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Multilocus Sequence Typing of Serially Collected Isolates of *Cryptococcus* from HIV-Infected Patients in South Africa

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Patients with cryptococcal meningitis in sub-Saharan Africa frequently relapse following treatment. The natural history and etiology of these recurrent episodes warrant investigation. Here, we used multilocus sequence typing (MLST) to compare the molecular genotypes of strains of *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from serial episodes of cryptococcal meningitis that were separated by at least 110 days. The most common MLST genotypes among the isolates were the dominant global clinical genotypes (M5 and M4) of molecular type VNI, as well as the VNI genotypes apparently restricted to southern Africa. In addition, there was considerable genetic diversity among these South African isolates, as 15% of the patients had unique genotypes. Eleven percent of the patients were reinfected with a genetically different strain following their initial diagnosis and treatment. However, the majority of serial episodes (89%) were caused by strains with the same genotype as the original strain. These results indicate that serial episodes of cryptococcosis in South Africa are frequently associated with persistence or relapse of the original infection. Using a reference broth microdilution method, we found that the serial isolates of 11% of the patients infected with strains of *C. neoformans* var. *grubii* with identical genotypes exhibited ≥4-fold increases in the MICs to fluconazole. Therefore, these recurrent episodes may have been precipitated by inadequate induction or consolidation of antifungal treatment and occasionally may have been due to increased resistance to fluconazole, which may have developed during the chronic infection.

Cryptococcus neoformans is an opportunistic pathogen that exhibits profound neurotropism and causes life-threatening disease among persons with HIV/AIDS (1–3). This encapsulated basidiomycetous yeast is widespread in the environment, where it is associated most commonly with avian excreta and tree hollows (4). Cryptococcosis is not contagious but is acquired by inhalation of organisms from the environment, and the most serious manifestation of disease is cryptococcal meningoencephalitis (CM). The U.S. Centers for Disease Control and Prevention (CDC) has estimated that approximately one million new infections (range, 371,700 to 1,544,000) occur each year, and the vast majority of these cases occur in sub-Saharan Africa, which has the largest prevalence of HIV-infected individuals (5). In sub-Saharan Africa, CM accounts for 13% to 40% of deaths among patients with HIV/AIDS, and according to CDC estimates, mortality from CM in the region may be similar to the death rates from tuberculosis and other tropical diseases (5). Most cases of CM are caused by *Cryptococcus neoformans*. The sibling pathogenic species, *Cryptococcus gattii*, exhibits a similar natural history but is considerably less prevalent than *C. neoformans* both clinically and environmentally (4).

Without treatment, CM is fatal. However, even when treatment is available, 20% to 60% of patients die from the disease, and many patients develop recurrent disease (6–8). The immune reconstitution inflammatory syndrome (IRIS), which occurs after initiation of antiretroviral therapy (ART) and is associated with an exuberant immune reaction to the residual pathogen or its antigens in the host and the sudden reappearance of symptoms, may account for up to 30% of recurrent episodes of disease (7, 9). The other common reason for recurrent cryptococcosis is inadequate treatment with the primary antifungal drug(s) and/or nonadherence to secondary fluconazole prophylaxis (7).

Although South Africa recognizes internationally accepted guidelines for treating cryptococcosis, there is significant variation in the care provided by individual facilities that treat patients with CM. The standard antifungal regimen for a first episode of CM includes a 2-week induction phase with amphotericin B (administered intravenously at 0.7 to 1.0 mg/kg/day) and flucytosine followed by a consolidation phase with 400 mg/day of oral fluconazole (3, 10). However, flucytosine is not available in South Africa, and during the period of this study, some public hospitals with limited resources treated patients with oral fluconazole from the outset (11, 12). Even in the areas with high standards of in-hospital medical care, adherence to prophylaxis therapy is exceptionally low, which results in recurrent disease and significant mortality (8). The actual rates of mortality from cryptococcosis are unknown due to the difficulties of following patients in the community; however, survival at 90 days has been estimated to be as low as 41% (13).

Surveillance data suggest that approximately 10% to 23% of patients in South Africa present with recurrent cryptococcosis (8, 13). Here we report the largest comparison to date of the treat-

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ment and molecular genotypes of serial isolates from patients with recurrent CM. Recurrent cryptococcosis can be caused by reinfection, persistence of the initial infecting strain, or relapse of an original strain or strains following clearance. Persistence and relapse are associated with suboptimal antifungal treatment, resistance to the antifungal treatment, or IRIS. Alternatively, a novel infecting strain can be acquired from the environment and cause a new episode of disease. Environmental sampling suggests that *C. neoformans* and *C. gattii* are widespread in the environment in South Africa, which results in constant exposure of immunosuppressed patients to these fungi (12, 14). Multilocus sequence typing (MLST) has been established as the gold standard for identifying the major molecular types or populations of *C. neoformans* and *C. gattii* (namely, VNI-VNIV, VNB, and VGI-VGIV) as well as the genotypes of individual strains (15, 16). A consensus set of seven primer pairs are utilized worldwide to determine the genotypes of isolates of the pathogenic *C. neoformans*/*C. gattii* species complex (15).

In this study, we determined the genotypes of initial and serial isolates of *C. neoformans* and *C. gattii* from patients with CM and estimated the proportion of relapses due to persistent or novel infections. We also measured the susceptibility of isolates of *C. neoformans* to fluconazole to investigate the development of antifungal drug resistance in serial isolates.

(Preliminary results of this study were presented at the 8th International Conference on *Cryptococcus* and Cryptococcosis, Charleston, SC, 1 to 5 May 2011.)

MATERIALS AND METHODS

Isolates. Clinical isolates were obtained from the Mycology Reference Laboratory at the National Institute for Communicable Diseases (NICD) in South Africa through an active, population-based, national surveillance program (the Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa [GERMS-SA]). At enhanced surveillance hospitals, nurse surveillance officers collected detailed case information, including HIV infection status, in-hospital antifungal treatment, and in-hospital outcome (survival or death). Serial isolate pairs or triads were identified from patients who were diagnosed with cryptococcal disease from 2005 through 2009 at an enhanced surveillance site and who had a second or third strain isolated >30 days after isolation of the initial strain. However, due to the large number of cases that met these criteria (841 isolates representing 402 cases), we selected only cases in which a second or third strain was isolated more than 110 days after the first strain. Because clearance of the initial infection from the cerebrospinal fluid was not documented, persistence and relapse could not be clinically differentiated. This national surveillance program was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg, and by other appropriate university and provincial ethics committees.

