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Multilocus Enzyme Typing of *Cryptococcus neoformans*

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Multilocus enzyme electrophoresis was adapted for subtyping *Cryptococcus neoformans*. The two cryptococcal varieties were clearly distinguishable. Isolates of the *C. neoformans* var. *neoformans* were sorted according to serotype and were sorted into four to five subtypes within each serotype. Nearly no two isolates of the *C. neoformans* var. *gattii* displayed the same enzyme electrophoretic type. This method may be a useful adjunct to current methods for classification and epidemiologic studies of cryptococci.

Cryptococcal meningitis, caused by the encapsulated yeast *Cryptococcus neoformans*, is a serious fungal disease in people with AIDS (8, 22, 32). More thorough study of the epidemiology of cryptococcosis is needed to define risk factors and to recommend appropriate prevention strategies. One obstacle to such epidemiologic studies has been the lack of sensitive and reliable methods to differentiate strains of *C. neoformans*. Serologic reactivity of the capsular polysaccharide and biochemical differences have historically been used for separating cryptococcal strains into the two varieties *neoformans* (serotypes A, D, and occasionally AD) and *gattii* (serotypes B and C) (2, 15). However, the identification of only five serotypes has limited the usefulness of capsule serology as a typing method. Several DNA-based subtyping systems, involving mitochondrial DNA probes (36), genomic DNA probes (24, 31, 37), direct DNA sequencing (3), and electrophoretic karyotyping (23, 25), have been reported. However, resolving power, discriminatory ability, and reproducibility have not consistently been reported.

Multilocus enzyme electrophoresis (MEE) has been employed in epidemiologic studies of many bacterial and fungal diseases (18, 19, 29, 38). In one previous study of cryptococci, variation among strains was seen in the electrophoretic mobilities of four enzymes (26). Our study evaluates the usefulness of MEE as a tool for subtyping *C. neoformans*.

(This work was presented in part at the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy, 1992 [2b].)

Thirty-four cryptococcal clinical isolates from the Centers for Disease Control Mycotic Diseases Branch culture collection included all five serotypes and various geographic origins. ATCC 48184 (9) was obtained from the American Type Culture Collection, and the acapsular mutant cap 67-2 (10) was obtained from Errol Reiss, Centers for Disease Control and Prevention. The identity of all cultures as *C. neoformans* was confirmed by standard biochemical methods (1). Yeast strains were maintained at 4°C on Sabouraud's dextrose agar (Difco Laboratories, Detroit, Mich.) slants. Serotyping was performed by indirect immunofluorescence with a combination of polyclonal and monoclonal reagents (13, 34).

Each isolate was transferred from stock into 25 ml of Sabouraud's dextrose broth (Difco) in a 50-ml flask containing sterile glass beads, which was then incubated at 30°C with shaking for 48 h. Five hundred microliters of this culture was used to inoculate each of two plates of Sabouraud's dextrose agar (Difco) containing 2.9% (wt/vol) NaCl (4), which were incubated at 30°C for 48 h. Growth was harvested by adding 5 ml of sterile water to each plate and scraping the growth from both plates into a single tube, which was centrifuged at 4,000 × *g* for 10 min at 4°C. The cell pellet was washed once with 5 ml of sterile water, recentrifuged as described above, and then frozen at −80°C until use.

Cytoplasmic extracts were prepared within 2 weeks of cell harvest. One milliliter of cold breaking buffer (10 mM Tris [pH 6], 1 mM EDTA) was added to each tube containing the pellet from two NaCl plates, and cells were allowed to thaw on ice. The cell suspension was then added to fill each of two 2-ml screw-capped Eppendorf tubes that had been previously filled with 1 ml of glass beads (0.5 mm in diameter). Each tube was placed on a mini-Bead-Beater (Biospec Products, Bartlesville, Okla.) and agitated for three 2-min pulses with chilling on ice between pulses. The tubes were then centrifuged for 15 min at 4°C in an Eppendorf centrifuge. Supernatants from each culture were pooled, and aliquots were frozen in an ethanol-dry ice bath. Aliquots were stored at −80°C and were used within 2 months of preparation.

