Global Trends in the Antifungal Susceptibility of *Cryptococcus neoformans* (1990 to 2004)

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The antifungal susceptibilities of 1,811 clinical isolates of *Cryptococcus neoformans* obtained from 100 laboratories in 5 geographic regions worldwide between 1990 and 2004 were determined. The MICs of amphotericin B, flucytosine, fluconazole, voriconazole, posaconazole, and ravuconazole were determined by the National Committee for Clinical Laboratory Standards broth microdilution method. Isolates were submitted to a central reference laboratory (University of Iowa) from study centers in Africa (5 centers, 395 isolates), Europe (14 centers, 102 isolates), Latin America (14 centers, 82 isolates), the Pacific region (7 centers, 50 isolates), and North America (60 centers, 1,182 isolates). Resistance to amphotericin B, flucytosine, and fluconazole was $\leq 1\%$ overall. Susceptibility to flucytosine (MIC, $\leq 4 \mu\text{g/ml}$) ranged from 35% in North America to 68% in Latin America. Similarly, only 75% of isolates from North America were susceptible to fluconazole (MIC, $\leq 8 \mu\text{g/ml}$) compared to 94 to 100% in the other regions. Isolates remained highly susceptible to amphotericin B (99% susceptibility at a MIC of $\leq 1 \mu\text{g/ml}$) over the entire 15-year period. Susceptibility to flucytosine (MIC, $\leq 4 \mu\text{g/ml}$) increased from 34% in 1990 to 1994 to 66% in 2000 to 2004. Susceptibility to fluconazole (MIC, $\leq 8 \mu\text{g/ml}$) increased from 72% in 1990 to 1994 to 96% in 2000 to 2004. Voriconazole, posaconazole, and ravuconazole all were very active (99% of isolates susceptible at MIC of $\leq 1 \mu\text{g/ml}$) against this geographically diverse collection of isolates. We conclude that in vitro resistance to antifungal agents used in the treatment of cryptococcosis remains uncommon among isolates of *C. neoformans* from five broad geographic regions and has not increased over a 15-year period.

Cryptococcosis is one of the leading community-acquired opportunistic mycoses (10, 11, 20, 21, 23, 29, 33). Although serious disease (e.g., meningitis and cryptococemia) may occur in healthy hosts, the majority of infections occur in patients with significant underlying predisposing factors, such as organ transplantation, hematologic malignancies, and advanced human immunodeficiency virus disease, particularly those not receiving highly active antiretroviral therapy (HAART) (20, 21, 33). Pharmacologic management of cryptococcal infections usually consists of primary therapy with amphotericin B, with or without flucytosine, followed by maintenance therapy, or in some instances life-long suppressive therapy, with fluconazole (43). High rates of fungal persistence and frequent disease relapse have sparked a growing concern among clinicians regarding the potential for the emergence of antifungal resistance among *Cryptococcus neoformans* (8, 11).

There are now several published reports of the emergence of resistance to amphotericin B, fluconazole, flucytosine, or itraconazole in *C. neoformans* during treatment (7, 9–11, 14, 15, 17, 24, 34, 42). Most of these reports involve resistance to fluconazole emerging in the setting of meningitis in AIDS patients after long treatments or prophylaxis with fluconazole (7, 8, 34). Recent reports from Cambodia (12, 44) and from India (16) have raised the concern of a more widespread increase in fluconazole resistance among *C. neoformans*, suggest-

ing the need for greater vigilance and more-widespread surveillance.

Although antifungal resistance surveillance with a focus on *Candida* spp. is now widespread (39, 40), similar programs providing temporal and geographic data regarding the antifungal susceptibility of *C. neoformans* isolates are quite limited. Studies by Brandt et al. (11), Davey et al. (17), Klepser and Pfaller (25), and Yildiran et al. (48) provide in vitro antifungal susceptibility data generated by National Committee for Clinical Laboratory Standards (NCCLS) reference MIC methods (30), indicating that in vitro resistance to commonly used antifungal agents (i.e., amphotericin B, flucytosine, fluconazole, and itraconazole) remains uncommon among *C. neoformans* and has not increased with time in the last decade in the United States and in the United Kingdom. In contrast, Sar et al. (44) report an increase in resistance to fluconazole from 2.5% to 14% between 2001 and 2002 in a hospital in Cambodia. Both Archibald et al. (6) and Datta et al. (16) stress the need for expanded international surveillance of antifungal susceptibility of cryptococcal isolates; however, the published literature thus far is limited to small studies of limited geographic scope conducted over rather short periods of time (2, 6, 12–14, 16, 18, 41, 44).

