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Serotyping of *Cryptococcus neoformans* by Dot Enzyme Assay

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A method is described for the serotyping of *Cryptococcus neoformans* based on direct analysis of culture supernatants for the major type-specific capsular antigen, glucuronoxylomannan. Factor sera prepared by absorption of polyclonal rabbit antisera (Iatron Laboratories, Inc., Tokyo, Japan) or selected anti-*C. neoformans* monoclonal antibodies were used in a dot enzyme assay to detect the presence of antigen.

The major capsular polysaccharide, glucuronoxylomannan (GXM), is the basis for defining the serotypes of *Cryptococcus neoformans* (4, 27–29). Anticapsular antibodies produced in rabbits immunized with killed whole cells were originally used to identify five serotypes (A, B, C, D, and A/D) (17, 30). Detailed structural analyses found that the observed serological specificities correlate with the structures determined for GXM derived from isolates of each serotype (1, 3, 9, 27–29). Subsequent studies have identified important epidemiological and clinical features of *C. neoformans* associated with capsular serotype (5, 11, 20, 21, 25). A major impediment to more widespread study of the role of serotype in the epidemiology and pathogenesis of cryptococcosis has been the lack of an assay that can identify the serotype of numerous isolates at a reasonable cost. Alternative means for serotyping new isolates include the use of a combination of selective media and differential staining of yeast cells by indirect immunofluorescence (13). Other investigators have examined genetic variations between serotypes and between isolates within a given serotype by using molecular methods such as karyotyping (23), DNA probes (24), restriction fragment length polymorphism (7, 12, 14), amplification of polymorphic DNA patterns by PCR (22), and multilocus enzyme electrophoresis (6). Despite the introduction of alternative techniques, no standardized method for serotyping of isolates has been adopted.

Our approach to determining serotype is based on a scheme of antigenic factors originally described by Ikeda et al. (18, 19). A panel of eight reciprocally absorbed antisera (factor sera) was used to define the distribution of eight putative antigenic factors among the cryptococcal serotypes (18, 19). The eight antigenic factors are either unique to a particular serotype (factor 5, B; factor 6, C; factor 7, A; factor 8, D) or shared by two or more serotypes (factor 1, A, B, C, and D; factor 2, A, B, and D; factor 3, A and D; factor 4, B and C) (18, 19). Polyclonal factor sera are available from a commercial source (Iatron Laboratories, Inc., Tokyo, Japan). More recently, monoclonal antibodies (MAbs) that have specificities similar to those of several factor sera have been described (MAb 439 ≈ factor 1; MAb 1255 ≈ factor 2; MAb 302 ≈ factor 3) (16, 26). In this paper, we describe an assay for the determination of cryptococcal serotype. The method uses small amounts of MAbs or commercially available antisera, thus making large-scale serotyping possible.

The isolates of *C. neoformans* used in this study are listed in Table 1. Factor sera (lot 910610; Iatron Laboratories, Inc.) were used as provided. MAbs 302, 1255, and 439 were described previously (16, 26). Checkerboard titrations with dilutions of antigen in twofold increments from 0.25 to 4.0 mg/ml and with factor sera in twofold increments from 1:20 to 1:160 (factor sera 7 and 8) or 1:100 to 1:800 (factor sera 1, 5, and 6) were used to determine the optimum conditions for the dot enzyme assay (DEA) experiments. MAbs (2 mg/ml) were titrated in twofold increments from 1:25 to 1:1,000 to determine the optimal concentrations for the DEA experiments. *C. neoformans* isolates were grown for 4 days at 37°C in 10 ml of a chemically defined medium containing 2% glucose (8). The cultures were autoclaved (15 min at 121°C), and the supernatants obtained after centrifugation were reserved. The culture supernatants were used routinely for DEA. Each antigen was diluted 1:10 in 0.06 M sodium carbonate buffer, pH 9.6, and then 1 μl of supernatant was spotted immediately on rectangular (0.5 by 5.0 cm) nitrocellulose membrane strips (Schleicher and Schuell) at 1-cm intervals. The strips were dried for 1 h at 24°C. Antigen-coated test strips were analyzed by a modification of a DEA method described previously (2). Briefly, nitrocellulose test strips were incubated for 1 h at 24°C with dilutions of factor serum or MAb in 1 ml of 0.01 M Na_2PO_4–0.14 M NaCl (pH 7.2) (phosphate-buffered saline) with 0.05% Tween 20 (PBS-T). Positive and negative control sera and control strips containing reference GXM were analyzed concurrently. The strips were then washed three times with PBS-T. After the final wash, the strips were incubated with 1 ml of a 1:1,000 dilution of peroxidase-labeled goat anti-rabbit immunoglobulin G (whole molecule) for factor sera or peroxidase-labeled goat anti-mouse immunoglobulin G (whole molecule) for MAbs. After incubation for 30 min at 4°C, the strips were

