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Evaluation of a New Method for Identification of *Cryptococcus neoformans* which Uses Serologic Tests Aided by Selected Biological Tests

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A new method for identifying *Cryptococcus neoformans* isolates and their serotypes by the slide agglutination test using five kinds of factor sera, with the aid of nitrate reduction, phenol oxidase, and growth at 37°C tests was evaluated by using 36 reference strains and 75 clinical isolates of *C. neoformans*. The results showed that the reference strains were identified exactly as they were labeled, and clinical isolates were identified as *C. neoformans* serotypes A, D, and AD. *C. neoformans* could be distinguished from other *Cryptococcus* species that cross-reacted with factor sera by their ability to grow at 37°C. These results indicate that the slide agglutination test combined the use of factor sera for isolates which grow at 37°C is a useful method for identification of *C. neoformans* and their serotypes and that the nitrate reduction test (negative in 100% of the isolates) and the phenol oxidase test (positive in approximately 95% of the isolates) can be used to confirm that the species is *C. neoformans*.

Cryptococcus neoformans is a frequent cause of invasive fungal disease in immunocompromised hosts (1). Although the detection of antigens or antibodies in body fluids in addition to clinical findings provides an important clue for diagnosis of cryptococcosis (6), isolation and identification of the causative yeasts are also desirable for diagnosis of the disease. It has become apparent that there are two variants and five serotypes of *C. neoformans*; *C. neoformans* var. *neoformans* is made up of serotypes A, D, and AD, and *C. neoformans* var. *gattii* is made up of serotypes B and C (7). Although the varieties can be distinguished by a medium containing canavanine, glycine, and bromthymol blue (8), their serotypes are indistinguishable by currently available methods. Ikeda et al. (5) established an antigenic formula consisting of eight antigenic factors for five serotypes of *C. neoformans* and prepared factor sera on the basis of the antigenic formula for the slide agglutination test. To date, however, evaluation of such factor sera for application in routine laboratory work identifying the *C. neoformans* isolates and their serotypes has not been attempted. To develop a serologic test for practical application, we established a new system for identification of *C. neoformans* and its serotypes by the slide agglutination test using five kinds of factor sera with the aid of a few relevant biochemical tests.

Cultures of *Cryptococcus* species and two *Candida* species which are closely related antigenically to *Cryptococcus* species were obtained from the American Type Culture Collection, National Institutes of Health, Centers for Disease Control, and Centraal Bureau voor Shimmelcultures. A total of 75 new isolates of *C. neoformans* from Japan were used for evaluation of the identification system. Each strain was grown on Sabouraud dextrose (2%) agar at 27°C for 48 h. Male New Zealand White rabbits were immunized with heat-killed cells or cells conjugated with bovine gamma globulin (Sigma Chemical Co., St. Louis, Mo.) (5). The antiserum (2 ml) was adsorbed with packed wet cells (1 ml) at 37°C for 2 h, left overnight at 4°C (13), and tested for antibody by the slide agglutination test (5), and then the degree (+, ++, +++) of agglutination was recorded (Fig. 1).

The identity of all the reference strains listed in Table 2 was reconfirmed on the basis of their biochemical characteristics, such as the assimilation of 12 sugars, urease test, nitrate reduction test, and the ability to grow at 37°C (11). Phenol oxidase activity was determined by a modified version of the method of Hopfer and Gröschel (4) by using DL-3,4-dihydroxylphenylalanine (DL-DOPA) as the substrate; 10 mM DL-DOPA in 100 mM sodium citrate (pH 6.0) was applied to disks (150 μl per disk).

Five kinds of factor sera were prepared on the basis of the antigenic formula (5). The adsorption systems necessary for preparation of the factor sera are shown in Table 1. The specificity of the factor sera was determined by the use of stock reference strains (Table 2). All 36 strains of *C. neoformans* were clearly divided into five serotypes, although *Cryptococcus* species other than *C. neoformans* also reacted with factor sera in various patterns. Although two *Candida* species which are positive for urease activity also reacted with the factor sera, strains of *Candida albicans* as well as

TABLE 1. Preparation of factor sera for serotyping *C. neoformans*[a]

Factor serum	Antiserum to serotype (antigenic pattern)	Adsorption with serotype (antigenic pattern)
1	C (1, 4, 6)	None
5	B (1, 2, 4, 5)	A (1, 2, 3, 7) + C (1, 4, 6)
6	C (1, 4, 6)	B (1, 2, 4, 5)
7	A (1, 2, 3, 7)	D (1, 2, 3, 8)
8	D (1, 2, 3, 8)	A (1, 2, 3, 7)

[a] Strains CDC551, NIH 112, NIH 18 and NIH 52 were used as serotype A, B, C, and D strains, respectively.