Starch gel electrophoresis was performed according to the method of Selander et al. (27), with Tris-citrate buffer at pH 8. Loading and electrophoresis conditions were as described by Selander et al. (27). Gels were stained for enzyme activities according to the method of Selander et al. (27). The 10 enzymes assayed were alcohol dehydrogenase (EC 1.1.1.1 [ADH]), glucose-6-phosphate dehydrogenase (EC 1.1.1.49 [G6D]), 6-phosphogluconate dehydrogenase (EC 1.1.1.44 [6PD]), glutamate dehydrogenase (EC 1.4.1.4 [GD2]), glutamate oxaloacetic transaminase (EC 2.6.1.1 [GOT]), malate dehydrogenase (EC 1.1.1.37 [MDH]), phosphoglucose isomerase (EC 5.3.1.9 [PGI]), phosphoglyceromutase (EC 2.7.5.1 [PGM]), phenylalanine-leucine peptidase (EC 3.4.X.X [PLp]), and leucine-alanine peptidase (EC 3.4.X.X [LAp]).

Each band of distinct gel mobility was numbered in order of increasing anodal migration. Electrophoretic mobility was determined by comparison with the mobility of a cryptococcal standard electrophoresed on the same gel. Each unique combination of electrophoretic variants was designated as an electrophoretic type (ET). Genetic diversity was determined by the method of Nei (21). Matrices of weighted distance coefficients were calculated (7), and dendrograms were

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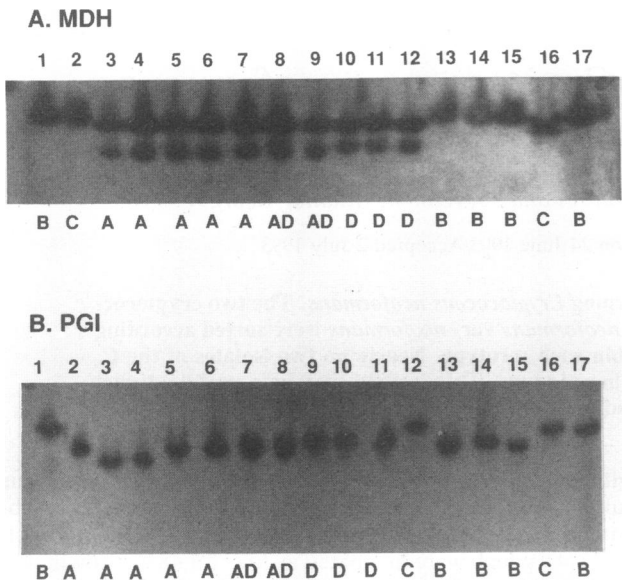

FIG. 1. Enzymatic mobility variants determined after starch gel electrophoresis of cytoplasmic extracts of *C. neoformans*. The cathode is at the top. (A) Gel stained for MDH activity. Lanes: 1, strain 4545; 2, 3181; 3, 4968; 4, 288-90; 5, MAS295; 6, MAS 7; 7, 4962; 8, ATCC 48184; 9, CBS 132; 10, 290-90; 11, 3176; 12, cap 67-2; 13, 4500; 14, 4495; 15, 4499; 16, 3184; 17, 4545 (same as lane 1). (B) PGI activity. Lanes: 1, strain 4499; 2, 4968; 3, 288-90; 4, MAS295; 5, MAS 7; 6, 4962; 7, ATCC 48184; 8, CBS132; 9, 290-90; 10, cap 67-2; 11, 3176; 12, 3181; 13, 4500; 14, 4495; 15, 4545; 16, 3184; 17, 4499 (same as lane 1). The serotype of each strain is listed underneath.

