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Cryptococcus neoformans is the most common cause of meningitis among adult South Africans with HIV infection/AIDS. Widespread use of fluconazole for treatment of cryptococcal meningitis and other HIV-associated opportunistic fungal infections in South Africa may lead to the emergence of isolates with reduced fluconazole susceptibility. MIC testing using a reference broth microdilution method was used to determine if isolates with reduced susceptibility to fluconazole or amphotericin B had emerged among cases of incident disease. Incident isolates were tested from two surveillance periods (2002-2003 and 2007-2008) when population-based surveillance was conducted in Gauteng Province, South Africa. These isolates were also tested for susceptibility to fluconazole, itraconazole, voriconazole, and posaconazole. Serially collected isolate pairs from cases at several large South African hospitals were also tested for susceptibility to fluconazole. Of the 487 incident isolates tested, only 3 (0.6%) demonstrated a fluconazole MIC of ≥16 µg/ml; all of these isolates were from 2002-2003. All incident isolates were inhibited by very low concentrations of amphotericin B and exhibited very low MICs to voriconazole and posaconazole. Of 67 cases with serially collected isolate pairs, only 1 case was detected where the isolate collected more than 30 days later had a fluconazole MIC value significantly higher than the MIC of the corresponding incident isolate. Although routine antifungal susceptibility testing of incident isolates is not currently recommended in clinical settings, it is still clearly important for public health to periodically monitor for the emergence of resistance.

Cryptococcus neoformans is the most common cause of meningitis among adult South Africans with HIV infection/AIDS (23). In South Africa, cryptococcal meningitis is often diagnosed among HIV-infected patients with advanced immunosuppression and poor prognostic factors such as a high fungal burden (22, 31) and is associated with high mortality (22). Although the fungicidal combination of amphotericin B and flucytosine is recommended for induction treatment (33), flucytosine is not available in countries with a high incidence of cryptococcosis (8), and the use of amphotericin B deoxycholate is limited by toxicity and the need for clinical and laboratory monitoring (8). Since 2000, fluconazole has been widely available in South Africa through the Diflucan Partnership Program for treatment of cryptococcal meningitis and esophageal candidiasis (41). Due to ease of administration and low toxicity, in sub-Saharan Africa fluconazole is often first-line treatment for cryptococcal meningitis, despite its fungistatic activity.

With widespread use of low-dose fluconazole (≤200 mg daily) for prophylaxis and treatment of candidiasis and other opportunistic fungal infections which may precede cryptococcal meningitis, it is possible that cryptococcal lineages with reduced susceptibility could arise by selective pressure and expand to cause incident cryptococcosis among persons with HIV infection/AIDS. However, isolates with reduced fluconazole susceptibility may be more likely to emerge in circumstances where patients have been treated with suboptimal induction-phase regimens (including fluconazole monotherapy at ≤400 mg daily) and where long-term, low-dose fluconazole (200 mg daily) is prescribed for suppression of disease (10). While we do not yet know enough about the relationship between elevated fluconazole MIC values and patient outcome to warrant routine testing, susceptibility testing of surveillance isolates can give us reliable data for trend analysis.

Long-term prophylaxis and, in some cases, induction therapy of cryptococcal meningitis in South Africa are still largely dependent upon fluconazole. In 2000, South African Department of Health guidelines recommended a relatively low fluconazole dose (400 mg daily) as an alternative to amphotericin B induction-phase treatment (39). Development of resistance to fluconazole would be devastating to the treatment of this disease, and so it is important for public health agencies to monitor for changes in susceptibility to this drug. In this study, two methods were used to monitor for changes in fluconazole susceptibility over time. In the first, incident cryptococcal isolates obtained through population-based surveillance from two time intervals (2002-2003 and 2007-2008) in Gauteng Province, South Africa, were tested to determine if median MIC values
to fluconazole and amphotericin B were elevated or had changed over time. In addition, the susceptibility to flucytosine, itraconazole, voriconazole, and posaconazole was assessed. In the second, serially collected isolate pairs from cases at several large sentinel hospitals within and outside Gauteng Province were tested for susceptibility to fluconazole.