Banked and stored clinical isolates of *Cryptococcus* species were subcultured at least twice on Sabouraud agar (Diagnostic Media Products [DMP], National Health Laboratory Service, Johannesburg, South Africa). Isolates were confirmed as *C. neoformans* using standard phenotypic tests, including development of brown-pigmented colonies on Staib's niger-seed medium (DMP) and a positive test for urease on urea-containing agar (DMP). *C. gattii* was distinguished from *C. neoformans* by growth and appearance on canavanine-glycine-bromothymol blue plates (17). Phenotype-based identifications were subsequently confirmed by analyses of DNA sequences as described below. In addition, DNA from a number of reference strains of *C. neoformans* were included in the phylogenetic analyses (see Table S1 in the supplemental material) (18, 19).

DNA isolation and MLST. Isolates were streaked onto plates of yeast peptone dextrose (YPD) agar and incubated at 30°C until single colonies were observed. A single colony from each isolate was transferred to a new YPD plate and incubated overnight prior to DNA extraction. DNA was isolated with the MasterPure yeast DNA purification kit (Epicentre, Biotechnologies, Madison, WI) according to the manufacturer's instructions. MLST was performed using seven consensus loci plus the *TEF1* locus, which is particularly helpful in differentiating closely related strains that are indistinguishable by the consensus loci (15, 18). The amplified products were cleaned with ExoSAP (USB Products, Affymetrix, Inc., Cleveland, OH).

Sequence analyses. Sequences were manually edited with Chromas Lite 2.01 (Techelysium, Brisbane, Australia) and verified automatically with Geneious 5.4.5 software (Biomatters, Ltd., Auckland, New Zealand). The forward and reverse amplicons were aligned and regions with low-quality sequencing data were removed. Sequences of the eight MLST loci were concatenated, aligned, and analyzed by the neighbor-joining (NJ) method, as previously described (19). Strains were considered to have the same genotype if 100% identity was observed among all eight loci. To establish their genotypic identity, the clinical isolates were compared with reference strains with previously characterized MLST genotypes (18–21).

When the incident and serial isolate(s) set from the same patient possessed different genotypes, the patient may have been subsequently reinfected with a new strain or initially infected with more than one strain. To investigate the latter possibility, we repeated the MLST genotyping on the initial sample without single-colony purification to detect evidence of mixed infection. For these experiments, DNA was extracted and PCR amplified with primers for the *PLB1* locus, which evinces more strain variation than the other MLST loci (18). We then compared these sequences with those of the single-colony purified analysis of the patient's initial culture. The presence of more than one haplotype would indicate that the incident isolate consisted of more than one strain.

Ploidy and mating type. The ploidy of each isolate was determined by measurement of DNA content using a fluorescence-activated cell sorter (FACS) as described previously (22). Mating types were determine by PCR and confirmed by mating type assays on V8 medium as described previously (21).

Antifungal susceptibility testing. Strains of *C. neoformans* var. *grubii* with identical MLST genotypes that were isolated from the same patient were tested for antifungal susceptibility. MICs to fluconazole (FLZ) were determined using the Clinical and Laboratory Standards Institute M27–A3 microdilution broth method; microtiter plates were prepared at the NICD (23). As clinical breakpoints are not available for FLZ, we compared the MICs of incident and serial isolates and defined resistance as a 4-fold increase in the MIC. Based on the distribution of MICs to FLZ among wild-type and clinical isolates, epidemiologic cutoff values (ECV) have been determined for nontyped isolates of *C. neoformans* (16 μg/ml FLZ) and isolates of the most prevalent molecular type VNI and for molecular type VNIII (8 μg/ml and 16 μg/ml, respectively) (24). MICs to amphotericin B (AMB) were not assessed because no resistance was observed in our recent analysis of nearly 500 isolates (23).

RESULTS

Patients. From 2005 through 2009, a national cryptococcal surveillance program in South Africa identified 26,850 cases of cryptococcosis. A total of 402 incident cases of cryptococcosis with a second or third strain isolated >30 days after isolation of the initial strain were identified at enhanced surveillance sites. For this study, we selected patients who had serial isolates that were collected >110 days after the initial isolates were obtained. Using these criteria, we selected 89 cases, and they were represented by 185 isolates. Most cases had two isolates, but eight cases were represented by three serially collected isolates. Among these cases, the time between incident and serial isolates ranged from 111 to 290 days. The study included patients who were diagnosed in each of the 5 years and sought hospital care in all nine South African

TABLE 1 Serotype, molecular type, ploidy, and mating type of the 185 incident and serial isolates of *Cryptococcus* obtained from cerebrospinal fluid specimens of 89 patients with cryptococcal meningitis

Species	Serotype and molecular type[a] (*n* [%] of isolates)	No. (%) of isolates according to: Ploidy		*MAT* allele			No. of patients[b]
		n	2*n*	*MAT*α	*MAT***a**	*MAT*α/**a**	
C. neoformans	A, VNI (139 [75])	134 (72)	5 (2.7)	139 (75)	0	0	64
	A, VNII (22 [12])	21 (11)	1 (0.5)	22 (12)	0	0	9
	A, VNB (9 [5])	9 (5)	0	8 (4)	1 (0.5)	0	3
	AD, VNIII (7 [3.8])	0	7 (3.8)	0	0	7 (3.8)	4
C. gattii	B, VGI (4 [2])	4 (2)	0	4 (2)	0	0	2
	C, VGIV (4 [2])	4 (2)	0	4 (2)	0	0	2

[a] No isolates of molecular types VNIV (serotype D), VGII, or VGIII were obtained.
[b] In addition, two patients had serial isolates of VNI and VNII, and three patients had serial isolates of VNI and VNB.

provinces. However, as expected, most patients sought care in the two most populous provinces, Gauteng and KwaZulu-Natal.