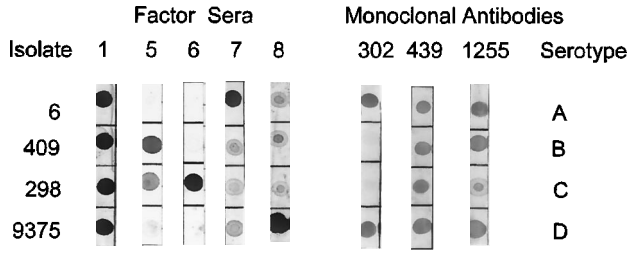

FIG. 1. DEA with serotype-specific factor sera or anti-*C. neoformans* MAbs against reference GXMs. Factor sera 1, 5, and 6 were used at a dilution of 1:200; factor sera 7 and 8 were used at a dilution of 1:100. The MAbs and concentrations used are as follows: 302 and 1255, 40 μg/ml; 439, 50 μg/ml. The DEA procedures were performed as described in the text.

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TABLE 1. Sources and serotyping of *C. neoformans* isolates

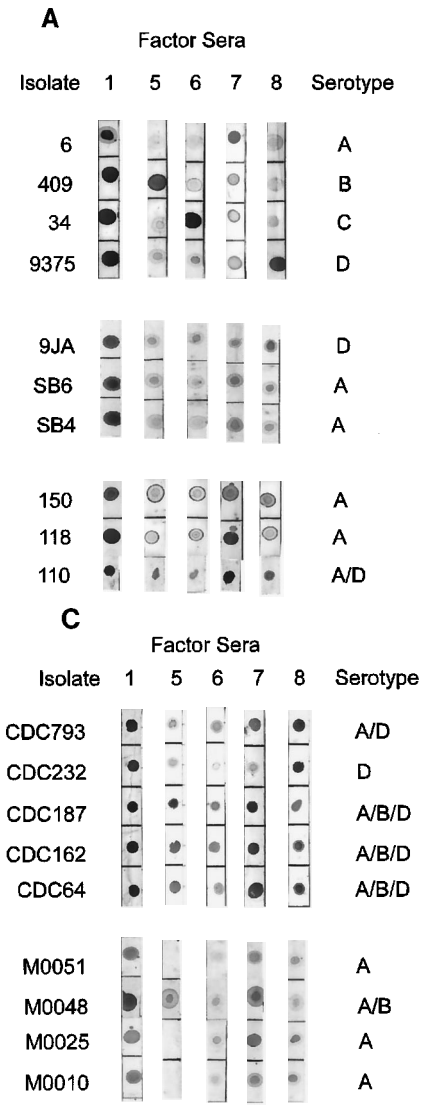
DEA isolate	Source	Serotype as determined by:		
		Source[a]	Filtrate	GXM[b]
6	T. G. Mitchell (Duke University)	A	A	A
SB6	A. Casadevall (N.Y.)	A	A	A
SB4	A. Casadevall	A	A	A
150	T. G. Mitchell	A	A	A
118	T. G. Mitchell	A	A	A
110	T. G. Mitchell	A	A/D	A/D
M00-51	T. G. Mitchell	A	A	A
M00-48	T. G. Mitchell	B/C	A/B	A/B
M00-25	T. G. Mitchell	A	A	A
M00-10	T. G. Mitchell	A	A	A
CDC-92-37	E. Brandt (ET3) (CDC)	A	A	A
CDC-92-187	E. Brandt (ET25)	A	A/B/D	A/B/D
CDC-92-162	E. Brandt (ET4)	A	A/B/D	A/B/D
CDC-92-64	E. Brandt (ET3)	A	A/B/D	A/B/D
4538 (H99)	J. Perfect (Duke University)	A	A	A
ATCC 32719	ATCC[c]	A	A/D	ND[d]
184[e]	J. W. Murphy (University of Oklahoma)	A	A	ND
F117	J. W. Murphy	A	A	ND
ATCC 6352	ATCC	A	A	ND
371	K. J. Kwon-Chung (NIH[f])	A	ND	A/D
9759	E. Reiss (CDC)	A	A	A
M00-21	T. G. Mitchell	A	A	A
M00-46	T. G. Mitchell	A	ND	A/B/D
M00-53	T. G. Mitchell	A	ND	A
M00-66	T. G. Mitchell	A	A	A
CBS 132	T. Shinoda (Japan)	A/D	A/D	A/D
CDC-92-793	E. Brandt (ET21)	A/D	A/D	A/D
409	K. J. Kwon-Chung	B	B	B
3939	E. Reiss	B	B	B
ATCC 34848	ATCC	B	B	ND
ATCC 32269	ATCC	B	B	ND
O869	J. W. Murphy	B	B	ND
184[e]	K. J. Kwon-Chung	B	B	B
ATCC 32269	ATCC	B	ND	B
34	K. J. Kwon-Chung	C	C	C
401	K. J. Kwon-Chung	C	C	C
298	K. J. Kwon-Chung	C	C	C
ATCC 24066	ATCC	C	B/C	B/C
9375	J. Shadomy (VA Med.)	D	D	D
9JA	A. Casadevall	D	D	D
CDC-92-232	E. Brandt (ET8)	D	D	D
433	K. J. Kwon-Chung	D	D	ND
127	E. S. Jacobson (VA Med.)	D	D	D
12	K. J. Kwon-Chung	D	D	D
3501	K. J. Kwon-Chung	D	ND	D
B3502	K. J. Kwon-Chung	D	ND	D
J11	A. Casadevall	D	ND	D

