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Comparison of Multilocus Enzyme Electrophoresis and Random Amplified Polymorphic DNA Analysis for Molecular Subtyping of *Cryptococcus neoformans*

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We evaluated multilocus enzyme electrophoresis (MEE) and random amplified polymorphic DNA (RAPD) for their usefulness in subtyping 344 *Cryptococcus neoformans* clinical isolates obtained from four U.S. metropolitan areas in 1992 to 1994. MEE and RAPD with five primers both discriminated between the two varieties of *C. neoformans*. MEE divided *C. neoformans* var. *neoformans* isolates into 15 enzyme electrophoretic subtypes (ETs) arranged in three complexes. The predominant ET 1 complex contained 10 ETs, with isolates from 70% of patients in 1 ET. RAPD with five primers further sorted this predominant ET into 19 subtypes, with 60% of isolates sorting into three RAPD types. The ET 8 MEE complex, containing three ETs, could not be divided further by RAPD. The ET 7 complex (two ETs) included isolates from all serotype AD patients. Although both MEE and RAPD identified isolates of *C. neoformans* var. *gattii*, neither distinguished between serotypes B and C. These results showed that the two *C. neoformans* varieties could be identified by MEE or RAPD profile as well as by biochemical methods. RAPD improved the discriminatory power of MEE for isolates within the ET 1 complex but with other ETs offered little additional sensitivity over MEE and was less sensitive than MEE with isolates of *C. neoformans* var. *gattii*. This information will be useful in identifying particular environmental sources of disease-causing exposures, in seeking clusters of cases, and in determining whether an infecting strain changes over time.

Cryptococcal meningitis, caused by the encapsulated yeast *Cryptococcus neoformans*, has emerged as a serious health problem in persons with AIDS (9, 21, 29). More thorough study of the epidemiology of cryptococcosis is needed to define exposure risk factors and consequently develop appropriate prevention strategies. An important component of these studies is the application of molecular subtyping to define the population structure of this organism, to identify particular environmental sources of disease-causing exposures, to seek clusters of cases, and to determine whether an infecting strain changes over time.

Multilocus enzyme electrophoresis (MEE) has been used in epidemiologic studies of many bacterial and fungal diseases (15, 25). We previously evaluated the utility of MEE in subtyping cryptococci (4). More recently, random amplified polymorphic DNA (RAPD) has been described as a potentially useful subtyping method (16, 34, 35). Several studies with bacteria and parasites have established concordance between results obtained by RAPD and by MEE (30, 33) and have suggested that RAPD may be more discriminatory than MEE for strain typing (30).

In this study, we evaluated and compared the relative usefulness of MEE and RAPD for subtyping cryptococci. We used *C. neoformans* clinical isolates obtained in 1992 to 1994 through a population-based active laboratory surveillance for cryptococcal disease in four U.S. metropolitan areas.

(This work was presented in part at the 94th General Meeting of the American Society for Microbiology, 23 to 27 May 1994 [4a].)

MATERIALS AND METHODS

Organisms and growth conditions. *C. neoformans* clinical isolates were obtained through the Centers for Disease Control Fungal Active Surveillance Project from four U.S. metropolitan areas (San Francisco, Calif., and Atlanta, Ga., in 1992 to 1994; Birmingham, Ala., and Houston, Tex., in 1993 and 1994 only). A case in this study was defined as isolation of *C. neoformans* from a single individual. Over 90% of the isolates were from blood or cerebrospinal fluid. The associated descriptive epidemiology is presented elsewhere (4a). Upon receipt at the Centers for Disease Control and Prevention, isolates were subcultured to Sabouraud dextrose agar slants and frozen in 20% glycerol at −20°C.

Every 10th isolate from San Francisco and Atlanta was selected for subtype analysis. All 1993 and most 1994 isolates from Houston and Alabama were subtyped. Frozen isolates were thawed and grown on Sabouraud dextrose agar at 37°C. Identification was confirmed by standard methods (1). Varietal assignment was made with canavanine-glycine-bromthymol blue agar (13). The serotypes of all serotype AD and all *C. neoformans* var. *gattii* isolates were confirmed by indirect immunofluorescence with a combination of polyclonal and monoclonal reagents (11, 31). Additional isolates included in the RAPD analysis were ATCC 48184 (serotype AD; electrophoretic type [ET] 6), CDC-B3181 and B3184 (serotype C; ET 13), and B3182 (serotype C; ET 16) (4, 10).