Seventy-three of the 85 patients (86%) had been tested for HIV infection, and all were seropositive. The HIV status of the other 12 patients was unknown. The prevalence of HIV/AIDS in South Africa is higher among women than men, and this patient cohort included more women ($n = 49$; 58%). The age distribution was also consistent with that of South Africans with HIV/AIDS (median, 35 years; range, 10 to 63 years). Reflecting the inadequate availability of ART during this period (12), only 9 of the 85 patients were known to be receiving ART, 62 patients were not receiving ART, and the other 14 patients had unknown treatment status. At the time of diagnosis, counts of circulating $CD4^+$ T lymphocytes were obtained for 50 of the 85 cases. Half of these patients (26 of 50) had $CD4^+$ cell counts of <50 cells/μl, and 10 of the tested patients had $CD4^+$ counts between 51 and 100 cells/μl. The counts of only three patients exceeded 200 $CD4^+$ cells/μl. In addition, 28 (33%) patients were being treated for tuberculosis, 39 were not undergoing treatment for tuberculosis, and 21 patients lacked a record of tuberculosis.

Isolates of *Cryptococcus*. As summarized in Table 1, 81 patients were infected with isolates of *C. neoformans* var. *grubii* (i.e., molecular type VNI, VNII, or VNB), 4 patients were infected with diploid hybrids of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (i.e., AD hybrids or molecular type VNIII), and 4 were infected with *C. gattii* (molecular type VGI or VGIV). Because the AD hybrids were few in number and problematic to analyze, the seven isolates and those patients infected with them were removed from the study. However, AD hybrids have been previously reported from South Africa (12). The remaining 85 patients with CM had a total of 178 incident and serial isolates. There were 170 isolates of *C. neoformans* var. *grubii* and 8 isolates of *C. gattii*. The isolates of *C. neoformans* var. *grubii* consisted of 139 strains of the globally predominant VNI molecular type, 22 isolates of the VNII molecular type, and 9 strains of the VNB molecular type. Consistent with previous surveys of the molecular types in South Africa, there were no isolates of VNIV (*C. neoformans* var. *neoformans*), VGII, or VGIII (12, 25).

Antifungal treatment of patients. Table 2 summarizes some of the demographic, clinical, molecular, and laboratory data associated with each patient and isolate. The treatments administered to these patients for CM varied considerably. Although most of the patients were treated with AMB and/or FLZ, the dosages and durations of their administration were not uniform. This inconsistency, which was largely attributable to drug availability and variable prescribing practices, was common during this period in South Africa (12). Of the 81 patients infected with *C. neoformans*, only 20 (24%) were treated with AMB followed by FLZ in hospital, and 14 (17%) of these patients received AMB for at least 7 days before receiving FLZ. Twenty-two patients (27%) received only AMB, and all but five of these patients were treated for 7 or more days. Twenty-nine patients (36%) were treated only with FLZ in hospital. Of the total of 49 patients who received FLZ, most (36 patients) were treated with 400 mg per day, 8 patients were treated with 200 mg per day, and 5 received 800 mg per day. Treatment with FLZ was initiated during hospitalization, as recorded in Table 2, and many of the patients probably continued to take FLZ after being discharged. However, patients were not followed after discharge, and information about the duration of FLZ treatment was not available. Four patients (cases 19, 34, 63, and 95) received neither AMB nor FLZ, and six patients (cases 13, 30, 41, 45, 47, and 75) lacked a history of treatment.

Multilocus sequence typing of incident and serially collected isolates. The MLST alleles at each locus of each isolate of *C. neoformans* var. *grubii* are depicted in Fig. S1A in the supplemental material. Variations in DNA sequences at these loci permitted the identification of multiple, distinct sequence types. We concatenated the sequences of each isolate at all eight loci and analyzed the phylogenetic relationships of these 170 isolates to one another as well as their relatedness to more than 50 previously genotyped strains from around the world (see Table S1 in the supplemental material) (18–21). The resulting neighbor-joining phylogram is presented in Fig. S1B in the supplemental material. Most genotypes were identical or differed only slightly (e.g., in one or more single nucleotide polymorphisms) from reference strain(s) with established genotypes (Table S1). As shown in Table 3, this comparative analysis enabled us to identify the genotypes of these isolates.

The overall diversity of these isolates is consistent with previous analyses of southern African isolates. The 139 isolates of molecular type VNI included 14 distinct multilocus sequence types or genotypes. The 22 isolates of molecular type VNII were represented by at least three genotypes, and there were six unique genotypes among the VNB isolates (Table 3). However, two of the three most prevalent VNI genotypes in this sample were identical to the globally dominant VNI genotypes, M5 and M4 (19, 26). The

TABLE 2 Serial isolates from cerebral spinal fluid specimens of 81 patients from South Africa with cryptococcal meningitis due to *C. neoformans* var. *grubii* (170 isolates) and four patients with *C. gattii* (eight isolates)