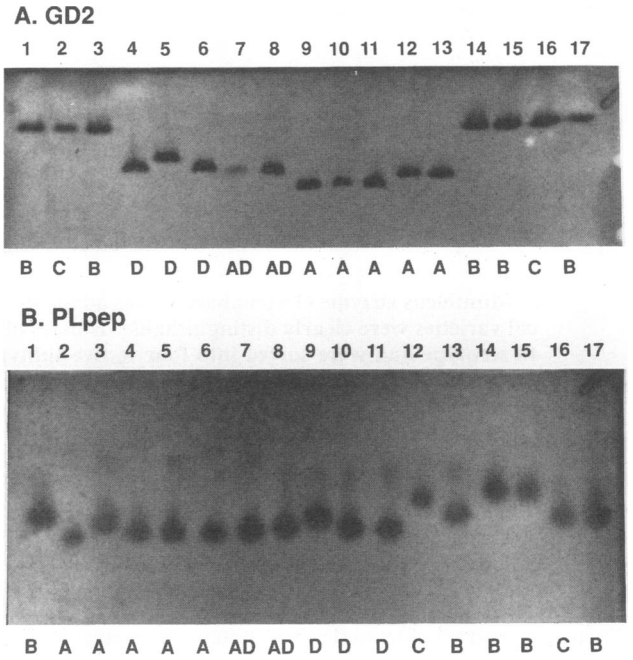

FIG. 2. Patterns of enzymatic activity after starch gel electrophoresis of *C. neoformans* extracts. (A) Gel stained for GD2 activity. Lanes: 1, strain 4499; 2, 3181; 3, 4500; 4, cap 67-2; 5, 3176; 6, 290-90; 7, CBS 132; 8, ATCC 48184; 9, 288-90; 10, MAS295; 11, MAS 7; 12, 4968; 13, 4962; 14, 4495; 15, 4545; 16, 3184; 17, 4499 (same as lane 1). (B) Phe-Leu peptidase (PLpep) activity. Lanes: 1, 4499; 2, 4968; 3, 288-90; 4, MAS295; 5, MAS 7; 6, 4962; 7, ATCC 48184; 8, CBS 132; 9, 290-90; 10, cap 67-2; 11, 3176; 12, 3181; 13, 4500; 14, 4495; 15, 4545; 16, 3184; 17, 4499 (same as lane 1).

generated from these data (28). The degree of relatedness between two strains is estimated by the relatedness index (RI) at the first node connecting the strains; the lower the RI, the more closely related are the two strains.

Representative MEE results are shown in Fig. 1 through 3 and are shown in dendrogram format in Fig. 4. Table 1 shows the ET for each isolate, and Table 2 lists the individual enzyme profile for each ET. At least one band of enzymatic activity was detected for every isolate tested. Bands that required longer than 15 min to develop were not analyzed. To assess the extent of nonspecific reactivity ("nothing dehydrogenase" [30]), a gel slice was incubated with staining reagents only. No nonspecific reactivity was detected (data not shown).

The two cryptococcal varieties could be readily distinguished from each other (Fig. 1A and 2A and Table 1) and are seen as two distinct clusters on the dendrogram (Fig. 4) separated at an RI of 0.94. Each variety displayed distinct mobility variants for the enzymes MDH, ADH, PGM, and GD2.

For the *C. neoformans* var. *neoformans*, serotype A and D isolates clustered separately on the dendrogram, separated at an RI of 0.58 (Fig. 4). The enzymes 6PD, MDH, PGI, and PGM could be used to discriminate between the two serotypes. MEE identified the acapsular mutant cap 67-2 as a serotype D strain. This strain was derived from a serotype D parent (10) and cannot be serotyped.

With the 10 enzymes used in this study, serotype A strains could be further divided into four enzyme types (Fig. 4). Addition of other enzymes did not define additional enzyme types. ET 1, the predominant subtype within the *C. neoformans* var. *neoformans*, included isolates from Atlanta, Ga.; San Francisco, Calif.; Brazil; Canada; and Zaire. When compared with ET 1, ET 2 displayed two mobility differences, ET 4 demonstrated four differences, and ET 3 displayed differences in 6 of the 10 enzymes tested. All of the

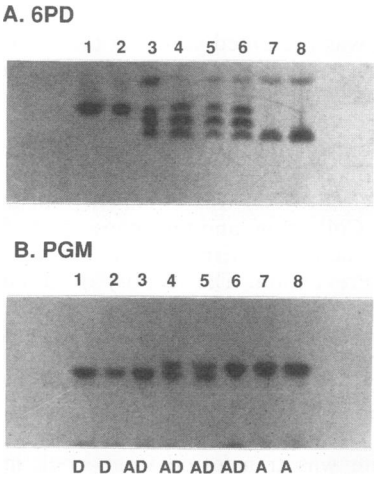

FIG. 3. Enzymatic mobility variants after starch gel electrophoresis of cytoplasmic extracts of *C. neoformans* serotype A, D, or AD. (A) Gel stained for 6PD activity. (B) Gel stained for PGM activity. Lanes: 1, strain 3176; 2, 290-90; 3, CBS 132; 4, 520-91; 5, MAS22; 6, ATCC 48184; 7, 4968; 8, 4962.

