requires carbon assimilation and other tests, which extend the period before the clinical report is provided to the physician.

Present conventional methods for the identification of *C. neoformans* begin by determining that the unknown yeast produces urease, does not form germ tubes, and grows at 35°C. Carbon and nitrate assimilation methods in liquid media (7, 8, 11) are cumbersome and time consuming, requiring 10 to 14 days to complete. Auxanographic assimilation methods on solid media are shorter, but still require several days. Commercially available rapid methods in kit form for yeast identification, primarily based upon carbon and nitrate assimilations, have decreased the time involved, but still require 2 to 3 days and in our experience are not as reliable for the cryptococci as they are for other yeasts. Mouse virulence (death), although providing strong identifying evidence, requires a minimum of 5 days for some *C. neoformans* isolates from cerebrospinal fluid (CSF), while some sputum and soil isolates may require more than 180 days.

Staib and Seeliger (9) described a medium which produces a brown pigment in *C. neoformans* colonies after a few days of growth. This selective, differential medium, containing an extract of *Guizotia abyssinica* seeds, has been modified by substituting caffeic acid for the *Guizotia* extract (6), and more recently by Hopfer and Gröschel (4), who added ferric citrate, decreasing the time for pigment formation to 6 h.

The present study was undertaken to develop a rapid serological method for the identification of *C. neoformans* isolates that could be carried out in almost any clinical microbiology laboratory. This rapid method consists of the application of a diagnostic serological test already available for the detection of cryptococcal polysaccharide ("antigen") in body fluids, i.e., CSF or serum (1), to "standard" carbohydrate extracts made from the unidentified yeast isolates. These extracts are processed in the same manner as CSF or serum in the serological test.

MATERIALS AND METHODS

Yeast isolates. One hundred and four known yeast isolates, maintained as stock cultures on Sabouraud dextrose agar under oil, were used in this study. All isolates were of clinical or soil origin and included: 54 *C. neoformans* (24 clinical isolates, 26 soil isolates, and

1 each of the four known capsule types, A, B, C, and D), 15 *C. laurentii*, 6 *C. albidus* subsp. *diffluens*, 4 *C. albidus* subsp. *albidus*, 2 *C. kuetzingii*, and 2 *C. terreus*; 9 *Candida albicans*, 3 *C. parapsilosis*, 3 *C. tropicalis*, and 1 *C. pseudotropicalis*; 2 *Rhodotorula* spp.; 1 *Saccharomyces* sp.; and 2 *Torulopsis glabrata*. All of these isolates were identified by currently accepted conventional identification criteria for yeasts (2, 7). About half the isolates of *C. neoformans* did not have a visible capsule in India ink.

Yeast carbohydrate extract. Each of the above yeast isolates was subcultured from stock slants to Sabouraud dextrose agar streak plates and incubated for 48 h at 25°C. A standard inoculum (0.001-ml platinum loop) from a single mature yeast colony was added to 3 ml of phenolized saline (1% phenol in normal saline) in a screw-cap tube. Tubes were capped, stirred on a Vortex mixer for 30 s, and centrifuged (1,500 rpm for 10 min) to sediment the yeast cells. The carbohydrate content of each yeast extract was estimated by the Molisch method (5) and adjusted to concentrations of 10, 5, and 1 µg/ml. Each of these dilutions of the carbohydrate-containing extracts was then tested for reactivity with the cryptococcal globulin-latex reagent. Subsequently the volume of phenolized saline was increased to 6 ml, which yields an extract containing less than 5 µg of carbohydrate per ml.