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MATERIALS AND METHODS

Population-based surveillance for cryptococcosis. Cases of laboratory-confirmed cryptococcosis were reported to the Mycology Reference Unit, National Institute for Communicable Diseases (NICD), in Johannesburg, South Africa, from 1 March 2002 through 28 February 2008. Active population-based surveillance was restricted to Gauteng Province from March 2002 through February 2004 (31); from January 2005, surveillance was expanded nationally (20). A case of incident cryptococcosis was defined as the first episode of laboratory-confirmed disease in a patient (encapsulated yeasts observed by microscopic examination of an India ink-stained fluid specimen or a positive cryptococcal antigen test or culture of Cryptococcus species from a specimen from any body site) diagnosed at a South African clinical laboratory.

For culture-confirmed cases, cryptococcal isolates were transported to the NICD and stored in brain heart infusion broth with 10% glycerol at −70°C. 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); surveillance was enhanced at four Gauteng hospitals from 2002 through 2006 and at an additional 14 hospitals across South Africa in 2005. Isolates were collected with minimal case demographic data at non-enhanced-surveillance hospitals. Ethics clearance for surveillance was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg, and from other university and provincial ethics committees.

Selection of isolates for antifungal susceptibility testing. Incident cases were included if the person had been diagnosed with a first episode of laboratory-confirmed cryptococcosis (i) at one of four enhanced-surveillance hospitals in Gauteng Province, (ii) from 1 March 2002 through 28 February 2003 (2002-2003) or from 1 March 2007 through 28 February 2008 (2007-2008), and (iii) where the incident isolate was stored by the NICD. We selected cases from these sites because continuous surveillance had been performed for 6 years. A subset of incident cases from each surveillance period was selected using a random-number generator. Incident cases were excluded if the isolate was nonviable, contaminated, or misplaced after storage or identified as Cryptococcus gattii or another cryptococcal species or if the case patient was known to be HIV uninfected or had been treated with antifungal drugs which suggested a prior episode of cryptococcosis.

Cases from whom isolates were serially collected more than 30 days apart were selected if (i) the case was diagnosed between 1 January and 31 December 2005 at 18 enhanced-surveillance hospitals across South Africa and (ii) the serially collected isolate pairs had been stored at NICD. We selected cases from 2005 because most patients were treated with low-dose fluconazole induction treatment (≤400 mg daily) during this period. Cases were excluded if the isolate was nonviable, contaminated, or misplaced after storage or was identified as C. gattii or another cryptococcal species.

Antifungal susceptibility testing. Isolates were tested by reference laboratories at the NICD and the Centers for Disease Control and Prevention (CDC, Atlanta, GA). Isolates were subcultured at least twice on Sabouraud dextrose agar (Diagnostica Medical Products-National Health Laboratory Service [DMP], Johannesburg, South Africa) after long-term storage to ensure optimal growth and purity. Isolates were confirmed to be C. neoformans using standard phenotypic tests, including development of brown-pigmented colonies on Stah1's niger seed agar (DMP) and a positive test for urease on urea-containing medium (DMP) (32). C. neoformans was distinguished from C. gattii using canavanine glycine bromothymol blue agar (DMP).

The MICs for six antifungal drugs (amphotericin B, fluconazole, flucytosine, voriconazole, posaconazole, and itraconazole) were determined for incident isolates. Fluconazole MICs were determined as outlined by Clinical and Laboratory Standards Institute (CLSI) standard M27-A3 (13), using broth microdilution panels prepared at the NICD. Fluconazole, flucytosine, voriconazole, posaconazole, and itraconazole MICs were determined at the CDC for a subset of incident isolates using custom broth microdilution panels prepared as outlined in standard M27-A3 by TREK Diagnostic Systems, Inc. (Cleveland, OH) (13). All broth microdilution panels were inoculated with RPMI 1640 medium (with glutamine and phenol red but without bicarbonate) (13). A subset of isolates was tested at both laboratories, and the results were found to be in essential agreement. Serially collected isolates were tested at the NICD using fluconazole with in-house panels (13). MIC values were determined visually following 72 h of incubation. The quality control isolates Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258 were run on all days of testing. The MICs for amphotericin B were determined by the Etest (bioMérieux S.A., Marcy l’Etoile, France) on RPMI 1640 medium plates containing 2% glucose, as recommended by the manufacturer. The Etest has been determined to be more discriminatory than the CLSI method for distinguishing isolates thought to be amphotericin B susceptible versus nonsusceptible on the basis of clinical data (28). Geometric mean MIC values were calculated for incident isolates for each surveillance period and compared using Student’s t test. For serially collected isolates, essential agreement was defined as MIC values within 2 dilutions of each other. Interpretive breakpoints were not assigned because there are no accepted breakpoints for Cryptococcus with any antifungal drug (12).