Case no.[a]	Sex[b]	Age (yr)	Province[c]	No. of days on treatment (dosage [mg/day])		Isolate no. (days after initial isolate)[e]	Molecular type	Geographic distribution	Genotype[f]	FLZ MIC (μg/ml)
				AMB[d]	FLZ					
1	F	24	MP	8 (45)	1 (400)	1	VNI	Global	M3b	4
						2 (238)	VNI	Global	M3b	4
2	F	31	GA	None	12 (400)	3	VNI	Global	M4b	0.5
						4 (121)	VNI	Global	M4b	1
3	M	63	GA	None	7 (200)	5	VNI	Global	M3b	NT
						6 (157)	VNB	African	M26a	NT
4	F	37	GA	Unknown	32 (400)	7	VNI	Global	M4	2
						8 (180)	VNI	Global	M4	4
5	M	11	KZ	16 (14)	None	9	VNII	Global	M7c	0.5
						10 (153)	VNII	Global	M7c	0.5
6	F	33	GA	Unknown	3 (400)	11	VNII	Global	M7c	NT
						12 (170)	VNI	Global	M5	NT
7	M	38	GA	None	8 (400)	13	VNB	African	M35a	1
						14 (137)	VNB	African	M35a	1
8	M	45	KZ	None	2 (400)	15	VNI	Global	M4	4
						16 (144)	VNI	Global	M4	16
9	F	10	KZ	None	7 (200)	17	VNII	Global	M7c	4
						18 (138)	VNII	Global	M7c	≥64
10	M	49	EC	2 (50)	17 (400)	19	VNI	Global	M4b	2
						20 (131)	VNI	Global	M4b	2
11	F	29	GA	None	17 (400)	21	VNI	Global	M3	4
						22 (155)	VNI	Global	M3	16
12	F	31	GA	None	7 (400)	23	VNI	Global	M5	4
						24 (121)	VNI	Global	M5	2
13	M	35	KZ	Unknown	Unknown	25	VNI	Global	M3b	8
						27 (286)	VNI	Global	M3b	8
14	M	27	NC	None	8 (400)	28	VNI	Global	M1c	2
						29 (145)	VNI	Global	M1c	4
15	F	24	GA	None	2 (800)	30	VNI	Global	M5	2
						31 (166)	VNI	Global	M5	4
						32 (223)	VNI	Global	M5	1
16	F	26	GA	None	1 (400)	33	VNI	African	M28a	4
						34 (169)	VNI	African	M28a	4
17	F	23	GA	2 (35)	3 (800)	35	VNI	African	M28a	NT
						36 (184)	VNI	Global	M5	NT
18	M	34	GA	None	5 (400)	37	VNI	African	M28a	1
						38 (171)	VNI	African	M28a	4
19	M	42	GA	None	None	39	VNII	Global	M7c	0.5
						40 (154)	VNII	Global	M7c	1
21	F	29	GA	12 (25)	None	45	VNI	Global	M5	4
						46 (266)	VNI	Global	M5	4
22	M	34	WC	13 (60)	7 (800)	47	VNI	Global	M3	4
						48 (151)	VNI	Global	M3	4
23	F	32	GA	None	2 (400)	49	VNI	Global	M3	4
						50 (221)	VNI	Global	M3	8
24	M	44	GA	None	3 (800)	51	VNI	Global	M3	8
						52 (191)	VNI	Global	M3	8
25	M	31	GA	None	3 (200)	53	VNI	Global	M5	2
						54 (149)	VNI	Global	M5	2
26	F	41	EC	7 (50)	7 (400)	55	VNI	African	M43	4
						56 (135)	VNI	African	M43	4
						57 (226)	VNI	African	M43	4
27	M	36	WC	12/14 (36)	13 (200)	58	VNI	Global	M4	4
						59 (228)	VNI	Global	M4	8
28	M	31	NC	10/11 (37.5)	None	50	VNB	African	M24a	1
						61 (129)	VNB	African	M24a	1

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TABLE 2 (Continued)

Case no.[a]	Sex[b]	Age (yr)	Province[c]	No. of days on treatment (dosage [mg/day])		Isolate no. (days after initial isolate)[e]	Molecular type	Geographic distribution	Genotype[f]	FLZ MIC (μg/ml)
				AMB[d]	FLZ					
29	M	23	KZ	None	10 (400)	62	VNI	African	M28a	4
						63 (272)	VNI	African	M28a	16
						64 (325)	VNI	African	M28a	8
30	F	49	KZ	Unknown	Unknown	65	VNI	Global	M4a	4
						66 (126)	VNI	Global	M4a	8
32	F	29	KZ	None	7 (400)	69	VNI	African	M28a	2
						70 (263)	VNI	Global	M5	1
33	M	30	GA	5/5 (50)	None	71	VNI	African	M32	2
						72 (124)	VNI	African	M32	8
						73 (187)	VNI	African	M32	8
34	F	23	EC	None	None	74	VNI	Global	M5	2
						75 (176)	VNI	Global	M5	2
35	M	56	GA	None	12 (400)	76	VNI	Global	M5	8
						77 (236)	VNI	Global	M5	2
36	F	32	GA	6/6 (50)	10 (400)	78	VNI	Global	M1	4
						79 (156)	VNI	Global	M1	4
						80 (254)	VNI	Global	M1	4
37	F	35	FS	12/13 (40)	None	81	VNI	Global	M3b	2
						82 (163)	VNI	Global	M3b	2
38	F	34	GA	13/13 (50)	None	83	VNI	Global	M4	2
						84 (141)	VNI	Global	M4	4
39	F	25	KZ	5/6 (50)	14 (200)	85	VNI	Global	M4	NT
						86 (225)	VNI	Global	M3	NT
40	M	26	KZ	5/5 (40)	None	87	VNII	Global	M7a	0.5
						88 (276)	VNII	Global	M7a	0.5
41	F	20	KZ	Unknown	Unknown	89	VNI	Global	M3	4
						90 (153)	VNI	Global	M3	8
43	F	26	MP	22/21 (36)	8 (400)	91	VNI	Global	M5	NT
						92 (174)	VNB	African	M24b	NT
44	M	33	GA	None	6 (400)	93	VNI	Global	M5	2
						94 (169)	VNI	Global	M5	2
45	M	36	KZ	Unknown	Unknown	99	VNII	Global	M7c	0.5
						100 (131)	VNII	Global	M7c	8
46	M	32	KZ	4/4 (45)	None	101	VNI	Global	M4	NT
						102 (233)	VNI	Global	M5	NT
47	M	24	GA	Unknown	Unknown	103	VNI	Global	M3b	4
						104 (153)	VNI	Global	M3b	2
55	M	22	KZ	14/14 (55)	None	120	VNI	Global	M5	4
						121 (142)	VNI	Global	M5	2
56	F	28	GA	None	15 (400)	122	VNI	African	M28a	4
						123 (209)	VNI	African	M28a	4
57	M	41	GA	1/1 (30)	26 (400)	124	VNI	African	M28a	4
						125 (152)	VNI	African	M28a	8
58	F	28	GA	13/13 (50)	4 (400)	126	VNI	African	M28a	4
						127 (131)	VNI	African	M28a	4
59	F	37	KZ	2/2 (30)	None	128	VNI	Global	M4a	NT
						129 (125)	VNI	Global	M4a	NT
61	M	30	MP	11/15 (36)	None	132	VNI	Global	M4	2
						133 (137)	VNI	Global	M4	2
62	F	30	KZ	7/7 (35)	None	134	VNI	Global	M5	2
						135 (127)	VNI	Global	M5	2
63	F	30	KZ	None	None	136	VNI	Global	M4	2
						137 (168)	VNI	Global	M4	4
64	F	32	KZ	13/15 (50)	None	138	VNII	Global	M7c	4
						139 (131)	VNI	Global	M3b	4
66	F	32	KZ	8/8 (50)	None	140	VNI	Global	M4	8
						141 (152)	VNI	Global	M4	8
68	F	20	KZ	7/7 (50)	None	144	VNI	Global	M3c	4
						147 (127)	VNI	Global	M3	8