At the University of Iowa, we have conducted surveillance of cryptococcosis (i.e., meningitis and cryptococemia) using a consistent protocol (consecutive incident isolates, one per patient, multiple institutions) and standardized reference quality identification and antifungal susceptibility testing methods from 1990 up to the present (25, 37, 38, 47). This cumulative experience allows us to examine change in the in vitro suscep-

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TABLE 1. Activities of amphotericin B, flucytosine, and fluconazole against 1,811 clinical isolates of *Cryptococcus neoformans* from five geographic regions^a

Area, no. of sites ^b (<i>n</i>)	Antifungal agent	Cumulative % inhibited at MIC (μg/ml) of:											
		0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	
Africa, 5 (395)	Amphotericin B	0	0	2	27	99 ^c	100						
	Flucytosine	0	0.3	0.3	0.5	2	15	60 ^d	95	99	99	99	
	Fluconazole		0	1	1	7	33	79	97 ^e	99	100		
Europe, 14 (102)	Amphotericin B	0	0	5	44	99 ^c	100						
	Flucytosine	0	0	0	0	4	15	51 ^d	88	99	100		
	Fluconazole		0	0	0	9	34	82	97 ^e	99	100		
Latin America, 14 (82)	Amphotericin B	0	0	7	54	100 ^c							
	Flucytosine	0	0	0	0	4	21	68 ^d	92	98	99	99	
	Fluconazole		0	0	2	9	34	79	94 ^e	96	99	100	
Pacific, 7 (50)	Amphotericin B	0	0	2	23	98 ^c	100						
	Flucytosine	0	0	2	2	6	32	66 ^d	98	100			
	Fluconazole		0	0	1	12	54	78	100 ^e				
North America, 60 (1,182)	Amphotericin B	0	0	1	27	99 ^c	100						
	Flucytosine	0.1	0.1	0.3	0.5	4	12	35 ^d	86	99	99	99	
	Fluconazole		0.2	0.4	1	4	17	43	75 ^e	94	98	99	
Total, 100 (1,811)	Amphotericin B	0	0	2	29	99 ^c	100						
	Flucytosine	0.1	0.1	0.3	0.5	4	14	44 ^d	89	99	99	99	
	Fluconazole		0.1	0.4	1	5	23	56	83 ^e	96	99	99	

^a Broth microdilution testing according to NCCLS M27-A2 (30).^b Number of study sites contributing isolates; n = no. of isolates.^c Percentage of isolates susceptible to amphotericin B at the breakpoint of ≤1 μg/ml.^d Percentage of isolates susceptible to flucytosine at the NCCLS breakpoint of ≤4 μg/ml.^e Percentage of isolates susceptible to fluconazole at the NCCLS breakpoint of ≤8 μg/ml.

tibility of *C. neoformans* to commonly used antifungal agents (amphotericin B, flucytosine, fluconazole) spanning a 15-year period and representing 100 medical centers in five geographic regions throughout the world. In addition, this diverse collection of clinically significant isolates provides the opportunity for a head-to-head comparison of the activities of the “expanded-spectrum” triazoles, voriconazole, posaconazole, and ravuconazole.

MATERIALS AND METHODS

Organisms. A total of 1,811 clinical isolates of *C. neoformans* obtained from 100 different medical centers in Africa (395 isolates from 5 sites), Europe (102 isolates, 14 sites), Latin America (82 isolates, 14 sites), the Pacific region (50

isolates, 7 sites), and North America (1,182 isolates, 60 sites) were tested (Table 1). The isolates represented consecutive incident isolates from patients with either cryptococcal meningitis or cryptococemia (one isolate per patient) collected at the respective medical centers between 1990 and 2004. The number of isolates submitted in each 5-year period was 882 between 1990 and 1994, 407 between 1995 and 1999, and 522 between 2000 and 2004 (Table 2).