MEE. MEE was carried out as described earlier (4). The 10 enzymes assayed were alcohol dehydrogenase (EC 1.1.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), glutamate dehydrogenase (EC 1.4.1.4), glutamate oxaloacetic transaminase (EC 2.6.1.1), malate dehydrogenase (EC 1.1.1.37), phosphoglucose isomerase (EC 5.3.1.9),

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TABLE 1. Enzyme profile of *C. neoformans*

ET	ST[a]	Relative mobility of[b]:									
		ADH	G6D	6PD	GD2	GOT	MDH	LAp	PLp	PGI	PGM
1	A	4	3	4	4	2/3[c]	2/4	4	4	3	2
2	A	4	1	4	5	2/3	2/4	4	4	3	2
3	A	4	1	4	5	1	2/4	2	3	5	2
4	A	4	1	4	5	1/2	2/4	4	4	5	2
6	AD	4	1	2/3/4	4	2/3	2/4	4	4	3	2
7	AD	4	1	2/3/4	4	2/3	2/4	4	4	3	2/3
8	D	4	1	2	2	2/3	2/3	3	3	2	3
9	D	4	1	2	2	2/3	2/3	2	3	2	3
12	D	4	1	2	2	2/3	2/3	4	4	2	3
18	B	5	3	3	1	1/4	1	1	1	2	4
20	A	4	3	4	4	2/3	2/4	2	3	3	2
21	AD	4	1/3	2/3/4	4	2/3	2/4	4	4	2/3	2/3
22	A	4	3	4	4	2	2/4	4	4	3	2
24	A	3	3	4	4	2/3	2/4	4	4	5	2
25	A	4	3	4	5	1	2/4	4	4	3	2/4
26	A	4	3	4	4	2/3	2/4	4	4	2	2
28	A	4	3	4	4	2/3	2	4	4	3	2
29	B	4	4	2	3	1/4	1/2	3	3	1	1
30	B	5	3	3	1	1/4	1/2	5	5	2	4

[a] ST, derived serotype.
[b] Abbreviations: ADH, alcohol dehydrogenase; G6D, glucose 6-phosphate dehydrogenase; 6PD, 6-phosphoglucanate dehydrogenase; GD2, glutamate dehydrogenase; GOT, glutamate oxaloacetic transaminase, MDH, malate dehydrogenase; LAp, leucine-alanine peptidase; PLp, phenylalanine-leucine peptidase; PGI, phosphoglucose isomerase; PGM, phosphoglyceromutase.
[c] Slash marks indicate detection of more than one mobility variant in isolates of that ET.

phosphoglyceromutase (EC 2.7.5.1), phenylalanine-leucine peptidase (EC 3.4.X.X), and leucine-alanine peptidase (EC 3.4.X.X).

RAPD. Cryptococcal DNA was prepared by using a modification of the methods of Spitzer and Spitzer (28) and Möller et al. (19). Briefly, log-phase cells grown in yeast-peptone-glucose broth containing 0.5 M NaCl (7) were harvested, suspended in TES buffer (0.1 M Tris [pH 8], 0.01 M EDTA, 0.01 M dithiothreitol, 2% sodium dodecyl sulfate [SDS]), and broken with glass beads in a mini-Bead Beater (Biospec Products, Bartlesville, Okla.). Proteinase K was then added to a final concentration of 100 μg/ml, and the extract was incubated at 55°C for 1 h with occasional mixing. The NaCl concentration was then adjusted to 1.4 M (final concentration), cetyltrimethyl ammonium bromide was added to a final concentration of 1%, and the solution was incubated at 65°C for 10 min. The procedure of Möller et al. was then followed as described previously (19). The final dried pellet was resuspended in 50 μl of Tris-EDTA. The DNA concentration of each sample was determined with a fluorometer (Hoefer, San Francisco, Calif.) with calf thymus DNA as a standard. DNA was stored at −20°C.

RAPD fingerprinting was performed with five 10-mer oligonucleotide primers (OPA 2, OPA 3, OPA 19, OPAO 9, and OPAO 15; Operon Technologies, Inc., Alameda, Calif.) according to the manufacturer's instructions. PCR core reagents and polymerase were purchased from Perkin-Elmer (Norwalk, Conn.). For each 25-μl reaction mixture, reactants and final concentrations included 1× PCR Buffer supplemented with $MgCl_2$ stock to a final Mg concentration of 2.5 mM, 100 μM (each) deoxynucleoside triphosphate, 5 pmol of RAPD primer, and 0.5 U of AmpliTaq DNA polymerase. Template DNA was diluted to 10 ng/μl just before use, and then 25 ng was added per reaction mixture. Diluted template was not reused. Amplification was carried out in a Perkin-Elmer Cetus GeneAmp 9600 system at 1 cycle of 95°C for 5 min, which was followed by 45 cycles of 1 min at 94°C, 1 min at 34°C, and 2 min at 72°C and which ended with a cycle of 10 min at 72°C. Amplicons were separated on 1.4% agarose gels in 1× TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA [pH 8.4]; Boehringer Mannheim) containing ethidium bromide (0.5 mg/ml) and photographed.

Statistical methods. For MEE, each band of distinct gel mobility was numbered in order of increasing anodal migration. Electrophoretic mobility was determined by comparison with the mobility of cryptococcal standards electrophoresed on the same gel. Each unique combination of electrophoretic variants was designated as an ET. Matrices of weighted distance coefficients were calculated (24), and dendrograms were generated from these data (27). The degree of relatedness between two strains was 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.

