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Trends in Antifungal Drug Susceptibility of *Cryptococcus neoformans* Isolates in the United States: 1992 to 1994 and 1996 to 1998

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The antifungal drug susceptibilities of two collections of *Cryptococcus neoformans* isolates obtained through active laboratory-based surveillance from 1992 to 1994 (368 isolates) and 1996 to 1998 (364 isolates) were determined. The MICs of fluconazole, itraconazole, and flucytosine were determined by the National Committee for Clinical Laboratory Standards broth microdilution method; amphotericin B MICs were determined by the E-test. Our results showed that the MIC ranges, the MICs at which 50% of isolates are inhibited (MIC_{50}s), and the MIC_{90}s of these four antifungal agents did not change from 1992 to 1998. In addition, very small numbers of isolates showed elevated MICs suggestive of in vitro resistance. The MICs of amphotericin B were elevated (≥2 μg/ml) for 2 isolates, and the MICs of flucytosine were elevated (≥32 μg/ml) for 14 isolates. Among the azoles, the fluconazole MIC was elevated (≥64 μg/ml) for 8 isolates and the itraconazole MIC (≥1 μg/ml) was elevated for 45 isolates. Analysis of 172 serial isolates from 71 patients showed little change in the fluconazole MIC over time. For isolates from 58 patients (82% of serial cases) there was either no change or a twofold change in the fluconazole MIC. In contrast, for isolates from seven patients (12% of serial cases) the increase in the MIC was at least fourfold. For isolates from another patient there was a 32-fold decrease in the fluconazole MIC over a 1-month period. We conclude that in vitro resistance to antifungal agents remains uncommon in *C. neoformans* and has not significantly changed with time during the past decade.

Cryptococcosis has been a leading cause of illness and death among persons with AIDS and is the single most common life-threatening fungal infection in these individuals. Meningitis is the most frequent clinical presentation, but widespread disseminated infection often occurs (11, 19, 22). Population-based active surveillance, conducted in four areas of the United States between 1992 and 1994, showed that 2 to 5% of persons with AIDS developed cryptococcosis per year (14). Although the incidence of cryptococcosis among persons with AIDS has declined since the introduction of highly active antiretroviral therapies, the mortality rate has remained unchanged at 10% (G. Ponce de Leon, M. Sattah, E. A. Graviss, M. Phelan, M. E. Brandt, D. Rimland, R. Hamill, and R. A. Hajjeh, Abstr. 37th Annu. Meet. Infect. Dis. Soc. Am., abstr. 406, 1999).

AIDS patients with cryptococcal meningitis who survive beyond the initial induction treatment generally require lifelong maintenance therapy to prevent relapses (11, 19, 22). Current regimens for treatment of the disease remain focused on amphotericin B, with or without flucytosine, for induction treatment, while fluconazole remains the agent of choice for long-term maintenance treatment. For patients in whom fluconazole cannot be used, itraconazole is an acceptable but less effective alternative (22). Although there is some evidence that rates of relapse of opportunistic infections are lower when patients are treated with potent antiretroviral therapy, present guidelines recommend that maintenance therapy for cryptococcal meningitis be administered for life (22). Studies to determine the timing and safety of fluconazole withdrawal among patients responding to highly active antiretroviral therapies have not been performed.

To date, there have been few published reports of the emergence of resistance to amphotericin B, fluconazole, or itraconazole in *Cryptococcus neoformans* during treatment (3, 4, 7, 9, 10, 17, 20; D. J. E. Marriott, R. Hardiman, S. Chen, J. L. Harkness, and R. Pennry, 3rd Int. Conf. Cryptococcus Cryptococcosis, abstr. 3.21, 1996; N. H. Smith, E. A. Graviss, R. Hashmey, M. Lozano-Chiu, J. H. Rex, R. Hammill, and S. Greenberg, 35th Annu. Meet. Infect. Dis. Soc. Am., abstr. 529, 1997). However, the long-term use of fluconazole as maintenance therapy in persons with AIDS has generated concern that less susceptible strains might begin to emerge. One study from the United States detected an upward shift in the MICs for blood and cerebrospinal fluid isolates of *C. neoformans* between 1991 and 1994 (S. L. Koletar, W. J. Buesching, and R. J. Fass, Abstr. 35th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E70, p. 98, 1995). This was not confirmed by Davey et al. (10) who compared the MIC ranges, the MICs at which 50% of isolates are inhibited (MIC_{50}s), and MIC_{90}s of