RESULTS

Incident cases of cryptococcosis. (i) Case selection and demographic characteristics. From 1 March 2002 through 28 February 2008, 8,439 cases of incident cryptococcosis were detected through population-based surveillance in Gauteng Province. The inclusion criteria for antifungal susceptibility testing were met by 1,033 cases of incident disease: 462 in 2002-2003 and 571 in 2007-2008. Of these cases, 391 and 280 from each period, respectively, were randomly selected. A total of 238 cases from 2002-2003 and 249 from 2007-2008 had viable isolates available for testing. Apart from more female patients in the selected group in 2007-2008, there were no significant differences in the baseline demographic characteristics of cases with and without viable isolates for each surveillance period (data not shown). Table 1 shows a comparison of baseline characteristics of cases with viable isolates from 2002-2003 versus 2007-2008. Patients were significantly more likely to be treated with amphotericin B than fluconazole or no drug in 2007-2008 than in 2002-2003 (Table 1). The case-fatality ratio was also significantly higher in 2007-2008 than in the earlier period (Table 1).

(ii) Antifungal susceptibility results. Amphotericin B, flucytosine, itraconazole, voriconazole, and posaconazole MICs were determined for 237 incident isolates; fluconazole MICs were determined for 487 incident isolates (Table 2 and Table 3). None of these isolates demonstrated fluconazole MIC values (MIC = 16 μg/ml); all of these isolates were from the earlier surveillance period (2002-2003) (Table 3). Three additional isolates from the earlier surveillance period had elevated itraconazole MIC values (MIC ≥ 1 μg/ml). As expected, all incident isolates were inhibited by low concentrations of amphotericin B (MIC90 = 0.19 μg/ml). Similarly, the MICs for voriconazole and posaconazole were low; for all tested isolates, the MICs were ≤0.25 μg/ml and ≤0.5 μg/ml for voriconazole and posaconazole, respectively. Despite no flucytosine use in South Africa during the surveillance period, 17 of 237 (7%) isolates had MIC values of 8 μg/ml or 16 μg/ml. There were no differences in MIC50 and MIC90 be-
isoles collected from 2002-2003 was 2.3 μg/ml, while the geometric mean MIC value for the isolates collected from 2007-2008 was 2.1 μg/ml (not statistically significant; P = 0.1).

Cases with serially collected isolates. (i) Case selection and demographic characteristics. From 1 January through 31 December 2005, 1,538 cases of incident cryptococcosis were detected at 18 enhanced-surveillance hospitals in seven South African provinces. The criteria for susceptibility testing were met by 67 cases diagnosed at 11 enhanced-surveillance hospitals in six provinces. The mean age of the case patients was 33 years (standard deviation, ±9.2 years), and 42 (63%) were female. Seventy-four percent (40/54) of patients received fluconazole monotherapy; 38 (95%) patients were treated with fluconazole at ≤400 mg per day in hospital for a median of 8 days (range, 1 to 32 days). Only 4 (6%) patients were receiving combination antiretroviral treatment (cART) at the time of incident diagnosis; a further 6 (8%) patients were known to have initiated cART postdiagnosis. The median time between the serially collected isolates was 70 days (range, 32 to 238 days).