[a] Serotype as provided by the submitter of the culture.
[b] Purified GXMs were available from previous studies (1, 8–10, 29).
[c] ATCC, American Type Culture Collection.
[d] ND, not done.
[e] Provided as *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* by J. W. Murphy and K. J. Kwon-Chung, respectively.
[f] NIH, National Institutes of Health.

washed three times with PBS-T and once with PBS. Finally, the strips were incubated for 15 min at 24°C with freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml) in PBS containing 0.006% hydrogen peroxide. The strips were washed four times with deionized water and dried.

In the event that equivocal results were produced by the analysis of culture filtrates, additional confirmatory assays were done with selected isolates by using antigen concentrated from culture filtrates by ultrafiltration or by precipitation with 3 volumes of ethanol. Total carbohydrate was determined for each concentrated sample by the phenol-sulfuric acid procedure (15). Antigen concentration was adjusted to 1 mg/ml for spotting nitrocellulose test strips.

Factor serum 1, which reacts with all serotypes, and factor sera 5, 6, 7, and 8, which react specifically with serotypes B, C, A, and D, respectively, were used as standard reagents for serotyping of *C. neoformans*. In addition, MAbs 302 (A and D specific), 1255 (A, B, and D specific), and 439 (panspecific) were used. A control experiment was done initially with purified reference GXMs (1, 8–10, 29) representative of the four serotypes as the test antigens (Fig. 1). "Reference" refers to isolates whose GXMs have molar ratios that approximate