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TABLE 1. In vitro susceptibilities of 732 isolates of *C. neoformans* to amphotericin B, flucytosine, fluconazole, and itraconazole

Antifungal agent	MICs (μg/ml) for:					
	Isolates collected from 1992 to 1994 (n = 368)			Isolates collected from 1996 to 1998 (n = 364)		
	Range	50%	90%	Range	50%	90%
Amphotericin B	0.25–1	1	1	0.25–2	1	1
Flucytosine	0.5–≥128	8	16	0.06–≥128	8	16
Fluconazole	0.25–≥64	8	16	0.12–≥64	4	16
Itraconazole	0.06–2	0.5	1	0.03–2	0.25	0.5

fluconazole and itraconazole for 143 British isolates of *C. neoformans* submitted to a national reference center between 1994 and 1996 with those for 77 isolates dating from 1971 to 1989. However, those investigators identified six patients for whom a fourfold or greater rise in the fluconazole MIC for the infecting isolates was associated with a relapse of cryptococcal meningitis. To address this question further, we determined the in vitro susceptibilities to amphotericin B, fluconazole, itraconazole, and flucytosine of 732 isolates of *C. neoformans* collected in four areas of the United States between 1992 to 1994 and 1996 to 1998 as part of a population-based active surveillance study. As part of this study, we also documented the MICs of fluconazole for serial isolates from 71 patients with persistent cryptococcal disease.

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MATERIALS AND METHODS

Isolates. *C. neoformans* clinical isolates were obtained as part of a population-based active surveillance program conducted by the Centers for Disease Control and Prevention (CDC) in four metropolitan areas of the United States (Atlanta, Ga.; San Francisco, Calif.; Houston, Tex.; and all major metropolitan areas of Alabama) between 1992 to 1994 and 1996 to 1998. A total of 732 isolates from 522 patients were tested in this study. Demographic information on many of these patients has been reported previously (14). Most of the patients (85%) were infected with the human immunodeficiency virus. No information on prior antifungal use was available for patients whose isolates were collected between 1992 and 1994. Of the 126 patients for whom this information was available between 1996 and 1998, only 25% had received fluconazole in the 3-month period prior to diagnosis. No information on treatment efficacy was available.

Species identification was confirmed at CDC (5, 6). Isolates were stored frozen at −20°C in 20% glycerol until the study was performed. For the collection obtained from 1992 to 1994, every 10th isolate from San Francisco and Atlanta, all isolates collected in 1993 and most isolates collected in 1994 from Alabama and Houston, and all serial isolates (2 or more isolates from an individual patient collected at least 1 month apart) were selected for antifungal susceptibility testing (5, 6). Among the isolates from this collection, 368 isolates from 266 patients were tested. Between 1996 and 1998, 364 isolates were collected from 298 patients in Atlanta and Houston. All these isolates were tested. Prior to antifungal susceptibility testing, each isolate was subcultured at least twice on potato dextrose agar plates to ensure purity and optimal growth.

Antifungal drugs. Standard powders of fluconazole, itraconazole, and flucytosine were supplied by Pfizer Pharmaceuticals Group, Central Research Division (Groton, Conn.); Janssen Research Foundation (Beerse, Belgium); and Hoffmann-La Roche, Inc. (Nutley, N.J.), respectively. Stock solutions were prepared in water (fluconazole and flucytosine) or dimethyl sulfoxide (itraconazole). Further dilutions of each antifungal agent were prepared with RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) which had been buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), as outlined in National Committee for Clinical Laboratory Standards (NCCLS) document M27-A (16). The drug dilutions were dispensed into 96-well microdilution plates that were then sealed and frozen at −70°C until needed.

Broth microdilution susceptibility test method. The MICs of fluconazole, itraconazole, and flucytosine were determined by the NCCLS broth microdilution method (16). The final concentrations of the antifungal agents ranged from 0.125 to 128 μg/ml for fluconazole and flucytosine and from 0.007 to 8 μg/ml for itraconazole. The yeast inoculum was adjusted to a concentration of 0.5×10^3 to 2.5×10^3 CFU/ml in RPMI 1640 medium, and an aliquot of 0.1 ml was added to each well of the microdilution plate. The plates were incubated at 35°C. The MIC endpoints were read visually following 48 and 72 h of incubation and were defined as the lowest concentration that produced an 80% reduction in growth (a prominent decrease in turbidity) compared with that of the drug-free growth control. The MIC results read at 48 and 72 h were in complete agreement. Thus, the MIC data read at 48 h are reported here.