Cryptococcal slide agglutination test. The slide agglutination test for the detection of *C. neoformans* capsular polysaccharide, as described by Bloomfield et al. (1) and using materials produced in our laboratory, was performed on each prepared yeast extract. These extracts were tested in the same way that CSF or serum from a patient is tested. In brief, serial twofold dilutions of each yeast extract were made from a diluent composed of glycine-buffered saline (pH 8.2) containing 0.1% bovine serum albumin. In the tests, 0.04 ml of each extract dilution was placed in one square of a glass slide (2 by 3 inches [ca. 5.1 by 7.6 cm], divided into six squares); 0.02 ml of the latex-globulin suspension (maximally reactive dilution of rabbit anti-*C. neoformans* globulin coated onto latex particles) was added to each square. The reagents in each square were mixed with an applicator stick. The slide was agitated for 2 min on a rotating apparatus (Arthur H. Thomas Co.) at 180 rpm. Positive and negative yeast extract controls were run on each batch of the latex-globulin suspension. Results were recorded as 0 to 4+ agglutination as originally described (1). The highest dilution exhibiting a 2+ agglutination was recorded as the titer for each extract.

A commercial Crypto-LA kit (International Biological Laboratories, Inc., Rockville, Md.) is available for the detection of *C. neoformans* capsular polysaccharide by the same technique. A kit was tested in comparison with the reagents produced in our laboratory for its efficacy in the rapid identification of *C. neoformans* yeast colonies. Tests were performed as outlined above, which conforms to the instructions provided with the kit.

Summarized procedure. The final procedure for the rapid serological identification of *C. neoformans* can be summarized as follows. (i) Pick one loop (0.001 ml) of growth from the yeast colony. (ii) Mix for 30 s

in 6.0 ml of phenolized saline in a Vortex mixer. (iii) Centrifuge to sediment yeast cells (supernatant will contain 1 to 5 µg of carbohydrate per ml by the Molisch method). (iv) Test supernatant for *C. neoformans* capsular polysaccharide by the latex agglutination test.

RESULTS

Cryptococcal latex agglutination reactions obtained from the 104 yeast extracts tested in this laboratory are recorded in Table 1. The titers recorded are the highest dilution of yeast extract that exhibited an agglutination of 2+. All 54 extracts (10 µg/ml) from *C. neoformans* isolates exhibited titers of 16 to 256. However, at the 10-µg/ml carbohydrate concentration, all 15 *C. laurentii* extracts also exhibited 2+ agglutination, but at dilutions of 1:16 or less. Of these 15 *C. laurentii* extracts, 3 exhibited titers of 16, while 11 reacted at 1:4 dilutions or less. Two extracts (10 µg/ml) from isolates of *Candida* spp. (one *C. albicans* and one *C. tropicalis*) reacted with the latex-globulin reagent at a dilution of 1:4 and 1:2, respectively. When the carbohydrate concentrations of the yeast extracts were tested at 5 µg/ml, 14 of the 15 *C. laurentii* isolates no longer demonstrated 2+ agglutination. The one *C. laurentii* extract still exhibiting agglutination was recorded as a 2+ agglutination only at a 1:2 dilution. The *C. neoformans* extracts all reacted strongly with high titers at 5-µg/ml concentrations. At a carbohydrate concentration of 1

TABLE 1. *Cryptococcal latex agglutination reactions obtained from 104 yeast extracts*

Yeast (no. of isolates tested)	Latex agglutination titer (no. of isolates) at carbohydrate concn (µg/ml) ^a of:		
	1	5	10
<i>C. neoformans</i>			
A (1)	4	32	32
B (1)	16	64	64
C (1)	8	16	32
D (1)	8	64	128
Human isolates (24)	4-32	16-64	16-128
Soil isolates (26)	8-32	16-128	16-256
<i>C. laurentii</i> (15)	0	2 (1)	2-16
<i>Cryptococcus</i> spp. (14)	0	0	2 (6)
<i>Candida albicans</i> (9)	0	0	4 (1)
<i>Candida</i> spp. (7)	0	0	2 (1)
<i>Rhodotorula</i> spp. (2)	0	0	0
<i>Saccharomyces</i> sp. (1)	0	0	0
<i>Torulopsis</i> spp. (2)	0	0	0

^a Carbohydrate concentration was determined by the Molisch method.