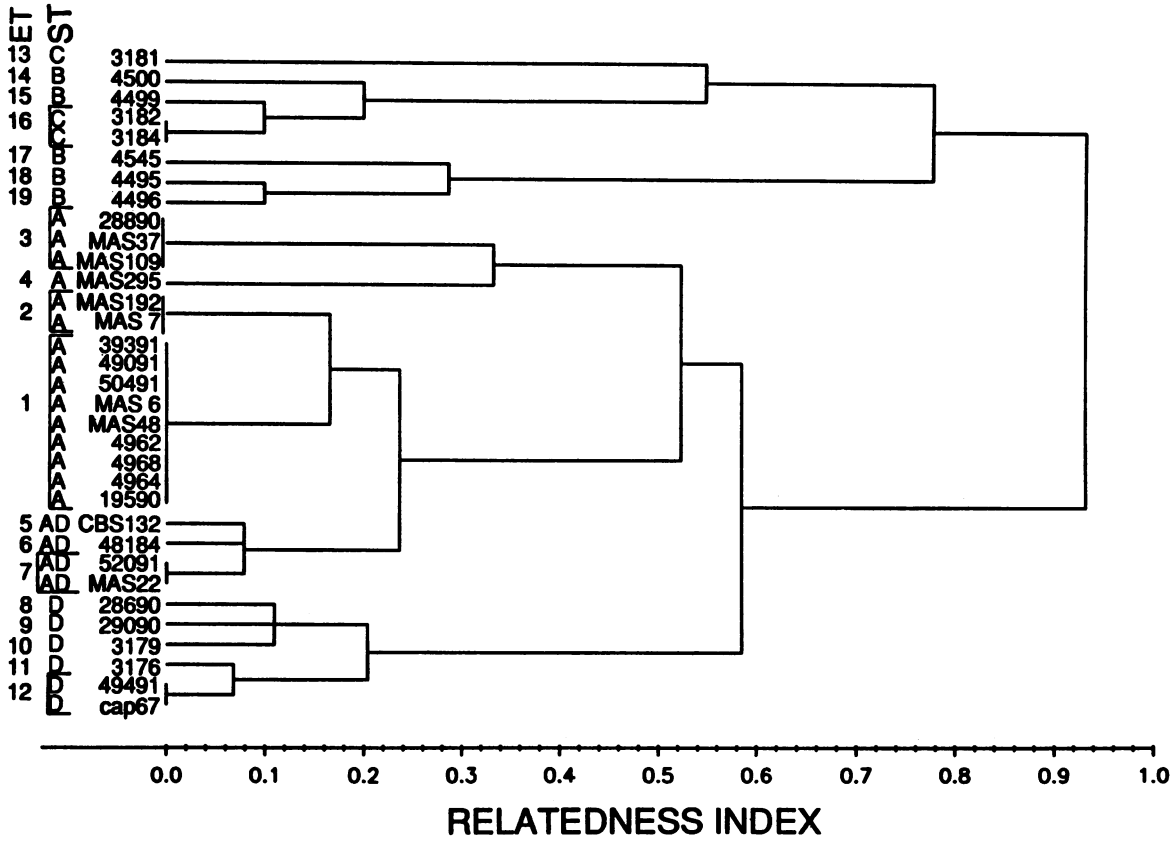

FIG. 4. Dendrogram showing strain relatedness among ETs of *C. neoformans*. ST, serotype.

serotype A strains were related to one another at an RI of 0.52. Serotype D isolates were divided into five groups, differing in mobility of GD2 (Fig. 2) or one of the two peptidases (Fig. 2). These isolates remain tightly clustered, however, displaying relatedness at an RI of 0.2.