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TABLE 2 (Continued)

Case no.[a]	Sex[b]	Age (yr)	Province[c]	No. of days on treatment (dosage [mg/day]) AMB[d]	FLZ	Isolate no. (days after initial isolate)[e]	Molecular type	Geographic distribution	Genotype[f]	FLZ MIC (μg/ml)
70	F	52	GA	None	28 (400)	150	VNB	African	M20a	NT
						151 (187)	VNI	Global	M3b	8
						152 (199)	VNI	Global	M3b	8
71	M	39	GA	Unknown	15 (400)	153	VNI	Global	M4	4
						154 (136)	VNI	Global	M4	2
72	F	34	GA	11 (50)	13 (400)	155	VNI	Global	M5	8
						156 (242)	VNI	Global	M5	16
74	F	41	GA	None	4 (400)	159	VNI	Global	M3	2
						160 (246)	VNI	Global	M3	4
75	F	30	WC	Unknown	Unknown	161	VNI	African	M28a	4
						162 (178)	VNI	African	M28a	2
76	M	39	LP	None	21 (400)	163	VNI	African	M28a	2
						164 (150)	VNI	African	M28a	4
77	F	40	WC	13 (32)	1 (400)	165	VNII	Global	M8a	1
						166 (182)	VNII	Global	M8a	4
						167 (290)	VNII	Global	M8a	1
78	F	28	GA	8 (40)	36 (400)	168	VNI	African	M28a	4
						169 (134)	VNI	African	M28a	2
79	F	28	GA	None	21 (400)	170	VNI	Global	M1	2
						171 (144)	VNI	Global	M1	4
80	F	28	GA	None	10 (400)	172	VNI	Global	M5	2
						173 (254)	VNI	Global	M5	4
81	M	49	GA	None	14 (400)	174	VNB	African	M24c	1
						175 (146)	VNB	African	M24c	32
82	F	29	WC	14 (50)	None	176	VNI	African	M41	8
						177 (182)	VNI	African	M41	4
83	F	10	WC	23 (18)	8 (200)	178	VNI	Global	M5	2
						179 (146)	VNI	Global	M5	≥64
84	F	27	GA	11 (40)	1 (400)	180	VNI	Global	M5	4
						181 (119)	VNI	Global	M5	8
85	F	53	KZ	3 (50)	None	182	VNI	Global	M4	1
						183 (131)	VNI	Global	M4	1
86	F	26	KZ	14 (50)	None	184	VNI	Global	M5	1
						185 (175)	VNI	Global	M5	2
87	F	29	KZ	10 (45)	None	186	VNI	Global	M3c	2
						187 (136)	VNI	Global	M3c	2
88	M	36	GA	7 (50)	10 (200)	188	VNI	Global	M5	1
						189 (184)	VNI	Global	M5	2
90	F	40	EC	1 (50)	18 (400)	192	VNII	Global	M7a	1
						193 (147)	VNII	Global	M7a	1
93	F	23	KZ	7 (30)	3 (400)	200	VNI	African	M28a	4
						201 (165)	VNI	African	M28a	16
94	M	49	KZ	10 (45)	None	202	VNII	Global	M7a	4
						203 (167)	VNII	Global	M7a	2
95	M	42	WC	None	None	204	VNI	Global	M4b	8
						205 (111)	VNI	Global	M4b	16
96	M	36	LP	12 (50)	6 (200)	206	VNI	Global	M3	2
						207 (197)	VNI	Global	M3	2
97	F	29	NW	14 (50)	None	208	VNI	Global	M5	4
						209 (159)	VNI	Global	M5	4
98	F	30	KZ	9 (50)	None	210	VNI	Global	M5	2
						211 (142)	VNI	Global	M5	2
99	M	25	GA	None	12 (800)	212	VNII	Global	M7c	0.5
						213 (174)	VNII	Global	M7c	0.5
						214 (243)	VNII	Global	M7c	1
31	M	34	KZ	4/4 (10)	None	67	VGIV	African	ST1	NT
						68 (124)	VGIV	African	ST1	NT
67	M	46	MP	12/13 (50)	None	142	VGIV	African	ST2	NT
						143 (135)	VGIV	African	ST2	NT

(Continued on following page)

TABLE 2 (Continued)

Case no.[a]	Sex[b]	Age (yr)	Province[c]	No. of days on treatment (dosage [mg/day])		Isolate no. (days after initial isolate)[e]	Molecular type	Geographic distribution	Genotype[f]	FLZ MIC (μg/ml)
				AMB[d]	FLZ					
69	F	39	MP	Unknown	3 (400)	148	VGI	Global	ST3	NT
						149 (134)	VGI	Global	ST3	NT
91	M	36	KZ	10 (50)	None	194	VGI	Global	ST4	NT
						195 (224)	VGI	Global	ST4	NT

[a] Incident and serial isolates of nine patients differed in their genotypes (the case numbers of those patients [3, 17, 32, 39, 43, 46, 64, 68, and 70] are in bold type). Among the 72 patients whose incident and serial isolates of *C. neoformans* var. *grubii* had the same genotype, in 11 cases, the MICs to FLZ of the serial isolates were ≥4-fold higher than the MIC of the incident isolate (the case numbers of those patients [8, 9, 11, 18, 29, 33, 45, 77, 81, 83, 93] are underlined).

[b] F, female; M, male.