$\mu\text{g/ml}$, only the *C. neoformans* extracts continued to react at any dilution. Titers were lower, but these extracts continued to react strongly (3+ to 4+) at the lower dilutions of 1:4 and 1:2.

No yeast extract, except *C. neoformans*, exhibited greater than a 2+ agglutination at dilutions of 1:16 or less at any carbohydrate concentration tested. The low dilutions (undiluted to 1:16) of *C. neoformans* extracts always exhibited strong 3+ to 4+ agglutinations.

In Table 2, the results from selected isolates of *C. neoformans* (two patient and one soil isolates) and *C. laurentii* (three soil isolates) demonstrate this difference in agglutination strength and pattern. At the highest carbohydrate concentration (10 $\mu\text{g/ml}$), none of the *C. laurentii* extracts showed an agglutination greater than 2+, whereas all of the *C. neoformans* extracts strongly agglutinated (3+ to 4+) the latex-globulin at lower dilutions. Decrease of polysaccharide concentrations below 5 $\mu\text{g/ml}$ resulted in reduced titers in all isolates, but *C. neoformans* continued to demonstrate 3+ to 4+ agglutina-

tion, while the *C. laurentii* isolates became negative.

One commercial cryptococcal latex kit (Crypto-LA) was tested in parallel with the latex-globulin reagent prepared in our laboratory on several selected yeast extracts (Table 3). The commercial reagent was tested on extracts from isolates of *C. neoformans* and from those *C. laurentii* extracts that had exhibited the strongest (2+) agglutination. Only one concentration (5 $\mu\text{g/ml}$) of carbohydrate was tested. In all *C. neoformans* isolates compared, the Crypto-LA kit materials exhibited 3+ to 4+ agglutination, with titers similar to those obtained using the latex-globulin reagent prepared in our laboratory. In eight isolates of *C. neoformans*, the commercial kit demonstrated titers one to two dilutions lower than the local product. Specificity was equal to our laboratory reagent, since no significant reaction was seen to occur with *C. laurentii* (only a titer of 2 in two isolates). The yeasts newly isolated from patients, included in Table 3, were identified as *C. neoformans* with

TABLE 2. Cryptococcal latex agglutination reactions obtained from selected isolates of *C. neoformans* and *C. laurentii*

Yeast ^a	Agglutination ^b at extract dilution:										Carbohy- drate concn (μg/ml) ^c
	0	2	4	8	16	32	64	128	256	512	
<i>C. neoformans</i>											
CP-114	4	4	4	3	2	2	1	±	0	0	10
	4	4	4	3	2	1	1	±	0	0	5
	3	3	2	1	±	0	0	0	0	0	1
CP-117	3	3	4	4	4	3	3	2	1	±	10
	4	4	4	3	3	2	1	0	0	0	5
	4	3	2	2	1	±	0	0	0	0	1
CS-26	3	4	4	4	4	4	3	3	2	1	10
	4	4	4	3	3	2	1	1	±	0	5
	4	4	3	2	1	±	0	0	0	0	1
<i>C. laurentii</i>											
S-87	2	2	2	2	1	±	0	0	0	0	10
	±	1	±	0	0	0	0	0	0	0	5
	0	0	0	0	0	0	0	0	0	0	1
S-56	±	1	2	2	2	1	±	0	0	0	10
	2	2	1	0	0	0	0	0	0	0	5
	0	0	0	0	0	0	0	0	0	0	1
S-9	2	2	2	1	±	0	0	0	0	0	10
	1	1	±	0	0	0	0	0	0	0	5
	±	0	0	0	0	0	0	0	0	0	1

^a CP, Patient isolates; CS, soil isolates; S, soil isolates.

^b Results were recorded as 0 to 4+ agglutination.

^c Carbohydrate concentration was determined by the Molisch method.