(ii) Antifungal susceptibility results. Among the cases with serially collected isolates, the fluconazole MIC<sub>50</sub> and MIC<sub>90</sub> for the incident isolates were 2 μg/ml and 16 μg/ml, respectively, and for the isolates collected >30 days later they were 2 μg/ml and 8 μg/ml, respectively. The fluconazole MIC remained the same for 34 (51%) isolate pairs, increased by 1 log<sub>2</sub> dilution for 11 (16%) isolate pairs, and increased by 2 log<sub>2</sub> dilutions for 7 (10%) isolate pairs (Table 4). For 1 case, the MIC increased significantly from 2 μg/ml to 32 μg/ml. This patient had been treated with low-dose fluconazole monotherapy at diagnosis of incident cryptococcosis; the second serial isolate was collected 5 months later. No clinical data were available for the second episode. The fluconazole MIC decreased for 14 (21%) pairs (Table 4). The incident isolates from 10 cases displayed elevated MIC values (16 μg/ml to 64 μg/ml), with six of these producing MIC values of 64 μg/ml. Isolates collected more than 30 days after the incident isolate displayed elevated MIC values among 12 cases, with only two isolates having MIC values of 64 μg/ml. Of note, among four cases where there was not essential agreement between the serially collected isolates, the MICs dropped significantly: from

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**TABLE 3. Fluconazole MICs for incident cryptococcal isolates from 2 surveillance periods: 1 March 2002 through 28 February 2003 and 1 March 2007 through 28 February 2008**

<table>
<thead>
<tr>
<th>Fluconazole MIC (μg/liter)</th>
<th>2002-2003</th>
<th>2007-2008</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>0.5</td>
<td>13 (5)</td>
<td>14 (5)</td>
<td>27 (6)</td>
</tr>
<tr>
<td>1</td>
<td>39 (16)</td>
<td>53 (21)</td>
<td>92 (19)</td>
</tr>
<tr>
<td>2</td>
<td>99 (42)</td>
<td>97 (39)</td>
<td>196 (40)</td>
</tr>
<tr>
<td>4</td>
<td>68 (29)</td>
<td>70 (28)</td>
<td>138 (28)</td>
</tr>
<tr>
<td>8</td>
<td>16 (7)</td>
<td>14 (6)</td>
<td>30 (6)</td>
</tr>
<tr>
<td>16</td>
<td>5 (1)</td>
<td>0 (0)</td>
<td>5 (0.6)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>238</td>
<td>249</td>
<td>487</td>
</tr>
</tbody>
</table>

* Chi-squared test, Mantel-Haenszel test, or Fisher's exact test.
**Discussion**

Incident cryptococcal isolates obtained through population-based surveillance in South Africa maintained low MIC values to the first-line antifungal drugs fluconazole and amphotericin B over a 6-year period, and very low MICs were determined for newer drugs, such as voriconazole and posaconazole. Despite infrequent use of these agents in South Africa, a small number of isolates were determined to have elevated MIC values to itraconazole and/or fluconazole. Nonsusceptibility to fluconazole, as defined by a 4-fold increase in the MIC (10, 33), was detected in only 1 case with serially collected isolates.

Historically, the determination of the MIC in the laboratory has been the method of choice for monitoring antifungal resistance. Although a standardized antifungal drug susceptibility testing method has been developed (13), antifungal resistance in Cryptococcus is difficult to define in the laboratory due to the absence of interpretive breakpoints. Attempts to correlate MIC with clinical outcome have produced mixed results. For example, in a small case series, an incident isolate with a fluconazole MIC of ≥16 μg/ml was associated with subsequent clinical failure among 5 of 25 (20%) patients (2); however, this finding was not replicated in other studies (15, 25). Nevertheless, if it is performed consistently over time, MIC testing can indicate shifts in susceptibility among isolates at a population level.