Serotype B and C isolates demonstrated greater diversity in ET patterns. With one exception, no two among the eight *C. neoformans* var. *gattii* isolates tested displayed the same ET profile. Heterogeneity among these isolates was demonstrated for all enzymes except GD2 (Fig. 2).

Serotype AD isolates occupied a dendrogram position between those of serotypes A and D (Fig. 4). All four isolates serotyped as AD could be distinguished from those of either serotype A or D by variation in the mobility of 6PD (Fig. 3). However, in the case of PGM (Fig. 3), either one or two bands were demonstrated with serotype AD isolates. With ATCC 48184, one PGM band was found which comigrated with that of all other serotype A isolates. For the stock isolate CBS 132, which has variously been classified as serotype D (11) or AD (12), the PGM band comigrated with that of all other serotype D isolates. Two recent clinical isolates each displayed two PGM bands, one comigrating with the band found in all other serotype A isolates and the other comigrating with that common to serotype D isolates (Fig. 3). These electrophoretic patterns remained unchanged even after recloning the strains, and also after 25 passages on Sabouraud's dextrose agar. These isolates were tested for self-fertility by plating on V-8 juice agar (16), malt agar (14), or American Type Culture Collection mating medium (11). No hyphal formation was observed after 14 days of incubation for any of the strains.

Intralaboratory reproducibility was examined by performing blinded MEE on two panels of samples that had been typed repeatedly over a 6-month period. The results agreed with previous MEE results; variety and enzyme type were correctly predicted for these samples.

Biologic reproducibility was addressed by passaging five isolates (one of each serotype) 25 times on Sabouraud's dextrose agar and performing MEE on cells from every fifth passage (data not shown). No differences were seen in enzyme profiles from the 1st to the 25th passages. No enzyme bands were lost or decreased in reactivity.

A primary goal of this study was to develop a laboratory typing scheme for *C. neoformans* that is based on fundamental biologic properties of the organism, that provides greater discrimination than serotyping, and that enhances descriptive epidemiologic information. Our results show that MEE meets the first criterion, since it appears to recognize varietal and serotype differences previously described for this species (2, 15, 16). Furthermore, our results suggest that cryptococcal varieties and serotypes can be defined by electrophoretic mobilities of characteristic cellular enzymes as well as by the composition of the polysaccharide capsule. Thus, MEE may serve as a useful complement to current genetic and serologic methods for classification of cryptococci.

One purpose of this study was to determine whether serotype A *C. neoformans*, the predominant serotype in the United States (2, 15), could be further divided into epidemiologically useful groupings. The predominance of ET 1 in approximately 70% of the serotype A samples tested, as well as the repeated sampling of ET 1 from geographic regions as diverse as Africa, Brazil, and Atlanta, Ga., suggests that

TABLE 1. *C. neoformans* isolates used in this study

Strain	Serotype	ET	Origin
Y195-90	A	1	Brazil
Y289-90	A	1	Canada
B4962	A	1	Zaire
B4964	A	1	Zaire
B4968	A	1	Zaire
Y367-91	A	1	Atlanta, Ga.
Y370-91	A	1	Atlanta
Y393-91	A	1	Atlanta
Y490-91	A	1	Atlanta
Y504-91	A	1	Atlanta
MAS 92-6	A	1	San Francisco, Calif.
MAS 92-48	A	1	San Francisco
MAS 92-7	A	2	San Francisco
MAS 92-192	A	2	Atlanta
Y288-90	A	3	Canada
MAS 92-37	A	3	Atlanta
MAS 92-109	A	3	Atlanta
MAS 92-295	A	4	San Francisco
CBS 132	AD	5	ATCC 32719
48184	AD	6	American Type Culture Collection
Y520-91	AD	7	Atlanta
MAS 92-22	AD	7	San Francisco
Y286-90	D	8	Canada
Y290-90	D	9	Canada
B3179	D	10	ATCC 24067
B3176	D	11	ATCC 34875
cap67-2	D	12	United States
Y494-91	D	12	Atlanta
B3181	C	13	ATCC 34883
B4500	B	14	Australia
B4499	B	15	Australia
B3182	C	16	ATCC 34880
B3184	C	16	ATCC 32608
B4545	B	17	United States
B4495	B	18	Australia
B4496	B	19	Australia