[c] Abbreviations of provinces: EC, Eastern Cape; FS, Free State; GA, Gauteng; KZ, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NW, North West; WC, Western Cape. Other abbreviations: AMB, amphotericin B; FLZ, fluconazole; MIC, minimum inhibitory concentration; NT, not tested.

[d] When 2 days are listed and separated by a slash, the first number indicates the number of doses administered, and the second number indicates the number of days for which treatment was prescribed.

[e] The nine isolates of *C. neoformans* (numbers 16, 18, 22, 63, 156, 175, 179, 201, and 205) with MICs to FLZ of ≥16 μg/ml are in bold type. All but six isolates are haploid; the six diploid (*2n*) isolates are numbers 18, 34, 35, 52, 120, and 121. With one exception, all the isolates possess the dominant *MAT*α mating type allele; African VNB isolate no. 150 has the rare *MAT***a** mating type.

[f] For explanations of the designations of MLST genotypes, refer to the text, Fig. S1B, and Table S1 in the supplemental material.

third most common genotype, M28a, is closely related to reference strain BT150 (see Table S1 and Figure S1B in the supplemental material), which belongs to the subpopulation that is genetically and ecologically related to the global strains of VNI but is restricted to southern Africa (19). At the other extreme, several unique genotypes were quite rare and isolated only from individual patients. Analyses of the clinical data in Table 2 revealed no association of cryptococcal genotypes with patients' gender, age, or provincial location.

A comparison of the molecular genotypes of strains from serial episodes due to *C. neoformans* var. *grubii* indicated that in 72 of the 81 cases, the genotypes of the initial and serially collected strains were identical, which is consistent with persistence or relapse of the original infection. Because 70% of the isolates were represented by the five most common genotypes (*viz.*, M5, M28a, M4, M3, and M7c), it is possible that some patients with identical serial isolates may have been reinfected with another isolate of the same genotype. However, nine patients (Table 2) (cases 7, 14, 26, 28, 33, 77, 81, 82, and 89) were infected with identical and unique serial isolates that were found in no other patient, and each of these cases was undoubtedly a relapse of the original infection. Conversely, in nine (11%) other cases, the initial and serial strains had different genotypes, which is consistent with reinfection. (The possibility of an original infection with different strains was excluded by reanalyzing the original cultures without single-colony purification.)

We considered the hypothesis that the length of time between collection of the incident and serial isolates was associated with the probability of reinfection with a new genotype. However, this possibility could not be addressed because the serial isolates were obtained only when CSF cultures were clinically warranted; they were not routinely obtained at regular intervals. Consequently, we observed no significant differences in times between the collections of serial isolates from patients who had identical (range, 111 to 286 days; median, 153 days; mean ± standard deviation [SD], 163 ± 41.8 days) or different genotypes (range, 127 to 263 days; median, 184 days; mean ± SD, 173 ± 44.5 days).

The isolates of *C. gattii* were obtained from four HIV-positive patients and included four strains of the VGI molecular type and four strains of the VGIV molecular type (Tables 1 and 2). Each patient with *C. gattii* had identical initial and serial isolates with unique genotypes (see Fig. S2 in the supplemental material).

Mating types. Mating type- and serotype-specific primers were used to determine the mating type of each isolate (Table 1). All eight isolates of *C. gattii*, and all but one isolate of *C. neoformans*, had the dominant *MAT*α mating type allele. The exception was an isolate of the African molecular type VNB (genotype M20a) with the rare *MAT***a** allele (Table 2) (isolate number 150), which was confirmed by mating assays in the laboratory. This isolate was responsible for the incident infection in patient 70; she was treated only with FLZ and subsequently relapsed with a global strain of VNI. (In addition, each of the seven AD hybrid strains possessed both *MAT*α and *MAT***a** alleles.)

Ploidy. The ploidy of *C. neoformans* has been reported to change during the course of infection (22, 27). The six diploid isolates of molecular type VNI or VNII (all *MAT*α/α) were obtained from five patients: the incident and serial isolates of patient 9 had the same relatively common genotype of VNII, but the incident isolate (Table 2) (isolate number 17) was haploid, and the serial isolate (isolate number 18), which was collected 138 days later, was diploid, and its MIC to FLZ had increased from 4 to ≥64 μg/ml. This was the only case in which a change in ploidy was accompanied by increased resistance to FLZ. The incident isolates of patients 16 and 24 were haploid VNI strains of the African genotype M28a and the global genotype M3, respectively. Their serial isolates were diploid, but there was no change in their genotypes or MICs to FLZ. The incident and serial isolates of patient 55 were identical diploid strains of the most prevalent global VNI genotype, M5. However, patient 17 was undoubtedly reinfected with a different strain of VNI. The genotype of her incident isolate (Table 2) (isolate number 35) was a diploid strain of the African VNI clade (genotype M28a), and the serial isolate (isolate number 36) was a haploid strain of the global VNI clade with the common M5 genotype.

Susceptibility of isolates of *C. neoformans* var. *grubii* to FLZ. The MIC to FLZ was assayed in isolates of *C. neoformans* var. *grubii* from patients whose incident and serial isolates had identical genotypes. Several isolates had relatively high MICs to FLZ. The persistence of these infections may have been attributable to

TABLE 3 MLST genotypes of 170 incident and serial clinical isolates of *C. neoformans* var. *grubii*