Candida parapsilosis ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control organisms and were included each time that a set of isolates was tested.

E-test method. The MICs of amphotericin B were determined in accordance with the manufacturer's instructions (18). The medium used was RPMI 1640 agar (1.5%) supplemented with 2% glucose and buffered to pH 7 with MOPS. The same yeast inoculum used for the broth-based tests was swabbed onto the surface of the agar plate and was allowed to dry for 15 min before the addition of the E-test strip. One E-test antimicrobial gradient strip containing amphotericin B (concentration range, 0.002 to 32 μg/ml) was placed on each plate. The plates were incubated at 35°C for 48 and 72 h, and the MIC was read as the drug concentration at which the border of the elliptical zone of complete inhibition intersected the strip.

RESULTS

Table 1 summarizes the in vitro susceptibilities of the 732 *C. neoformans* isolates to amphotericin B, flucytosine, fluconazole, and itraconazole. The results are reported as MIC ranges, MIC_{50}s, and MIC_{90}s. A broad range of MICs of flucytosine, fluconazole, and itraconazole was observed, but the MIC_{50}s and MIC_{90}s of the four antifungal drugs for the isolates did not change by more than 1 $\log_2$ dilution between the isolates collected from 1992 to 1994 and those collected from 1996 to 1998. No differences in the MICs of any of the four antifungals by geographic region were seen (data not shown). The amphotericin B MIC was not ≥2 μg/ml for any of the 368 isolates of *C. neoformans* collected between 1992 and 1994, whereas the amphotericin B MIC was ≥2 μg/ml for 2 of 364 isolates (0.6%) collected between 1996 and 1998. For six isolates (1.6%) collected from 1992 to 1994 and eight isolates (2.2%) collected from 1996 to 1998, flucytosine MICs were ≥32 μg/ml.

Tables 2 and 3 summarize the in vitro susceptibilities of 522 incident isolates of *C. neoformans* to fluconazole and itraconazole, respectively. The fluconazole MIC for the incident surveillance isolate was ≥64 μg/ml for isolates from 6 of the 253 patients (2.4%) detected between 1992 and 1994 and for iso-

TABLE 2. In vitro susceptibilities of 522 incident isolates of *C. neoformans* to fluconazole

MIC (μg/ml)	No. (%) of patients		
	1992 to 1994	1996 to 1998	Total
<8	89	142	231 (44)
8	91	98	189 (37)
16	62	23	85 (16)
32	5	4	9 (1.6)
64	3	2	5 (1)
128	1	0	1 (0.1)
256	2	0	2 (0.3)
Total	253	269	522

TABLE 3. In vitro susceptibilities of 522 incident isolates of *C. neoformans* to itraconazole

MIC (μg/ml)	No. (%) of patients		
	1992 to 1994	1996 to 1998	Total
0.03	0	1	1 (0.2)
0.06	1	14	15 (2.9)
0.12	11	35	46 (8.8)
0.25	89	111	200 (38.3)
0.5	120	95	215 (41.2)
1	31	12	43 (8.2)
2	1	1	2 (0.4)
Total	253	269	522

lates from 2 of the 269 patients (0.7%) detected between 1996 and 1998. The itraconazole MICs for the incident isolate were ≥1 μg/ml for the isolates from 32 patients (12.6%) detected between 1992 and 1994 and for the isolates from 13 patients (4.8%) detected between 1996 and 1998. Cross-resistance to both azole drugs (fluconazole MICs, ≥64 μg/ml; itraconazole MICs, ≥1 μg/ml) was demonstrated in 11 isolates from 8 patients.