The isolates were identified by standard methods (22) and were stored as water suspensions until used. Upon receipt at the University of Iowa, the isolates were subcultured onto potato dextrose agar not containing antibiotics (Remel, Lenexa, KS) and CHROMagar *Candida* medium (Hardy Laboratories, Santa Maria, CA) to ensure viability and purity. Identifications were reconfirmed with Vitek and API yeast identification systems (bioMérieux, St. Louis, MO).

Antifungal agents. Standard antifungal powders of amphotericin B (Sigma), flucytosine (Sigma), fluconazole (Pfizer, New York, NY), voriconazole (Pfizer), posaconazole (Schering-Plough, Kenilworth, NJ), and ravuconazole (Bristol-

TABLE 2. Activities of amphotericin B, flucytosine, and fluconazole against 1,811 clinical isolates of *Cryptococcus neoformans* over 15 years, 1990 to 2004^a

Antifungal agent	Time (yr) ^b	No. of isolates tested	Cumulative % inhibited at MIC (μg/ml) of:											
			0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	
Amphotericin B	90–94	882	0	0	1	24	99 ^c	100						
	95–99	407	0	0	2	28	99 ^c	100						
	00–04	522	0	0	3	38	99 ^c	100						
	90–04	1,811	0	0	2	29	99 ^c	100						
Flucytosine	90–94	822	0	0	0.2	0.2	3	10	34 ^d	88	99	99	99	
	95–99	407	0.2	0.2	0.2	1	4	13	38 ^d	84	98	98	99	
	00–04	522	0	0.2	0.6	1	4	22	66 ^d	94	99	99	99	
	90–04	1,811	0.1	0.1	0.3	0.5	4	14	44 ^d	89	99	99	99	
Fluconazole	90–94	882		0	0.2	0.2	2	13	39	72 ^e	93	98	99	
	95–99	407		0.5	0.5	1	6	22	58	89 ^e	97	98	100	
	00–04	522		0	1	2	10	41	82	96 ^e	99	99	99	
	90–04	1,811		0.1	0.4	1	5	23	56	83 ^e	96	99	99	

^a Broth microdilution testing according to NCCLS M27-A2 (30).^b Times in years from 1990 to 1994, 1995 to 1999, 2000 to 2004, and 1990 to 2004, respectively.^c Percentage of isolates susceptible to amphotericin B at the breakpoint of ≤1 μg/ml.^d Percentage of isolates susceptible to flucytosine at the NCCLS breakpoint of ≤4 μg/ml.^e Percentage of isolates susceptible to fluconazole at the NCCLS breakpoint of ≤8 μg/ml.

Myers Squibb, Princeton, NJ) were obtained from their respective manufacturers. Stock solutions were prepared in polyethylene glycol (posaconazole), dimethyl sulfoxide (amphotericin B, voriconazole, and ravuconazole), or water (fluconazole and flucytosine). Serial twofold dilutions were prepared exactly as outlined in NCCLS document M27-A2 (30). Final dilutions were made in RPMI 1640 medium (Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid buffer (Sigma). The final concentration of solvent did not exceed 1% in any well. Aliquots (0.1 ml) of each antifungal agent at a 2× final concentration were dispensed into wells of plastic microdilution trays by using a Quick Spense II System (Dynatech Laboratories, Chantilly, VA). The trays were sealed and frozen at -70°C until they were used.

Antifungal susceptibility studies. Broth microdilution testing was performed in accordance with the guidelines in NCCLS document M27-A2 (30), using RPMI 1640 medium, a final inoculum concentration of $(1.5 \pm 1.0) \times 10^3$ cells/ml, and incubation at 35°C for 72 h. The final concentrations of the antifungal agents were 0.12 to 128 $\mu\text{g/ml}$ for fluconazole, 0.06 to 128 $\mu\text{g/ml}$ for flucytosine, and 0.007 to 8 $\mu\text{g/ml}$ for all other agents. Drug-free and yeast-free controls were included.