Genetic diversity (h) was determined by the method of Nei (20). Genetic diversity was calculated separately for each enzyme locus and as the mean allelic diversity over all loci (20, 23). Genetic diversity for a locus is calculated by the formula $h = (1 - \Sigma x_i^2)(n/n - 1)$, where x_i is the frequency of the ith allele and n is the number of ETs (20, 23). Mean diversity per locus is the arithmetic average of h over all loci assayed (23, 24). (An explanation of these calculations is given in reference 23.)

For each sample, five RAPD fingerprints were generated, one with each primer. A BioImage whole band analyzer (Millipore, Ann Arbor, Mich.) was used to scan RAPD gels. The derived molecular weights (in base pairs) were then used to compute similarities on the basis of matching comigrating fragment positions between pairs of RAPD patterns by using the Dice coefficient (S_D) (6). When two profiles were compared, a match was recorded if the normalized molecular weight of the fragment in the first profile was within a window of ±5% of the molecular weight of a fragment in the second profile. Overall similarity between any given pair of samples was derived by combining the number of matches from each separate pair of fingerprints. The similarity values were then clustered by the unweighted pair group method with the arithmetic averages technique (27). A dendrogram was generated from these data as described above.

RESULTS

Varietal and serotype designations. MEE subtypes were determined for 344 isolates from 247 patients. Isolates from 244 patients (98% of all patients) were *C. neoformans* var. *neoformans*, and isolates from 3 patients (2% of all patients) were *C. neoformans* var. *gattii*. Within *C. neoformans* var. *neoformans*, isolates from 13 patients (5% of all patients) were serologically confirmed as serotype AD. All three *C. neoformans* var. *gattii* isolates were serotype B.

MEE subtype designations. MEE separated *C. neoformans* var. *neoformans* isolates into 15 ETs (Table 1), arranged on the MEE dendrogram (Fig. 1) in three complexes: ET 1, ET 7, and ET 8 complexes. Isolates from 222 patients (91% of *C. neoformans* var. *neoformans* patients) could be placed into the ET 1 complex, composed of ETs 1 to 4, 20, 22, 24, 25, 26, and 28. The isolates comprising these ETs were all related to one another at an RI of 0.44 on the dendrogram (Fig. 1).

This complex could be further divided into two smaller clusters, the ET 1 and ET 2 clusters. The ET 1 cluster contained ET 1 (168 cases) and the related ETs 20, 22, 24, 25, 26, and 28 (1 case each). The smaller ET 2 cluster included ET 2 (25 cases), ET 3 (13 cases), and ET 4 (11 cases). The ET 1 and ET 2 clusters were related to each other at an RI value of 0.44. The

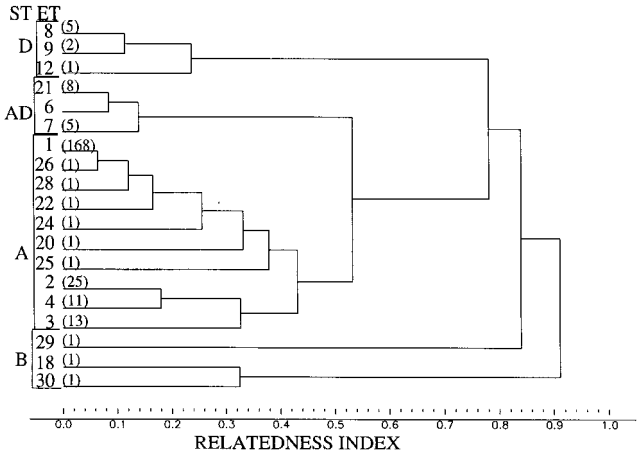

FIG. 1. Dendrogram showing strain relatedness among ETs of *C. neoformans* determined by MEE with starch gel electrophoresis. A total of 344 isolates from 247 patients are represented. The numbers in parentheses show the number of cases with that ET. ET 6 is represented by ATCC 48184 (serotype AD). ST, derived serotype.

majority of isolates (240 isolates from 168 patients; 69% of all *C. neoformans* var. *neoformans* patients) sorted into ET 1.

The ET 8 complex, formed by ET 8 (five cases), ET 9 (two cases), and ET 12 (one case), was distinct from the ET 1 complex (Fig. 1). ETs 8, 9, and 12 were closely related to one another (RI of 0.24) but were less closely related to the ET 1 complex, at an RI of 0.78.

Serotype AD isolates (13 cases) formed the ET 7 complex (ETs 7 and 21). These isolates were closely related to the serotype AD strain ATCC 48184 (10), which is shown for comparison (Fig. 1, ET 6).

Serotype B isolates displayed greater diversity among themselves than did isolates of *C. neoformans* var. *neoformans*. ET 18 (one case) and ET 30 (one case) clustered together (RI of 0.32). ET 29 (one case) was less closely related to these two isolates (RI of 0.9).

In 41 cases, at least two samples were collected from a single individual. MEE patterns among multiple samples from the same patient remained identical, regardless of the timing of sample collection.