TABLE 3. Comparison of cryptococcal latex agglutination reactions obtained with locally produced and commercial reagents

Yeast	Latex agglutination titer ^b	
	VAH/OCO	Commercial
<i>C. neoformans</i> ^a		
A	32	16
B	64	32
C	16	16
D	64	32
CP-114	64	64
CP-117	32	32
CP-130	64	64
CS-26	128	64
CS-19	128	32
CS-20	128	64
W.C.	1,024	512
N.R.	128	64
J.T.	8	8
	2	2
<i>C. laurentii</i>	2	2
(10 isolates)	(1 isolate)	(2 isolates)

^a CP, Patient isolates; CS, soil isolates; W.C., N.R., J.T., new patient isolates.

^b Carbohydrate concentration was 5 μ g/ml. VAH/OCO, Veterans Administration Hospital, Oklahoma City, Oklahoma.

this rapid identification method by both our laboratory's reagent and the commercial reagent. These yeasts were subsequently confirmed as *C. neoformans* by conventional procedures.

DISCUSSION

Although many media have been developed to streamline the primary isolation of cryptococci from clinical samples, no methods have been developed to provide early serological identification of *C. neoformans*. Conventional definitive identification of *C. neoformans* utilizes demonstration of mouse virulence and carbon and nitrate assimilations. These procedures are both cumbersome and time consuming.

This study reports a rapid serological method for the presumptive identification of *C. neoformans*. This serological test appears entirely specific when the yeast colony extracts are prepared so that the carbohydrate concentrations are 5 μ g/ml or less. As seen in Table 1, some extracts from *C. laurentii* and *Candida* spp. showed weak agglutination at concentrations of 10 μ g/ml. However, the degree of agglutination even at this higher concentration was low, never reaching 3+ to 4+ as did the *C. neoformans* extracts (Table 2). Possible cross-reactions were

avoided by using a carbohydrate extract containing less than 5 μ g/ml. Extracts from yeasts other than *C. neoformans* did not react at these concentrations (Table 1, 1 and 5 μ g/ml).

In the rapid identification procedure, a standard extract (0.001 ml of yeast in 6 ml of phenolized saline) containing 1 to 5 μ g of carbohydrate per ml was prepared from 104 yeast isolates (including 54 *C. neoformans*). Non-encapsulated isolates yielded the lower amounts (1 μ g/ml) of carbohydrate. This extract was tested for *C. neoformans* antigen by the Bloomfield et al. (1) latex agglutination test. All *C. neoformans* tested gave strong agglutinations (3+ to 4+), whereas none of the other yeasts gave strong reactions. This method required 20 min to perform, exclusive of the Molisch carbohydrate determination, and provided a simple, rapid, and accurate means of identifying *C. neoformans* yeast colonies isolated from clinical, soil, or other specimens.

The cryptococcal material, which reacts with the latex-globulin reagent and which may be found in CSF, serum, etc. of patients with cryptococcosis, is a soluble, haptenic polysaccharide derived from the capsule of *C. neoformans*. The latex-globulin reagent prepared in this laboratory showed a 2+ agglutination reaction with purified cryptococcal capsular polysaccharide in concentrations as low as 0.03 μ g/ml.

Preparation of the latex-globulin reagent requires techniques (animal immunization, protein fractionation, etc.) not available to most clinical microbiology laboratories. However, a commercial cryptococcal latex kit provides any clinical laboratory with a valuable diagnostic tool, both for the detection of circulating cryptococcal polysaccharide antigen in CSF or serum and for the rapid presumptive identification of a yeast isolate as *C. neoformans*, as described in this paper. For early additional identification of this organism, we recommend that one of the presently available differential media (3, 9, 10) be used. For therapeutic decisions, however, we believe the accuracy of the rapid procedure described in this paper has been sufficiently established. Even though we found not one false reaction, we recommend that the results of this test be verified by conventional culture methods (e.g., ref. 8) until additional reports confirm the reliability of this test.

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