Following incubation, the MICs of fluconazole, flucytosine, voriconazole, posaconazole, and ravuconazole were read as the lowest concentration at which a prominent decrease (approximately 50%) in turbidity relative to the turbidity of the growth control was observed (30). Amphotericin B MICs were determined to be at 100% inhibition relative to the growth control. Quality control was performed by testing the NCCLS-recommended strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (30).

The interpretive criteria for susceptibility to fluconazole and flucytosine were those published by the NCCLS (30): for fluconazole, susceptible, $\leq 8 \mu\text{g/ml}$; susceptible dose dependent, 16 to 32 $\mu\text{g/ml}$; resistant, $\geq 64 \mu\text{g/ml}$; for flucytosine, susceptible, $\leq 4 \mu\text{g/ml}$; intermediate, 8 to 16 $\mu\text{g/ml}$; resistant, $\geq 32 \mu\text{g/ml}$. Interpretive criteria have not yet been approved for amphotericin B, voriconazole, posaconazole, or ravuconazole. As suggested by Nguyen and Yu (32) and Lozano-Chiu et al. (26), isolates for which amphotericin B MICs were $\geq 2 \mu\text{g/ml}$ were considered resistant ($\leq 1 \mu\text{g/ml}$, susceptible). For purposes of comparison and because preliminary pharmacokinetic data indicated that levels of voriconazole, posaconazole, and ravuconazole achievable in serum may range from 2 to 6 $\mu\text{g/ml}$ (3–5, 19, 36), we have used a breakpoint for susceptibility of $\leq 1 \mu\text{g/ml}$ for all three agents.

RESULTS AND DISCUSSION

During the 15-year study period (1990 to 2004), a total of 1,811 clinical isolates (episodes) of *C. neoformans* were submitted by 100 study centers in Africa, Europe, Latin America, the Pacific, and North America (Table 1). Resistance to the three commonly used antifungal agents, amphotericin B, flucytosine, and fluconazole, was $\leq 1\%$ overall (Table 1). Between 98 and 100% of isolates were susceptible (MIC, $\leq 1 \mu\text{g/ml}$) to amphotericin B irrespective of geographic region. In contrast, susceptibility to flucytosine (MIC, $\leq 4 \mu\text{g/ml}$) ranged from 35% in North America to 68% in Latin America (Table 1). Likewise, only 75% of isolates from North America were susceptible to fluconazole (MIC, $\leq 8 \mu\text{g/ml}$), compared to 94 to 100% in the other regions.

Aller et al. (1) have shown that a more-positive clinical response to fluconazole maintenance therapy could be expected when the fluconazole MIC is $< 16 \mu\text{g/ml}$ than when the MIC is $\geq 16 \mu\text{g/ml}$ for the infecting *C. neoformans* strain. Using this “surrogate breakpoint,” 25% of North American isolates demonstrate decreased susceptibility (MIC, $\geq 16 \mu\text{g/ml}$) compared to 0 to 6% of isolates from the other four geographic regions (Table 1).

Table 2 summarizes the in vitro susceptibilities to the three antifungal agents in use throughout the 15-year period (amphotericin B, flucytosine, and fluconazole) of *C. neoformans* from all areas stratified by 5-year time intervals. Isolates remained highly susceptible to amphotericin B (99% susceptible

at MIC of $\leq 1 \mu\text{g/ml}$) over the entire 15-year period. In contrast, susceptibility to flucytosine (MIC, $\leq 4 \mu\text{g/ml}$) increased from 34% in 1990 to 1994 to 38% in 1995 to 1999 and 66% in 2000 to 2004 (Table 2). A similar trend was seen with fluconazole, where the percentage of isolates susceptible at a MIC of $\leq 8 \mu\text{g/ml}$ increased from 72% in 1990 to 1994 to 89% in 1995 to 1999 and 96% in 2000 to 2004 (Table 2). High-level resistance to flucytosine (MIC, $\geq 32 \mu\text{g/ml}$) and to fluconazole (MIC, $\geq 64 \mu\text{g/ml}$) remained essentially unchanged at 1 to 2% over the entire study period, indicating that the apparent improvement in susceptibility to flucytosine and fluconazole over time was due to a shift of isolates from the intermediate or susceptible dose-dependent category, respectively, to the susceptible category.