Genetic diversity. Genetic diversity at each enzyme locus and the mean genetic diversity for all 10 enzymes are shown in Table 2. Genetic diversity was calculated separately for isolates of each major ET complex, as well as for all strains within *C. neoformans* var. *neoformans*. The ET 8 complex and the ET 7 complex (serotype AD) each displayed lower mean genetic diversity (0.16 for the ET 8 complex and 0.18 for the ET 7 complex) than did the ET 1 complex (0.35). However, all of these values were lower than that for serotype B (*C. neoformans* var. *gattii*) at 0.66.

RAPD. Selected results of RAPD with five primers are shown in Fig. 2 and 3 and in dendrogram format in Fig. 4. All templates were amplified twice. Forty primers (kits OPA and OPAO; Operon Technologies) were initially analyzed with a small panel of cryptococcal templates, but with numerous primers, either no amplicons or a DNA smear was generated by using the reaction conditions described above. The five primers selected gave clear, reproducible patterns with cryptococcal templates.

This combination of five primers discriminated between the two varieties of *C. neoformans* in terms of the nature and intensity of amplicon patterns (Fig. 2; compare lanes 1 to 11 with lanes 12 to 18).

Within *C. neoformans* var. *neoformans*, common bands could be identified that were shared by members of either the ET 1 complex or the ET 8 complex. For example, in Fig. 2A, bands in the 1,400- to 1,800-bp region were absent from most members of the ET 1 complex, except for the ET 1 isolate in lane 2. Taken together, the results from all five primers could be used to distinguish between the ET 1 complex and the ET 8 complex (Fig. 4).

The four ET 7 complex (serotype AD) isolates displayed amplicon patterns generally resembling those of either the ET 1 complex or the ET 8 complex, depending on the primer used (Fig. 2). With primers OPA 19 (Fig. 2A) and OPA 3, the patterns displayed more similarity to those of the ET 8 complex. However, with primers OPAO 9 (Fig. 2B) and OPA 2 (data not shown), patterns of ET 7 complex (serotype AD) isolates resembled those of the ET 1 complex. When RAPD results with all five primers were combined, ET 7 complex (serotype AD) isolates clustered in a dendrogram position between the ET 1 and ET 8 complexes (Fig. 4).

Heterogeneity among ET 7 complex (serotype AD) isolates could also be shown. For example, when tested with primer OPA 19 (Fig. 2A), the ET 7 isolate (lane 5) displayed a predominant 1,090-bp band not seen in any of the other ET 7 complex isolates tested. This heterogeneity also extended to isolates of the ET 8 complex. The ET 8 and ET 9 isolates (lanes 9 and 11) were identical to each other when tested with all five primers, but they showed identity with the third member of the ET 8 complex (ET 12, lane 10) only when tested with primers OPA 2 and OPA 3 (data not shown). This correlates with the results obtained by MEE, which show ET 8 and ET 9 more closely related to each other than to ET 12 (compare Fig. 1 with Fig. 4).

RAPD with five primers also distinguished between the two MEE-defined clusters of the ET 1 complex (Fig. 3 and 4). Within the ET 1 cluster, isolates of ETs 20, 22, 24, 25, 26, and 28 generally resembled those of ET 1 (compare lanes 1 through 12 in Fig. 3). Members of the ET 1 cluster could be distinguished from those of the ET 2 cluster (ETs 2, 3, and 4)

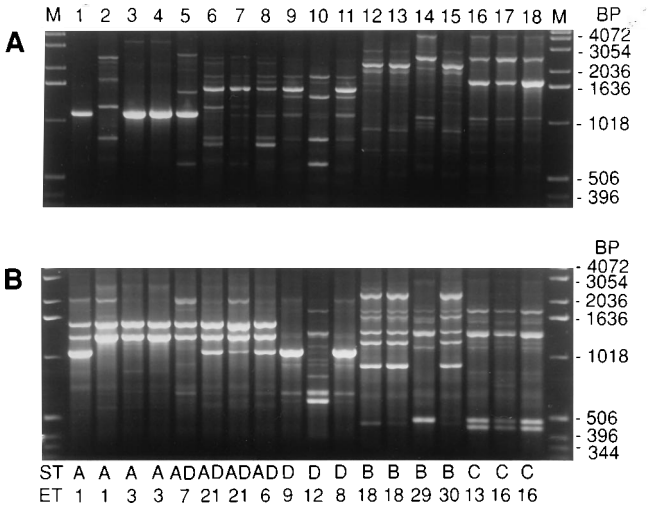

FIG. 2. DNA fingerprint patterns of *C. neoformans* subtypes after PCR with selected 10-mer primers. (A) Primer OPA 19; (B) primer OPAO 9. A DNA ladder (Bethesda Research Laboratories) was used as the molecular size standard. Derived serotypes (STs) and ETs are displayed underneath each isolate. BP, base pairs.