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TABLE 2. Serologic and biological characteristics of authentic strains of *C. neoformans* and related species

Species	Strain	Agglutination with the following factor serum[a]:					Phenol oxidase[b]	Nitrate reduction	Growth at 37°C	Test result	
		1	5	6	7	8				Species	Serotype
Cryptococcus species											
C. neoformans	ATCC 24064	+++	−	−	+++	−	+++	−	+	*C. neoformans*	A
	ATCC 2526	+++	−	−	+++	−	+	−	+	*C. neoformans*	A
	ATCC 10226	+++	−	−	+++	−	++	−	+	*C. neoformans*	A
	ATCC 13690	+++	−	−	+++	−	+++	−	+	*C. neoformans*	A
	ATCC 14115	+++	−	−	+++	−	+++	−	+	*C. neoformans*	A
	ATCC 28205	+++	−	−	+++	−	+++	−	+	*C. neoformans*	A
	ATCC 28737	+++	−	−	+++	−	+++	−	+	*C. neoformans*	A
	ATCC 28738	+++	−	−	+++	−	+++	−	+	*C. neoformans*	A
	ATCC 24065	+++	+++	−	−	−	−	−	+	*C. neoformans*	B
	ATCC 4414	+++	+++	−	−	−	−	−	+	*C. neoformans*	B
	ATCC 24066	+++	−	+++	−	−	++	−	+	*C. neoformans*	C
	ATCC 24067	+++	−	−	−	+++	++	−	+	*C. neoformans*	D
	CDC312	++	−	++	−	−	+	−	++	*C. neoformans*	C
	CDC551	++	−	−	++	−	−	−	++	*C. neoformans*	A
	CDCB3171	++	++	−	−	−	++	−	++	*C. neoformans*	B
	CDCB3172	++	++	−	−	−	−	−	++	*C. neoformans*	B
	CDCB3173	+++	++	−	−	−	−	−	++	*C. neoformans*	B
	CDCB3174	+++	++	−	−	−	+	−	++	*C. neoformans*	B
	CDCB3175	++	++	−	−	−	+	−	++	*C. neoformans*	B
	CDCB3181	++	−	++	−	−	±	−	++	*C. neoformans*	C
	CDCB3182	++	−	++	−	−	−	−	++	*C. neoformans*	C
	CDCB3183	++	−	++	−	−	−	−	++	*C. neoformans*	C
	CDCB3184	++	−	++	−	−	+	−	++	*C. neoformans*	C
	CDCB3185	++	−	++	−	−	−	−	++	*C. neoformans*	C
	CDCB3186	++	−	++	−	−	−	−	++	*C. neoformans*	C
	CDCB3187	++	−	++	−	−	+	−	++	*C. neoformans*	C
	CDCB3501	++	−	−	−	+	+++	−	++	*C. neoformans*	D
	CDCB3502	++	−	−	−	+	+	−	++	*C. neoformans*	D
	NIH 112	++	++	−	−	−	−	−	++	*C. neoformans*	B
	NIH 444	++	+	−	−	−	+++	−	++	*C. neoformans*	B
	NIH 18	+	−	++	−	−	+	−	++	*C. neoformans*	C
	NIH 119	++	−	++	−	−	−	−	++	*C. neoformans*	C
	NIH 312	++	−	++	−	−	+	−	+	*C. neoformans*	C
	NIH 52	++	−	−	−	+	+	−	++	*C. neoformans*	D
	NIH 68	++	−	−	++	+	+++	−	+	*C. neoformans*	AD
	CBS 132	++	−	−	+	+	+++	−	++	*C. neoformans*	AD
C. albidus var. *aerius*	ATCC 10665	+++	−	+++	−	−	−	+	−	Unknown	Untypeable
C. albidus var. *albidus*	ATCC 10666	+++	−	−	−	−	−	+++	−	Unknown	Untypeable
C. albidus var. *albidus*	ATCC 22460	+++	−	−	+++	−	−	+++	−	Unknown	A
C. albidus var. *diffluens*	ATCC 12307	+++	−	−	−	−	−	+++	−	Unknown	Untypeable
C. diffluens var. *uruguaiensis*	ATCC 24612	+++	−	−	−	−	−	+++	−	Unknown	Untypeable
C. ater	ATCC 14247	−	−	−	−	−	−	++	−	Unknown	Untypeable
C. gastricus	ATCC 24225	−	−	−	−	−	−	−	−	Unknown	Untypeable
C. kuetzingii	ATCC 22025	+++	−	−	−	−	−	+++	−	Unknown	Untypeable
C. laurentii	ATCC 18803	+++	−	++	−	−	−	−	−	Unknown	Untypeable
C. luteolus	ATCC 32044	++	−	−	−	−	−	−	−	Unknown	Untypeable
C. uniguttulatus	ATCC 24227	−	−	−	−	−	−	−	−	Unknown	Untypeable
	ATCC 32047	−	−	−	−	−	−	−	−	Unknown	Untypeable
	ATCC 32048	+	−	−	−	−	−	−	−	Unknown	Untypeable
Candida species											
C. curvata	ATCC 10567	+++	−	−	−	−	−	−	−	Unknown	Untypeable
C. humicola	ATCC 9949	+++	−	−	−	−	−	−	−	Unknown	Untypeable
C. albicans	ATCC 10259	−	−	−	−	−	−	−	+	Unknown	Untypeable

[a] Symbols, −, +, ++, and +++, refer to Fig. 1.
[b] DL-DOPA was used as a substrate.