MEE discriminates poorly among the serotype A isolates tested in this study. These same data also suggest that the *C. neoformans* var. *neoformans* possesses a clonal population structure (33) not seen in *C. neoformans* var. *gattii*, in which greater genetic diversity was noted. Other investigators have used genotype-based methods to subtype serotype A isolates (31, 37), but the extent of discrimination was not clear because small numbers of isolates were tested. This serotype A population structure of related clonal lines may limit the extent to which further subtype distinctions can be made. Alternatively, genotype-based methods may provide greater discrimination than phenotype-based MEE in subtyping serotype A isolates; this possibility has been suggested in another clonal population (35). We are currently investigating this point.

Our analysis of serotype AD isolates extends previous serotyping and genetic studies of this haploid yeast. For the enzyme 6PD, all four serotype AD isolates tested display an electrophoretic pattern resembling that seen for codominant expression of two subunits sorting into enzymatically active dimers (20). This result suggests that AD strains may express the products of two alleles for this enzyme. Diploidy has been previously suggested (14) as the reason for self-fertility in occasional clinical isolates previously described (6). We could not demonstrate self-fertility in our AD strains. However, the double-band pattern for PGM seen with recent clinical AD samples but not with the stock AD isolates supports the possibility of unstable diploidy in these serotype AD strains. This unstable diploid feature has been previously described in self-fertile isolates (14). Genetic backcrossing could confirm these possibilities. We have been unable to mate our tester strains, probably because of loss of mating ability after years of agar passage. This situation has been previously described with *C. neoformans* (17).

We show that MEE typing is not only straightforward to perform with cryptococci but also possesses intralaboratory and biologic reproducibility, as demonstrated by analysis of blinded samples and serially passaged isolates. All isolates

TABLE 2. Enzyme profile of *C. neoformans* ETs

ET	Relative mobility of:									
	ADH	G6D	6PD	GD2	GOT	MDH	LAp	PLp	PGI	PGM
1	4	3	4	4	2/3[a]	2/4	4	4	3	2
2	4	1	4	5	2/3	2/4	4	4	3	2
3	4	1	4	5	1	2/4	2	3	5	2
4	4	1	4	5	1/2	2/4	4	4	5	2
5	4	1	2/3/4	4	2/3	2/4	4	4	3	3
6	4	1	2/3/4	4	2/3	2/4	4	4	3	2
7	4	1	2/3/4	4	2/3	2/4	4	4	3	2/3
8	4	1	2	2	2/3	2/3	3	3	2	3
9	4	1	2	2	2/3	2/3	2	3	2	3
10	4	1	2	2	2/3	2/3	4	3	2	3
11	4	1	2	6	2/3	2/3	4	4	2	3
12	4	1	2	2	2/3	2/3	4	4	2	3
13	3	4	2	1	1/5	1/6[b]	5	5	1	1
14	2	4	2	1	1/4	1/6[b]	2	3	3	4
15	2	4	2	1	1/4	1	2	3	1	1
16	2	4	2	1	1/4	1/6[b]	2	3	1	1
17	6	3	3	1	1/4	1	1	1	3	4
18	5	3	3	1	1/4	1	1	1	2	4
19	5	3	3	1	1/4	1/6[b]	1	1	2	4

[a] Slash marks indicate detection of more than one mobility variant.
[b] Band 6 is found only when freshly prepared extracts are tested.

tested in this study could be assigned a varietal and subtype identity, including an acapsular strain that could not be serotyped. Preparation of extracts is much simplified compared with purification of DNA from cryptococci, which can be very difficult because of the high degree of expression of capsular polysaccharide in some fresh clinical samples (2a) and the high concentration of extracellular DNases characteristic of this organism (5).

We plan to apply laboratory strain typing data in analysis of cryptococcal isolates we are collecting through an active surveillance for cryptococcal disease. These studies may provide information valuable for the design of appropriate prevention and control strategies for cryptococcal disease.

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