TABLE 2. Genetic diversity at 10 enzyme loci and mean genetic diversity for *C. neoformans*[a]

Category (no. of ETs)	Genetic diversity at locus[b]										Mean genetic diversity
	ADH	G6D	6PD	GD2	GOT	MDH	LAp	PLp	PGI	PGM	
All isolates (19)	0.46	0.65	0.69	0.71	0.56	0.69	0.56	0.53	0.77	0.68	0.63
C. neoformans var. *neoformans* ET complex											
1 (10)	0.2	0.46	0	0.53	0.64	0.20	0.35	0.35	0.60	0.20	0.35
8 (3)	0	0	0	0	0	0	1.0	0.66	0	0	0.16
7 (3)	0	0.66	0	0	0	0	0	0	0.5	0.66	0.18
All *C. neoformans* var. *neoformans* (17)	0.4	0.63	0.60	0.60	0.41	0.58	0.40	0.38	0.77	0.56	0.53
C. neoformans var. *gattii* serotype B (3)	0.6	0.66	0.66	0.66	0	0.66	1.0	1.0	0.66	0.66	0.66

[a] Genetic diversity and mean genetic diversity were calculated as described in the text.
[b] Abbreviations: ADH, alcohol dehydrogenase; G6D, glucose 6-phosphate dehydrogenase; 6PD, 6-phosphogluconate dehydrogenase; GD2, glutamate dehydrogenase; GOT, glutamate oxaloacetic transaminase; MDH, malate dehydrogenase; LAp, leucine-alanine peptidase; PLp, phenylalanine-leucine peptidase; PGI, phosphoglucose isomerase; PGM, phosphoglyceromutase.

when any primer except OPA 19 was used (Fig. 3; compare lanes 1 to 12 with lanes 13 to 27). Within the ET 2 cluster, ET 2 isolates (lanes 13 to 20) could be distinguished from those of ET 3 (lanes 21 to 25) and ET 4 (lanes 26 and 27) with all primers except OPA 19.

Various degrees of heterogeneity with RAPD were seen within isolates of the same ET. On the basis of results with all five primers, 67 ET 1 isolates from 57 patients were further subdivided into 19 RAPD subtypes, with three predominant RAPD types accounting for 59% of the isolates. Twelve of these RAPD types contained one isolate each. Isolates from 17 of 18 ET 2 patients displayed total identity when tested with all five primers (Fig. 4). RAPD patterns from all ET 3 isolates (eight cases) were completely identical to one another (Fig. 3, lanes 21 to 25). ET 4 isolates (five cases) displayed minor differences (Fig. 3, lanes 26 and 27). One group (three cases) was totally indistinguishable from ET 3, while the other group (two cases) was similar to ET 3, except that one additional band was detected after amplification with either OPA 2 or OPA 3 (Fig. 3B; compare lanes 26 and 27).

Within *C. neoformans* var. *gattii*, the two isolates of ET 18 (serotype B) were identical to each other (Fig. 2A and B, lanes 12 and 13) and to the ET 30 isolate (lanes 15) with all five primers. On the other hand, ET 29 (lanes 14) more closely resembled the three serotype C isolates, which were all identical, (lanes 16 to 18). The ET 29 RAPD pattern differed in that, after amplification with any of four primers, it lacked at least one band found in the serotype C pattern. This mirrors the relatedness shown with MEE (Fig. 1), where ET 18 and 30 were more closely related to one another than they were to ET 29. However, within *C. neoformans* var. *gattii*, MEE appeared to offer greater discrimination among isolates than did RAPD. This is seen in the RAPD failure to discriminate between ETs 13 and 16 (serotype C) (Fig. 2, lanes 16 to 18) and between ETs 18 and 30 (serotype B) (lanes 12, 13, and 15).

Reproducibility. The reproducibility of MEE was determined by blinded analysis of multiple panels as described earlier (4). MEE patterns from a single sample remained identical when tested in multiple blinded and unblinded experiments.

Reproducibility of RAPD was assessed by repeatedly ampli-

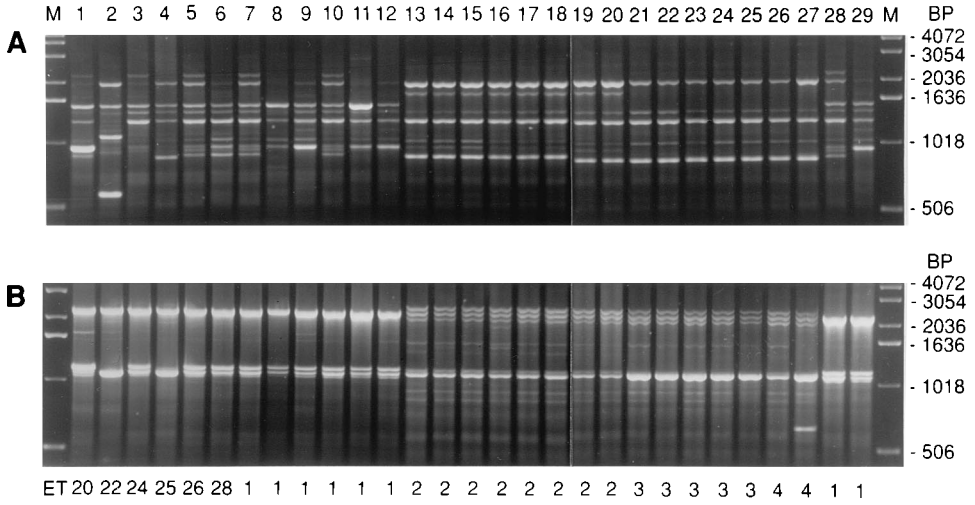

FIG. 3. RAPD fingerprint patterns of *C. neoformans* enzyme genotypes. (A) Primer OPAO 15; (B) primer OPA 2. ETs are displayed underneath each isolate. BP, base pairs.