TABLE 3. Identification of clinical and environmental isolates by the new system

No. of isolates	Agglutination with the following factor serum:					Phenol oxidase	Nitrate reduction	Growth at 37°C	Test result	
	1	5	6	7	8				Species	Serotype
55	++	−	−	+	−	+	−	+	*C. neoformans*	A
2	++	−	−	+	−	−	−	+	*C. neoformans*	A
8	++	−	−	+	+	+	−	+	*C. neoformans*	AD
10	++	−	−	−	+	+	−	+	*C. neoformans*	D

other medically important *Candida* species did not show positive reactions (data not shown).

To supplement the serologic test for differentiating *C. neoformans* from other *Cryptococcus* species, selected biological and biochemical characteristics of these species were examined (Table 2). All 36 strains of *C. neoformans* showed characteristics of *C. neoformans* in the 37°C growth test and the nitrate reduction test. However, in the phenol oxidase test 11 strains (30%) were negative. On the other hand, although *Cryptococcus* species other than *C. neoformans* exhibited variable results by the nitrate reduction test, they could be simply distinguished from *C. neoformans* by their ability to grow at 37°C. These results suggest that the combined use of the factor sera and the 37°C growth test is the most reliable procedure.

A total of 75 new strains of *C. neoformans* which had been isolated from clinical and environmental materials and identified as *C. neoformans* by conventional methods were tested by a new method using factor sera in combination with growth at 37°C, phenol oxidase, and nitrate reduction tests (Table 3). The results showed that the 75 isolates were identified as *C. neoformans* serotype A (76%), D (13%), and AD (11%). All the isolates exhibited *C. neoformans* characteristics in the nitrate reduction and 37°C growth tests, while two isolates of serotype A (4%) were negative in the phenol oxidase test. These results indicate that a serologic test with five factor sera is a simple and practical method for identifying clinical isolates of *C. neoformans* as well as their serotypes, and two biochemical tests (phenol oxidase test and nitrate reduction test) can be used for the confirmation of the above test, if necessary.

The present study was undertaken to apply the factor sera to identification of *C. neoformans* along with selected biological procedures. Although the antigenic formula for five serotypes consists of eight antigenic factors (5), we found that the use of only five factor sera (factor sera 1, 5, 6, 7, and 8) is satisfactory for routine use. Although, to our knowledge, there may not be a correlation between serotype and virulence in *C. neoformans*, serotyping of *C. neoformans* isolates may have epidemiological significance in the clinical investigation.

The characteristics of *C. neoformans* that differentiate it from other yeast species in clinical specimens are its polysaccharide capsule and urease activity (2). However, isolates lacking a capsule or urease activity have been reported (12). Although melanin formation is reported to be a characteristic of *C. neoformans*, we found some phenol oxidase-negative *C. neoformans* isolates, even though several substrates which are known to produce melanin by the action of the enzyme were used (data not shown). However, it seems that there may not be a correlation between the lack of phenol oxidase activity and the serotype.

The ability of *C. neoformans* isolates to grow at 37°C (11) was found to be a characteristic property for differentiating *C. neoformans* isolates from other nonpathogenic *Cryptococcus* species in this system. Although some isolates of *Cryptococcus albidus* are reportedly able to grow at 37°C (9) and to show the same agglutinating pattern as that of *C. neoformans* serotype A (Table 2), the nitrate reduction test can be used for differentiating *C. albidus* from *C. neoformans* (Table 2).

Several systems based on biological and biochemical characteristics of the yeasts are available commercially for the identification of clinical isolates (3, 10). Our system reported here has an advantage over these systems in serotyping ability. Moreover, some isolates which had been placed in the category of "unknown" by the API 20C system could be identified as *C. neoformans* and to the serotype level by our system (data not shown).

In conclusion, a set of five kinds of factor sera could be useful for a kit that can be used for simple identification of isolates of *C. neoformans* and their serotypes with the aid of selected biochemical tests.

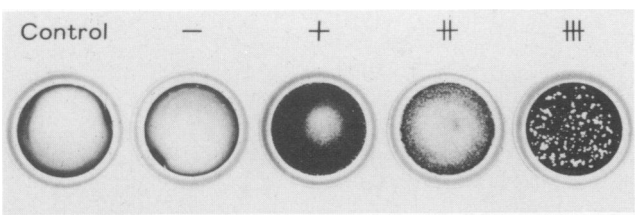

FIG. 1. Characteristics and degree of slide agglutination of *C. neoformans* with factor serum.

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