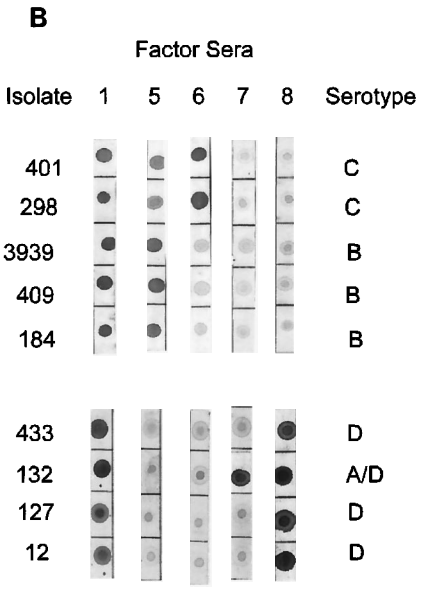


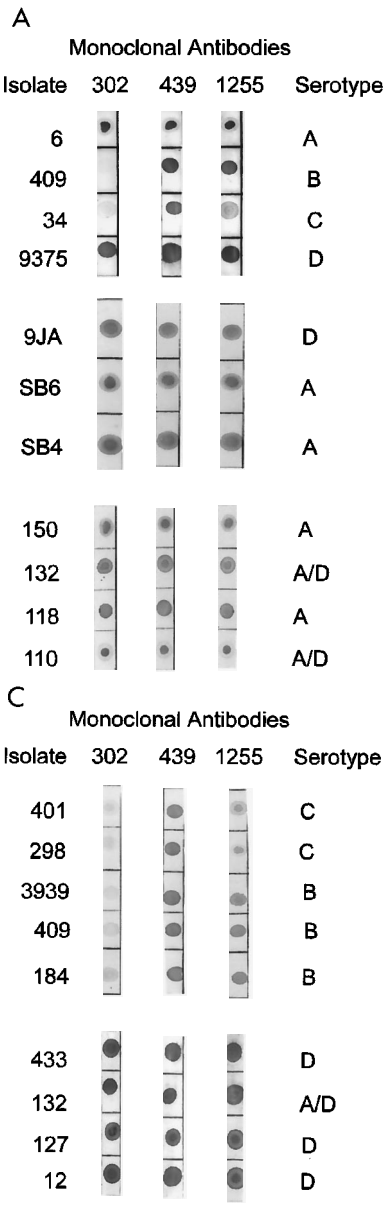
FIG. 2. DEA with factor sera against culture supernatants from selected isolates of *C. neoformans*. The appropriate dilution of each factor serum described in the legend to Fig. 1 was added to strips spotted with the diluted or concentrated culture supernatant of each isolate. The DEA procedures were performed as described in the text.

those suggested for serotypes A, B, C, and D by Bhattacharjee et al. (4). Figure 1 illustrates the idealized DEA reaction pattern if no heterogeneity exists within the previously defined serotypes. Next, DEA serotyping using culture filtrates was evaluated in a study of a group of chemically and serologically defined isolates of *C. neoformans* (Table 1). The same procedure was also done with a panel of purified GXMs available from our previous studies (Table 1). Culture supernatants were reactive to factor serum 1 (Fig. 2). The results obtained with factor sera 5 and 6 were used to differentiate serotypes B and C (Fig. 2A and B). The serotype C isolate 34 gave a strong spot only with factor serum 6, whereas serotype C isolates 298 and 401 gave light spots with factor 5 in addition to the strong spot with factor serum 6. This is due to a near absence of serotype B epitopes in isolate 34 and the presence of detectable serotype B epitopes in isolates 298 and 401 (1, 10).

Isolates 3939, 409, and 184 (Fig. 2B) gave strong spots with factor serum 5 and gave no spot with factor serum 6. These isolates are assigned to serotype B. The results for other culture supernatants that were typed as serotype B are given in Table 1. Isolates of serotype B and serotype C showed no reactivity to factor serum 7 or factor serum 8. Factor serum 7 was the weakest of the reagents used in this study. This resulted in marginal staining with some isolates. Some isolates that were factor 7 or factor 8 positive were found to give low-intensity dots with factors 5 and/or 6. This was particularly true for several Centers for Disease Control and Prevention (CDC) isolates from enzyme type (ET) groups 3 and 4 (6). CDC-92-793 (ET21) reacted to both factor sera 7 and 8 with the same intensity, showing that it is of serotype A/D. Isolates CDC-92-162 (ET4), CDC-92-187 (ET25), and CDC-92-64 (ET3) also gave positive dots with factor sera 5, 7, and 8. However, here the intensity of the dot with factor 5 or 8 was less than the intensity observed with factor 7, and the background with factor serum 6 was higher than the normally observed negative threshold. These isolates were assigned to serotype A/B/D. CDC-92-232 (ET8) was reactive to factor serum 8; therefore, this isolate is of serotype D. Representative results are shown in Fig. 2C and Table 1. These serotype assignments were not always consistent with those made by multilocus enzyme typing (6). The DEA results with the CDC group of isolates correlate with the heterogeneity of their structures observed by ^{1}H nuclear magnetic resonance spectroscopic analysis (unpublished observations).

Serotype D and A/D isolates tend to produce less capsule; this was particularly evident for serotype A/D isolate CBS 132 (Fig. 2B). Consequently, lyophilized retentates obtained by ultrafiltration of the culture supernatants were used to prepare confirmatory test strips (Table 1). Isolates 433, 127, and 12 gave spots with factor serum 8 (Fig. 2B). These isolates were assigned to serotype D. Other GXMs identified as serotype D are identified in Table 1.