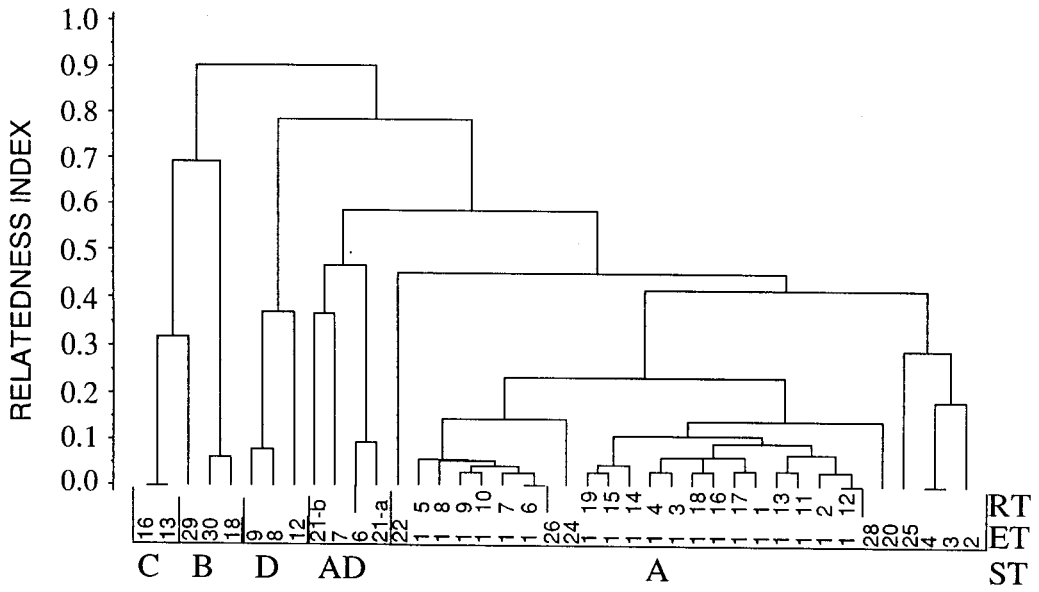

FIG. 4. Dendrogram showing strain relatedness among RAPD groups of *C. neoformans*. RAPD was performed with five 10-mer primers as described. Isolates (listed on the abscissa) are designated by ET and derived serotype (ST). Isolates 21-a and 21-b refer to the ET 21 isolates described in Fig. 2, lanes 6 and 7, respectively. Sixty-seven ET 1 isolates from 57 patients are represented. RT, RAPD type.

fying DNA templates prepared at different times from each of several stock cultures. Only bands that were consistently amplified were scored (data not shown). Additionally, variations in assay conditions were monitored by amplifying and electrophoresing two standard templates on every agarose gel. Standards were selected for each primer as the two cryptococcal templates that generated the most disparate amplicon patterns with that primer. For each PCR, unknowns were not scored unless all previously defined amplicons in the standards were clearly revealed on every gel. Finally, MEE and blinded PCR were performed with DNAs from seven pairs of cerebrospinal fluid-blood cultures collected from the same patient during the same episode of disease (not more than 24 h apart). In each case, the two samples generated identical MEE and amplicon patterns (data not shown).

DISCUSSION

This study was undertaken to define and evaluate methods useful for the molecular subtyping of *C. neoformans*. We have previously shown that multilocus genotype correlates with variety and serotype (A, AD, D, and B or C) (4). This study extends our work by defining the utility of RAPD as a subtyping tool for cryptococci, by showing the concordance between MEE and RAPD, and by using RAPD to enhance the discriminatory power of MEE within the predominant ET 1 complex.

In general, RAPD offers a sensitive and straightforward system for subtyping *C. neoformans*. Pattern-matching software can be used to facilitate comparison among large numbers of isolates and to estimate degrees of relatedness among members of a population. As with MEE, poorly or nonencapsulated isolates can be subtyped. Great attention must be paid, however, to ensuring reproducibility of results (3, 8, 17). We have tried to address this issue by adhering carefully to standardized reaction conditions, by quantitating DNA in template preparations, and by using standard strains.