Molecular type	Major clade	Key reference strain	Geographic distribution	Genotype[a]	Isolate no.	No. of isolates	Isolate MICs to FLZ of ≥8 μg/ml[b] (%)
VNI global	A1/M1	A1-35-8	Argentina, Australia, France, Malawi, South Africa, USA	M1	78, 79, 80, 170, 171	5	0
				M1c	28, 29	2	
	A3/M3	A3-1-1	Belgium, France, Italy, South Africa, Uganda, USA	M3	21, **22**, 47, 48, 49, 50, 51, 52, 86, 89, 90, 147, 159, 160, 206, 207	16	34.5
				M3c	144	1	
		UG2471	Belgium, Brazil, Botswana, India, South Africa, Uganda	M3b	1, 2, 5, 25, 27, 81, 82, 103, 104, 139, 151, 152	12	
				M3c	186, 187	2	
	A4/M4	BR795	Belgium, Botswana, Brazil, India, South Africa, Tanzania, Uganda	M4	7, 8, 15, **16**, 58, 59, 83, 84, 85, 101, 132, 133, 136, 137, 140, 141, 153, 154, 182, 183	20	23.1
		C27	Democratic Republic of the Congo, India, South Africa, USA	M4a	65, 66, 128, 129	4	
		A4-1-12	France, South Africa, USA	M4b	3, 4, 19, 20, 204, **205**	6	
	A5/M5	A5-35-17	Belgium, Botswana, China, Italy, Japan, Malawi, South Africa, USA	M5	12, 23, 24, 30, 31, 32, 36, 45, 46, 53, 54, 70, 74, 75, 76, 77, 91, 93, 94. 102, 120, 121, 134, 135, 155, **156**, 172, 173, 178, **179**, 180, 181, 184, 185, 188, 189, 208, 209, 210, 211	40	13.9
VNI African	SA VNI	BT150	Botswana, South Africa	M28a	33, 34, 35, 37, 38, 62, **63**, 64, 69, 122, 123, 124, 125, 126, 127, 161, 162, 163, 164, 168, 169, 200, **201**	23	23.3
		BT9		M43	55, 56, 57	3	
		BT15		M32	71, 72, 73	3	
		BT121		M41	176, 177	2	
VNII global	VNII	8-1	South Africa, USA	M7a	87, 88, 192, 193, 202, 203	6	9.5
		C45		M7c	9, 10, 11, 17, **18**, 39, 40, 99, 100, 138, 212, 213, 214	13	
		C44		M8a	165, 166, 167	3	
VNB African	VNB	BT33	Botswana, South Africa	M26a	6	1	16.7
		BT88		M35a	13, 14	2	
		BT35		M24a	60, 61	2	
				M24b	92	1	
				M24c	174, **175**	2	
		BT206		M20a	150	1	

[a] The MLST allelic profile of each isolate can be observed in Figure S1A in the supplemental material. As shown by the subsequent phylogenetic analysis in Figure S1B, most strains were identical or closely related to reference strains (also see Table S1 in the supplemental material) that were previously genotyped by amplified fragment length polymorphisms (AFLP) and MLST methods and designated with an A and M genotype, respectively (12, 18, 19, and 26). Strains with minor allelic variations are denoted with a lowercase suffix (a, b, etc.). Genotypes within the SA VNI and VNB clades have been found only in southern Africa. Most of the VNII strains in this study are closely related to previously genotyped global isolates of VNII, but the database of genotyped VNII isolates is too small to be certain of their distribution.

[b] Of the 170 isolates, 155 were tested for susceptibility to FLZ, and 31 (20%) had an MIC of ≥8 μg/ml. Nine isolates (5.2%) had an MIC to FLZ of ≥16 μg/ml, and they are in bold type (isolate numbers 16, 18, 22, 63, 156, 175, 179, 201, and 205). The last column indicates the percentage of tested isolates within each major clade that had an MIC to FLZ of ≥8 μg/ml.

insufficient treatment and/or the acquisition of resistance within the patient. Of 155 isolates that were tested, 31 (20%) had MICs to FLZ of ≥8 μg/ml, and nine (5.8%) had MICs to FLZ of ≥16 μg/ml. The latter were all relapse isolates, and they were distributed among seven different genotypes (see the bolded isolate numbers in Tables 2 and 3). Of the 128 tested isolates of molecular type VNI, 28 (21.9%) had MICs of ≥8 μg/ml. The last column of Table 3 shows the percentages of tested isolates in each major clade with MICs of ≥8 μg/ml For example, 10 of the 29 (34.5%) tested isolates within the global VNI clade of A3/M3 genotypes had MICs of ≥8 μg/ml, but only 5 of 36 tested isolates within the A5/M5 clade had MICs of ≥8 μg/ml.

A common metric of acquired resistance to FLZ is a ≥4-fold increase in the MIC. The serial isolates of 11 patients (15%) were significantly more resistant (i.e., with MICs at ≥4-fold higher) than their genetically identical incident isolates (see Table 1, underlined patient numbers 8, 9, 11, 18, 29, 33, 45, 77, 81, 83, and 93). Because eight of these patients (cases 8, 9, 11, 18, 29, 45, 83, and 93) were infected with strains that possessed more prevalent genotypes (M3, M4, M5, M7c, and M28a), it is also possible that during the time between obtaining the incident and serial isolates, some of these patients could have become reinfected with a more resistant strain of the same genotype. However, three patients (cases 33, 77, and 81) were infected with strains of relatively rare

genotypes (M32, M8a, and M24c, respectively). Considering their comparative rarity, these cases of infection with more resistant serial isolates were most likely not reinfections but relapses caused by the proliferation of resistant clones during the course of chronic infection.

As noted above, because of the stochastic timing of serial isolations, it was not possible to assess whether the number of days between incident and serial isolations was associated with the acquisition of resistance to FLZ. Indeed, there were no appreciable differences in the timing of their collection from the 11 patients with FLZ-resistant serial isolates (range, 124 to 272 days; median, 146 days; mean ± SD, 151 ± 39.7 days) and the other patients for whom MIC values were available (range, 111 to 286 days; median, 153 days; mean ± SD, 165 ± 42.2 days). Consequently, it is impossible to conclude from these data that exposure to FLZ poses a risk of resistance.

DISCUSSION

Recurrent cryptococcosis is well documented among HIV-infected patients in South Africa (8, 12, 13, 28). However, genetic relationships between the incident and recurrent strains have not been previously evaluated. We used the MLST method to genotype the initial or incident isolates and serially collected recurrent isolates that were obtained >110 days after the incident isolate. Seventy-two of 81 patients with *C. neoformans* var. *grubii* and four patients with *C. gattii* had identical MLST genotypes. There are three alternative explanations for the isolation of strains with identical genotypes from serial disease episodes. First, these cases may represent persistence of the disease, in which the cryptococcal strain causing the initial infection was not eradicated due to inadequate treatment, treatment failure, IRIS, or resistance of the isolate to antifungal drugs. IRIS is less likely to have developed in these patients because only very few received ART (29). Second, even among cases where eradication was achieved by antifungal treatment, relapse for the same reasons may also be possible. Third, it is also possible that patients were reinfected by a strain with an identical genotype. This possibility is difficult to refute because most patients were infected with relatively prevalent genotypes However, strains with rare genotypes (i.e., genotypes that occurred only once in the entire collection) were isolated from serial episodes of nine patients with *C. neoformans* var. *grubii* and all four patients with *C. gattii*. In these cases, reinfection with strains of the same rare genotypes would require regular exposure to these strains, which is not consistent with the reported distribution of genotypes in the environment (18, 19, 25).