Isolates M00-51, M00-25, and M00-10 were assigned to serotype A on the basis of the formation of spots with factor serum 7 (Fig. 2C). Isolate M00-48 also showed a low-intensity spot with factor serum 5; this isolate was assigned to serotype A/B. Results with other M00-purified GXMs are given in Table 1. The serotypes of these isolates were provided to us after the study was completed.



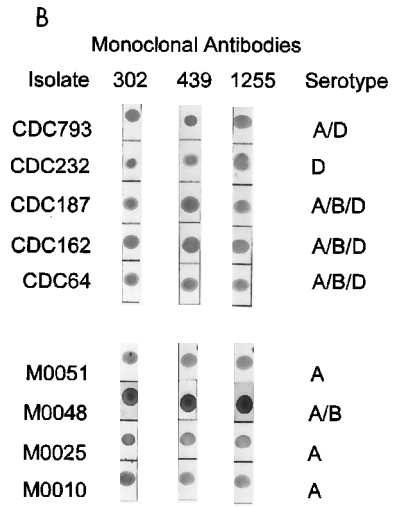


FIG. 3. DEA with anti-*C. neoformans* MAbs against culture supernatants from all isolates. The appropriate dilution of each MAb described in the legend to Fig. 1 was added to strips spotted with the diluted or concentrated culture supernatant of each isolate. The DEA procedures were performed as described in the text. *C. neoformans* var. *neoformans* cannot be subdivided into serotype A and D with available MAbs. The serotype A and D designations are taken from Fig. 2.

A demonstration study was done with antigen prepared by washing *C. neoformans* colonies cultured on Sabouraud dextrose agar plates. Cell suspensions were prepared by washing the plates with sterile saline (10 ml). The suspensions were autoclaved and centrifuged, and the supernatants were analyzed by DEA as described above. The same conclusions and serotype assignments were made when colony washes from isolates CDC-92-232, M00-51, 110, SB4, 9JA, 401, 298, 3939, and 409 were used as the source of the antigen (data not shown). This shows that it is practical to substitute colony washes if culture filtrates are not immediately available.

The DEA serotyping analysis was repeated with a selected set of MAbs and the same series of antigens (Fig. 3). The results with the MAbs were more uniform and gave data that were easier to interpret, compared with the factor sera results. Unfortunately, serotype-specific MAbs have not been attainable. With a combination of MAbs it is possible to differentiate *Cryptococcus neoformans* var. *gattii* and *C. neoformans* var. *neoformans*, since MAb 302 is A and D specific. *C. neoformans* var. *gattii* can be subdivided into serotypes B and C by comparing the specificities of MAb 439 (panspecific), MAb 302 (no reaction with B and C), and MAb 1255 (A, B, and D specific). For example, isolate 298 reacts with panspecific MAb 439 but does not react with MAbs 302 and 1255 (Fig. 3C). However, *C. neoformans* var. *neoformans* could not be subdivided with any combination of available MAbs (Fig. 3).

Our results show that isolates of *C. neoformans* can be identified, differentiated by variety, and serotyped without antigen purification. Some variation in the intensity of spots between serotypes and within isolates of each serotype was observed. These differences among isolates are due partly to the variability in the amounts of capsular polysaccharide shed into the liquid medium during growth and partly to microheterogeneity in capsule structure. In DEA, 5 to 10 μl of factor serum in 1 ml of buffer is used for a strip having five antigen spots. The Iatron factor sera are marketed for use in yeast cell agglutination assays, and the directions for the use of the factor serum indicate that 50 to 100 μl of reagent antibody is applied to whole-cell suspensions (19). In contrast, the amount of factor serum required for a single agglutination test can be used to develop 20 DEA strips, each containing five test antigen spots. DEA done with Iatron factor sera is an objective and cost-effective method for serotyping *C. neoformans* isolates. The availability of a rapid and simple method such as DEA using the Iatron reagents may facilitate more detailed characterization of *C. neoformans* isolates for clinical, epidemiological, and research investigations. This method can be implemented immediately without having to undertake the production of type-specific rabbit antisera. The epidemiological value of serotyping and subtyping remains to be determined. However, the association of various cryptococcal serotypes with specific geographic locations (20), possible variability in the virulence of different serotypes (25), the apparent disappearance of serotype B isolates from clinical isolates observed in recent years, and the significant number of serotype D isolates reported in France (14) suggest that further studies of cryptococcal serotype will contribute to our understanding of this pathogen.

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