We previously demonstrated that, within *C. neoformans* var. *neoformans*, varieties and serotypes can be defined by MEE profile as well as by the structure of the polysaccharide capsule (4, 5). Within *C. neoformans* var. *neoformans*, members of either serotype A, AD, or D display an identical electrophoretic variant or variants for 6-phosphogluconate dehydrogenase and variants for phosphoglyceromutase and malate dehydrogenase also correlate with serotype A or D (4). In a similar manner, consideration of MEE genetic diversity data (Table 2) can be used in this study to derive serotypes for members of the *C. neoformans* var. *neoformans* complex, the ET 1 complex (serotype A like), the ET 8 complex (serotype D like), and the ET 7 complex (serotype AD like). Furthermore, varieties and multilocus genotypes can also be correlated with the RAPD profile. With this primer combination, we could identify variety- and ET-associated differences in the nature and intensity of amplicon patterns. Our data concur with those from previous analyses of cryptococcal mitochondrial DNA restriction patterns (32) or middle-repetitive DNA sequences (22) in recognizing that detection of particular polymorphisms at various genetic loci can be correlated with expression of variety and serotype either biochemically or serologically. Furthermore, the use of non-serologically based subtyping methods introduces the ability to identify subtypes within members of the same serotype.

Both RAPD and MEE recognized two clusters within the predominant ET 1 (serotype A-like) complex. Members of the ET 2 cluster (ETs 2, 3, and 4) were more closely related to one another than they were to the predominant ET 1 cluster (ETs 1, 20, 22, 24, 25, 26, and 28). Within the ET 2 cluster (about 20% of ET 1 complex isolates), RAPD showed only marginal improvement in further dividing isolates. Nearly all ET 2, ET 3, or ET 4 isolates were identical to one another, regardless of the subtyping method used. On the other hand, RAPD was useful in subdividing the ET 1 cluster (80% of ET 1 complex isolates) into nearly 20 subtypes. Both RAPD and MEE recognized two related lineages of the ET 8 (serotype D-like) complex, one lineage represented by ET 8 and 9 and the other represented by ET 12.

Although both MEE and RAPD identified isolates of *C. neoformans* var. *gattii*, MEE displayed greater sensitivity at subtyping these isolates. RAPD with these five primers could not distinguish between ETs 18 and 30 (serotype B), while

MEE revealed differences in 2 of the 10 enzymes tested. RAPD also failed to distinguish among three serotype C isolates that MEE divided into two ETs. We (4) and others (18) have noted an inability to discriminate between serotypes B and C in both MEE- and PCR-based subtyping studies. Our studies (Fig. 1 and reference 4) suggest that *C. neoformans* var. *gattii* can be divided into at least two lineages: one including serotype C (ETs 13 and 16) and some serotype B isolates (ETs 14, 15, and 29) and the other containing serotype B isolates only (ETs 17 to 19 and 30) (4). It is possible that the failure of previous PCR-based methods to discriminate between serotype B and C isolates may be due to the selection of isolates from only the B and C common line for analysis, thus inadvertently excluding isolates representing ETs 17 to 19 and 30 (4). Inclusion of more isolates and isolates from both lineages may improve B and C discriminatory ability.

Serotype A has been identified as the most prevalent serotype of *C. neoformans* in clinical isolates from the United States in previous epidemiologic surveys in 1977 (74.6% of the total [2]) and again in 1984 (80.6% of the total [12]). In this study, in which most isolates were obtained from AIDS patients and samples were collected through active laboratory-based rather than passive surveillance, members of the ET 1 complex (serotype A like) comprise 90% of total cases. The high incidence of cryptococcosis due to serotype A in AIDS patients has been previously noted (14, 26). The percentage of serotype AD isolates in this study parallels those of earlier surveys: 4% in 1977 (2), 4.4% in 1984 (12), and 5% in this study. Analysis of over 200 isolates in this study has not essentially altered the ET distribution reported earlier (4), in which about 70% of clinical isolates were ET 1. It is not clear whether the ET distribution of this organism in nature is similar to that of clinical isolates. It is possible that particular *C. neoformans* environmental subtypes may be disproportionately selected to undergo clonal expansion in a susceptible patient population. If true, this hypothesis would suggest that *C. neoformans* strains differ in their virulence potential and that prevention efforts should be targeted toward these more virulent strains.

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REFERENCES

1. **Balows, A., W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.).** 1991. Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
2. **Bennett, J. E., K. J. Kwon-Chung, and D. H. Howard.** 1977. Epidemiologic differences among serotypes of *Cryptococcus neoformans*. Am. J. Epidemiol. **105:**582–586.
3. **Böttger, E. C.** 1990. Frequent contamination of *Taq* polymerase with DNA. Clin. Chem. **36:**1258–1259.
4. **Brandt, M. E., S. L. Bragg, and R. W. Pinner.** 1993. Multilocus enzyme typing of *Cryptococcus neoformans*. J. Clin. Microbiol. **31:**2819–2823.

4a. **Brandt, M. E., L. Hutwagner, R. J. Kuykendall, S. L. Bragg, R. W. Pinner, and Cryptococcal Disease Active Surveillance Group.** 1994. Active surveillance for cryptococcal disease: molecular epidemiology of *Cryptococcus neoformans*, abstr. F-66, p. 600. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.