Resistance of strains of *C. neoformans* var. *grubii* to FLZ may contribute to recurrent disease among South African patients. MIC testing by the CLSI broth microdilution method demonstrated that recurrent isolates from 11 patients became at least 4-fold more resistant to FLZ than did the original strains isolated from the same patients (see underlined cases in Table 2). Twenty-two percent of the tested isolates had MICs of ≥8 μg/ml, and 5.8% had MICs of ≥16 μg/ml. Our recent epidemiological survey of 487 incident isolates of *C. neoformans*, obtained from patients with serial cases of cryptococcal meningitis separated by at least 30 days, indicated that only 0.6% of strains had MICs of ≥16 μg/ml (23), which was consistent with other reports that <1% of all strains in Africa had MICs of ≥16 μg/ml (30). In this study, the higher proportion and number of resistant strains were isolated from a biased selection of patients with serial episodes separated by at least 110 days. These results indicate that changes in susceptibility levels may occur during chronic infection, and the emergence of resistant strains may occasionally contribute to relapse of the disease.

FLZ is a fungistatic drug, and exposure to FLZ could have selected resistant mutants that subsequently proliferated, leading to clinical relapse. Mechanisms of resistance include altered expression of certain genes (e.g., *ERG1* and *AFR1*), efflux pumps, and gene duplications. It is known that *in vitro* growth of *C. neoformans* in the presence of sublethal concentrations of FLZ induces the selection of resistant colonies with elevated MICs to FLZ (31, 32), and a similar increase was recently shown to occur in infected mice that were treated with FLZ (33). However, 40 of the 49 patients who received FLZ in the hospital relapsed with the same isolate, but there was no significant increase in the MIC to FLZ. Unfortunately, no follow-up records were available to document which, if any, patients received treatment with FLZ after they were discharged from hospital.

The other factors that contribute to the recurrence of cryptococcosis among South African patients are limited access to treatment and inadequate treatment (7, 8, 12). Although none of the patients received Infectious Diseases Society of America (IDSA)- and World Health Organization (WHO)-recommended induction therapy with AMB and flucytosine, most received AMB followed by FLZ. Indeed, some patients received ample dosages of AMB and/or FLZ (e.g., patients 4, 22, 27, 58, and 78 in Table 2), and their relapse strains had the same genotypes and MICs to FLZ as the original isolates. Clearly, the original infecting strain may persist despite apparently adequate treatment and the absence of *in vitro* antifungal resistance. These observations emphasize the need for patients to be monitored for any signs of relapse after completion of the initial treatment.

The incident and serial isolates of nine patients had differing genotypes. There are three nonexclusive explanations for the isolation of strains with different MLST genotypes from serial episodes of cryptococcosis. It is possible that these cases represent subsequent new infections that were acquired from the environment. Our data suggest that *C. neoformans* var. *grubii* is common in the environment in southern Africa (12, 26), and people who do not receive or adhere to ART may become infected again with different strains. It is also possible that patients were simultaneously infected with more than one strain. Recent evidence indicates that approximately 30% of cryptococcal infections in France are caused by multiple strains (27). To test for any evidence of mixed infections in our samples, we obtained DNA from the entire population of cryptococcal cells isolated from each patient (not from single colonies) and performed MLST to detect any evidence of more than a one strain. If a multiple infection were present, we would have observed mixed MLST patterns similar to those obtained from the direct sequencing of the AD hybrid strains. However, identical MLST patterns were obtained from the individual colonies and mixed populations of cells, suggesting that mixed infections with multiple strains are not common in South Africa. Alternatively, it is possible that a single strain may have been selected from the original cerebrospinal fluid (CSF) culture for long-term storage.

Results of molecular epidemiological analysis of strains in this study are consistent with our previous observations and confirm the high genetic diversity among strains of *C. neoformans* var. *grubii* in South Africa (18, 19, 25, 26). Although MLST genotyping

has been somewhat standardized (15), many studies vary the protocol or employ other methods, and there is no universal nomenclature for designating VNI genotypes. However, where similar methods have been used or genotyping strains have been analyzed phylogenetically for similarity to reference strains with known genotypes, the global dominance of a small number of VNI genotypes has been demonstrated. For example, MLST analyses of several hundred VNI isolates from the United States and a dozen other countries yielded only a few distinct genotypes (designated M1 to M5), of which genotype M5 was the most prevalent (18, 20). Subsequent investigations have shown that the M5 is the dominant genotype among clinical isolates from China, Japan, Thailand, and South Korea (34–37). In contrast to the limited genetic diversity observed among global isolates of VNI, two subpopulations of VNI coexist in southern Africa: (i) strains with SA VNI genotypes that are closely related to the global genotypes of VNI (see Fig. S1B in the supplemental material) and associated with the same environmental niches (e.g., avian feces), but are more diverse and apparently confined to southern Africa, and (ii) a genetically diverse, endemic population (VNB genotypes) that is associated with indigenous African trees (19). Predictably, the majority of the patients reported here were infected with strains of the globally dominant VNI genotypes of M5 and M4, as well as the southern African SA VNI genotypes (19).

To our knowledge, this study represents the first molecular epidemiological investigation of strains isolated from serial episodes of cryptococcosis in Africa. The results indicate that the majority of infections were caused by strains with identical molecular genotypes and suggest persistence or relapse of the original infecting strain rather than independent infections with multiple strains. Within this highly selected group, there were also relatively high percentages of strains with reduced susceptibilities to fluconazole. These results highlight the importance of properly treating cryptococcosis and monitoring patients after the completion of treatment.

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