A total of 172 serial isolates of *C. neoformans* collected at least 1 month apart were received from 71 individual patients: 37 patients (52%) from the period from 1992 to 1994 and 34 patients (48%) from the period from 1996 to 1998 (Table 4). The median number of isolates per patient was 3 (range, 2 to 11 isolates). The mean time between collection of any two isolates was 5.8 months (range, 1 to 10 months). When these isolates were compared, the fluconazole MICs for isolates from 58 patients (82% of serial cases) showed either no change (33 patients) or a 1 $\log_2$ dilution change (25 patients) over time periods that ranged from 1 to 10 months. A fourfold or greater increase in the fluconazole MIC was seen for isolates from seven patients (12% of serial cases). For isolates from four of these patients, a fourfold increase in the MIC was demonstrated over time. For the isolates from the other three patients, one of whom has been described earlier (7), a change in the MIC of at least eightfold (3 $\log_2$ dilutions) appeared to represent acquisition of in vitro resistance. However, a 4-fold decrease in the MIC was seen for isolates from five other patients and a 32-fold decrease in the MIC (from 4 to 0.12 μg/ml) was seen over a 1-month period for isolates from a sixth patient. In the absence of strain typing information, we cannot be certain whether these decreased MICs represent a loss of resistance by a resident strain of *C. neoformans* or the replacement of a resistant strain by a second susceptible one. The amphotericin B MIC did not increase more than twofold for any of the cases over time. For isolates from three patients, the MIC of flucytosine increased more than fourfold (from 8 to >128 μg/ml), and for two isolates taken 5 months apart from one patient, the MIC dropped by the same amount (from >128 to 8 μg/ml).

DISCUSSION

Treatment failure attributable to the development of amphotericin B resistance by *C. neoformans* appears to be an uncommon problem (20; Marriott et al., 3rd Int. Conf. Cryptococcus Cryptococcosis; Smith et al., 35th Annu. Meet. Infect. Dis. Soc. Am.). One of the possible reasons for the small number of published reports may be the lack of reliable methods for susceptibility testing of *C. neoformans*. Neither the NCCLS broth macrodilution reference method (16) nor the alternative broth microdilution method (13) recommended in the M27-A document has proved ideal for the detection of resistance to amphotericin B (15). In contrast, the E-test method, performed on glucose-supplemented RPMI 1640 agar, has provided an excellent means of discrimination between susceptible and resistant strains of *C. neoformans* (15). Using this method, we found that there had been no change in the MIC ranges, MIC_{50}s, or MIC_{90}s of amphotericin B for *C. neoformans* between 1992 to 1994 and 1996 to 1998 (Table 1). Although the NCCLS M27-A document does not define MIC breakpoints for amphotericin B resistance, it has been suggested that isolates for which MICs are ≥2 μg/ml should be regarded as resistant. If this definition is adopted, our findings (Table 1) indicate that less than 1% of *C. neoformans* isolates obtained between 1996 and 1998 were resistant to this agent.

Although less than 2% of *C. neoformans* isolates are resistant to flucytosine prior to treatment (24), a justified fear of the emergence of resistance during treatment with this drug alone and reports of favorable interactions in tests with *C. neoformans* in vitro and in vivo have led to its use in combination with amphotericin B in patients with cryptococcosis (22). In this study, we found that there had been almost no change in the MIC ranges, MIC_{50}s, and MIC_{90}s of flucytosine for *C. neoformans* between 1992 to 1994 and 1996 to 1998 (Table 1). The NCCLS M27-A document (16) recommends that isolates for which MICs are ≥32 μg/ml be regarded as resistant to flucytosine. By this definition, the rate of flucytosine resistance by *C. neoformans* ranged from 1.6% among isolates collected from 1992 to 1994 to 2.2% among those collected from 1996 to 1998.

Various methods have been developed for testing the susceptibility of *C. neoformans* to azole antifungal agents, including fluconazole and itraconazole (2, 10, 12, 13, 21, 23). The NCCLS reference method (16) has proved problematic, as has

TABLE 4. In vitro susceptibilities to fluconazole of serial isolates of *C. neoformans* from 71 patients with cryptococcosis

Change in MIC	No. of patients		
	1992 to 1994	1996 to 1998	Total
No change	17	16	33
Twofold change			
Increase	15	10	25
Decrease	0	0	0
Fourfold change			
Increase	3	1	4
Decrease	2	3	5
Eightfold change			
Increase	0	2	2
Decrease	0	0	0
Greater than eightfold change			
Increase	1	0	1
Decrease	0	1	1
Total	38	33	71

the alternative broth microdilution method (13) described in the M27-A document (21). Nevertheless, regardless of the particular test method used, there is some evidence that elevated MICs of fluconazole are correlated with a diminished response in animal models of cryptococcal meningitis (8, 25) and that a high or rising MIC of fluconazole is sometimes associated with treatment failure in human immunodeficiency virus-infected persons with cryptococcosis (1, 3, 4, 7, 9, 10, 17).