5. **Cherniak, R., and J. B. Sundstrom.** 1994. Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. Infect. Immun. **62:**1507–1512.
6. **Dice, L. R.** 1945. Measures of the amount of ecological association between species. Ecology **26:**297–302.
7. **Dykstra, M. A., L. Friedman, and J. W. Murphy.** 1977. Capsule size of *Cryptococcus neoformans*: control and relationship to virulence. Infect. Immun. **16:**129–135.
8. **Ellsworth, D. L., K. D. Rittenhouse, and R. L. Honeycutt.** 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. BioTechniques **14:**214–216.
9. **Grant, I. H., and D. Armstrong.** 1988. Fungal infections in AIDS: cryptococcosis. Infect. Dis. Clin. N. Am. **2:**457–464.
10. **Ikeda, R., T. Shinoda, Y. Fukazawa, and L. Kaufman.** 1982. Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. J. Clin. Microbiol. **16:**22–29.
11. **Kaplan, W., S. L. Bragg, S. Crane, and D. G. Ahearn.** 1981. Serotyping *Cryptococcus neoformans* by immunofluorescence. J. Clin. Microbiol. **14:**313–317.
12. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am. J. Epidemiol. **120:**123–130.
13. **Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett.** 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J. Clin. Microbiol. **15:**535–537.
14. **Kwon-Chung, K. J., A. Varma, and D. H. Howard.** 1990. Ecology of *Cryptococcus neoformans* and prevalence of its two varieties in AIDS and non-AIDS associated cryptococcosis, p. 103–113. *In* H. Vanden Bossche (ed.), Mycoses in AIDS patients. Plenum Press, New York.
15. **Lehmann, P. F., B. J. Kemker, C.-B. Hsiao, and S. Dev.** 1989. Isoenzyme biotypes of *Candida* species. J. Clin. Microbiol. **27:**2514–2521.
16. **Lehmann, P. F., D. Lin, and B. A. Lasker.** 1992. Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. J. Clin. Microbiol. **30:**3249–3254.
17. **MacPherson, J. M., P. E. Eckstein, G. J. Scoles, and A. A. Gajadhar.** 1993. Variability of the random amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. Mol. Cell. Probes **7:**293–299.
18. **Meyer, W., T. G. Mitchell, E. Z. Freedman, and R. Vilgalys.** 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J. Clin. Microbiol. **31:**2274–2280.
19. **Möller, E. M., G. Bahnweg, H. Sandermann, and H. H. Geiger.** 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. **20:**6115–6116.
20. **Nei, M.** 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics **89:**583–590.
21. **Panther, L. A., and M. A. Sande.** 1990. Cryptococcal meningitis in the acquired immunodeficiency syndrome. Semin. Respir. Infect. **5:**138–145.
22. **Polacheck, I., G. Lebens, and J. B. Hicks.** 1992. Development of DNA probes for early diagnosis and epidemiological study of cryptococcosis in AIDS patients. J. Clin. Microbiol. **30:**925–930.
23. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51:**873–884.
24. **Selander, R. K., R. M. McKinney, T. S. Whittam, W. F. Bibb, D. J. Brenner, F. S. Nolte, and P. E. Pattison.** 1985. Genetic structure of populations of *Legionella pneumophila*. J. Bacteriol. **163:**1021–1037.
25. **Selander, R. K., J. M. Musser, D. A. Caugant, M. N. Gilmour, and T. S. Whittam.** 1987. Population genetics of pathogenic bacteria. Microb. Pathog. **3:**1–7.
26. **Shimizu, R. Y., D. H. Howard, and M. N. Clancy.** 1986. The variety of *Cryptococcus neoformans* in patients with AIDS. J. Infect. Dis. **154:**1042.
27. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy, the principles and practice of numerical classification, p. 230–234. W. H. Freeman and Co., San Francisco.
28. **Spitzer, E. D., and S. G. Spitzer.** 1992. Use of a dispersed repetitive DNA element to distinguish clinical isolates of *Cryptococcus neoformans*. J. Clin. Microbiol. **30:**1094–1097.
29. **Sugar, A. M.** 1991. Overview: cryptococcosis in the patient with AIDS. Mycopathologia **114:**153–157.
30. **Tibayrenc, M., K. Neubauer, C. Barnabe, F. Guerrini, D. Skarecky, and F. J. Ayala.** 1993. Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. Proc. Natl. Acad. Sci. USA **90:**1335–1339.
31. **Todaro-Luck, F., E. Reiss, R. Cherniak, and L. Kaufman.** 1989. Characterization of *Cryptococcus neoformans* capsular glucuronoxylomannan polysaccharide with monoclonal antibodies. Infect. Immun. **57:**3882–3887.
32. **Varma, A., and K. J. Kwon-Chung.** 1989. Restriction fragment polymorphism in mitochondrial DNA of *Cryptococcus neoformans*. J. Gen. Microbiol. **135:**3353–3362.
33. **Wang, G., T. S. Whittam, C. M. Berg, and D. E. Berg.** 1993. RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. Nucleic Acids Res. **21:**5930–5933.
34. **Welsh, J., and M. McClelland.** 1991. Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwide combinations of primers. Nucleic Acids Res. **19:**5275–5279.
35. **Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1991. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18:**6531–6535.