These data address the concerns of Archibald et al. (6) and Datta et al. (16) regarding the need for expanded surveillance involving international sites (Table 1) and confirm the observations of Brandt et al. (11), Davey et al. (17), and Yildiran et al. (48) that high-level resistance to commonly used antifungal agents among isolates of *C. neoformans* is uncommon and has not increased over time (Table 2). Notably, we have shown that isolates of *C. neoformans* from North America are considerably less susceptible to both flucytosine and fluconazole than isolates from other geographic regions. The reasons for these differences are not immediately apparent; however, greater accessibility and exposure to both of these agents in North America versus other regions may account for some of the difference.

The apparent improvement in susceptibility of *C. neoformans* over time to both flucytosine and fluconazole is notable and may reflect in part a decrease in the overall drug pressure due to these agents concomitant with a decrease in cryptococcosis among human immunodeficiency virus-infected individuals receiving HAART. A similar improvement in fluconazole susceptibility among *Candida albicans* isolates from AIDS patients has been noted following the introduction of HAART (27). It was suggested that decreased use of antifungal agents in patients treated with effective antiretroviral therapy may have contributed to the decrease in fluconazole-resistant strains colonizing and infecting these individuals (27).

Although the overall picture regarding the susceptibility of *C. neoformans* to the standard therapeutic agents shown in Tables 1 and 2 is quite positive, it is reasonable to explore new and alternative therapies for cryptococcosis (33). The three new extended-spectrum triazoles, voriconazole, posaconazole, and ravuconazole, all have potent in vitro activity against this large, globally diverse collection of *C. neoformans* isolates (Table 3). Overall, 99% of all isolates tested were susceptible to these three agents at a MIC of $\leq 1 \mu\text{g/ml}$ (Table 3). The rare strains for which the MICs for fluconazole were $\geq 64 \mu\text{g/ml}$ also exhibited MICs of 2 to 4 $\mu\text{g/ml}$ for the three extended-spectrum triazoles (data not shown). This potent activity suggests that these new agents may be useful as alternative therapies for cryptococcosis. Notably, both voriconazole and posaconazole have been shown to be useful in treating other central nervous system mycoses (28, 31, 35, 45, 46).

In summary, we have confirmed that in vitro resistance to standard antifungal agents used in the treatment of cryptococcosis remains uncommon among isolates of *C. neoformans* from five broad geographic regions and has not increased over

TABLE 3. In vitro susceptibilities of *Cryptococcus neoformans* to voriconazole, posaconazole, and ravuconazole^a

Antifungal agent	No. of isolates tested	Cumulative % inhibited at MIC (μ g/ml) of:									
		0.007	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4
Voriconazole	1,800	2	8	26	62	89	97	99	99	99	100
Posaconazole	1,646	0	0.4	2	12	62	82	97	99	100	
Ravuconazole	1,048	2	8	36	61	82	95	98	99	99	100

^a Broth microdilution testing according to NCCLS M27-A2 (30).

a 15-year period. We have shown that clinical isolates of *C. neoformans* from Africa, Europe, Latin America, and the Pacific regions are more susceptible to both flucytosine and fluconazole than isolates from North America. The susceptibility trend over time, however, indicates an overall shift to greater susceptibility of isolates to both flucytosine and fluconazole, possibly influenced by effective antiretroviral therapy and a decreased incidence of cryptococcosis. Finally, we provide the most extensive evidence to date of the excellent potencies of voriconazole, posaconazole, and ravuconazole against clinical isolates of *C. neoformans*. Despite these favorable findings, continued surveillance for emerging resistance may be warranted given the global importance of this organism as an opportunistic pathogen and the ever-increasing use of antifungal agents in the immunocompromised patient population.

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