The distribution of azole antifungal MICs in *C. neoformans* has been assessed in several prior studies. Koletar et al. reported that, in their small collection of clinical isolates, the MIC_{50}s of fluconazole increased over time, from ≤ 2 μg/ml in 1991 to >32 μg/ml in 1994 (Koletar et al., 35th ICAAC). The fluconazole MIC was not ≥32 μg/ml for any of 11 isolates tested in 1991, whereas the fluconazole MIC was ≥32 μg/ml for 11 of 20 isolates tested in 1994. Most of the 1994 isolates for which MICs were higher were from patients who had previously received fluconazole. Davey et al. (10) compared 143 British isolates of *C. neoformans* submitted to a national reference center between 1994 and 1996 with 77 isolates dating from 1971 to 1989. The results showed that the MIC ranges, MIC_{50}s, and MIC_{90}s of fluconazole and itraconazole had remained unchanged, despite the widespread use of triazoles for long-term maintenance of AIDS-associated cryptococcal meningitis. Our findings (Table 1) confirm and extend those of Davey et al. (10). Moreover, because the *C. neoformans* isolates that we tested were collected as part of a population-based surveillance study, our results may be more representative than those of the British study.

Although it is clear that relapses in patients with AIDS-associated cryptococcosis are often due to deterioration of the host immune function rather than to changes in MICs (26), published case reports demonstrate the potential for variation in fluconazole MICs and indicate that resistance can develop during treatment in some patients. Birley et al. (4) reported on two cases of relapsed meningitis in which there was evidence of rising MICs of fluconazole, the agent which had been used for maintenance treatment. Davey et al. (10) found 8- to 32-fold rises in the fluconazole MICs for paired blood or cerebrospinal fluid isolates of *C. neoformans* recovered from six patients with recurrent cryptococcal meningitis, all of whom had received treatment with fluconazole. Aller et al. (1) obtained paired isolates from six patients, three of whom were cured and three of whom died with recurrent cryptococcal meningitis. A 2-fold rise in the fluconazole MIC was detected for isolates from the patients who survived, but 4- to 16-fold rises were seen for the isolates from the patients who died. For the isolate from one patient whom we have described previously, a 16-fold rise in the fluconazole MIC was demonstrated over 18 months (7). In addition, there have been at least three other case reports in which a high or rising MIC of fluconazole was associated with clinical relapse in patients with AIDS-associated cryptococcal meningitis (3, 9, 17).

One reason for the low level of azole resistance detected in this study might be the infrequent use of these antifungal agents among the population under investigation. A previous case-control study conducted in Atlanta and San Francisco from 1992 to 1994 showed that only 24% of patients had received fluconazole during the 3-month period prior to enrollment (14). Similarly, in the latter part of this study, only 25% of patients from whom isolates were collected between 1996 and 1998 had received fluconazole in the 3-month period prior to diagnosis (unpublished observations). However, it is also notable that in this study, for only 13 of the 71 patients from whom follow-up isolates of *C. neoformans* were available for testing, fourfold or greater changes in the fluconazole MICs were found for later isolates compared with the MICs for the incident isolates. This was despite the fact that, presumably, many of these individuals were receiving maintenance therapy with fluconazole.

In conclusion, our results indicate that there has been no significant shift in the MICs of amphotericin B and fluconazole for *C. neoformans*, despite the widespread use of these agents in persons with AIDS. Although the majority of isolates of *C. neoformans* tested in this study appeared to be susceptible to fluconazole, continued surveillance for emerging resistance may be warranted on a national and an international basis given the widespread use of this agent for long-term maintenance treatment in persons with AIDS.

APPENDIX

Members of the CDC Cryptococcal Disease Active Surveillance Group who participated in this study were David Stephens, Monica Farley, Wendy Baughman, Chris Lao, Jodie Otte, Matthew Sattah, and Christopher Harvey (Atlanta); Edward A. Graviss (Houston); Carolynn Thomas (Alabama); and Gretchen Rothrock, Bharat Pattni, and Pam Daily (San Francisco).

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