The distribution of 200-mg fluconazole tablets through the Diflucan Partnership Program in South Africa has doubled from approximately 1.8 million doses in 2002 to 3.8 million doses in 2008 (Pfizer, South Africa, personal communication). Despite this, the finding that almost all incident cryptococcal isolates had a fluconazole MIC of ≤16 μg/ml was not surprising. A large global study found that the fluconazole MIC of incident isolates did not change substantially over a 15-year period (1990-2004), despite increased use of fluconazole (35). In addition, Brandt et al. tested 143 incident C. neoformans (serotype A) isolates from the same population-based surveillance system in Gauteng Province (2002-2003) with the reference broth microdilution method; the MIC range, MIC50, and MIC90 for fluconazole were 0.5 μg/ml to 2 μg/ml, and 4 μg/ml, respectively, which are almost identical to the results that we obtained (9). In contrast, a report from Cambodia described incident isolates with increased fluconazole MICs over a 2-year period when they were tested with the Etest method (38). The findings from the latter study are difficult to explain. Fluconazole-resistant Candida species have been shown to emerge in areas where primary fluconazole prophylaxis is used as a preventative strategy (3), but Candida is a colonizer that can replicate as a commensal in the host and can inhabit many different body sites to avoid maximum exposure to antifungal drugs. Dormant cryptococcal strains, which are hypothesized to have established a latent infection many years previously, reactivate primarily in the milieu of advanced HIV-associated T-cell immunodeficiency and cause disseminated disease (18). Even in the setting of primary fluconazole prophylaxis, incident C. neoformans isolates with reduced susceptibility to fluconazole have been infrequently documented (4, 29); this may be directly related to the fact that they do not actively replicate in the host as commensal organisms prior to onset of disease.

Voriconazole and posaconazole have consistently been shown to have good activity against C. neoformans (1, 9, 11, 36). However, the use of voriconazole and posaconazole is still restricted to salvage settings (33), as no clinical trials have been undertaken to compare these agents to first-line drugs and these agents remain prohibitively expensive for use in resource-limited settings. In our study, the three incident isolates with reduced fluconazole susceptibility (MIC = 16 μg/ml) had relatively low MICs to voriconazole. However, fungistaticazole drugs would still not be the first choice for treatment of incident cryptococcosis.

Even in the absence of interpretive breakpoints, MIC testing may be more helpful to document the emergence of resistance over time among patients with serially collected isolates if the same test method is used and isolate pairs are tested in parallel. In our surveillance, we found that, in most cases, serially collected isolates displayed fluconazole MIC results within 2 dilutions, indicating essential agreement. Similarly, an Ugandan study which compared fluconazole broth microdilution MICs of serially collected isolates from 17 patients found no evidence of a stepwise increase in MIC over a 2- to 10-week period (37). In contrast, a prospective, observational study from Cape Town, South Africa (2003 to 2005), found that 16 of 20 (80%) patients with culture-confirmed relapse disease had isolates with reduced fluconazole susceptibility, as determined by the Etest method (6). Bicanic et al. suggested that the high prevalence of fluconazole nonsusceptibility among Cape Town isolates was associated with low-dose fluconazole induction treatment (400 mg daily) and concurrent rifampin use (6). A Cambodian study also reported that the MIC to fluconazole, as determined by Etest, increased significantly from the year 2000 to 2002 (38). In contrast, we found only one case where the serially collected isolate had an MIC value significantly elevated above the MIC of the incident isolate, despite most patients receiving low-dose fluconazole induction treatment (400 mg daily). Similarly, Brandt et al., who also determined the fluconazole MIC for isolates from Gauteng surveillance (2002-2003) serially collected more than 30 days apart, detected an increase in fluconazole MIC values of at least 3 log₂ dilutions over time for only 2 of 30 cases (9).
Differences in MIC testing methods may have contributed to some of these reported differences. Several reports indicate that Cryptococcus MIC values to the azoles that have been generated by Etest are higher than those generated by broth microdilution testing when they are performed in parallel (15, 17, 30, 34, 40). While we used a broth dilution method for MIC determination, Bicanic et al. used the Etest method, where endpoint determination for azoles is technically more difficult to establish and may be more subjective (6). There is still some question as to whether the results of various testing methodologies can be directly compared. In addition, Bicanic et al. defined resistance to fluconazole as a single (relapse episode) isolate with an MIC of ≥16 µg/ml; isolate pairs were not tested in parallel (6).

We believe that there are other, more common reasons for recurrent disease, such as nonadherence to suppressive fluconazole treatment (14), development of the immune reconstitution inflammatory syndrome (IRIS) following initiation of cART (7, 26), or suboptimal induction-phase treatment. Recently, Jarvis et al. described patients with symptomatic relapse disease at the same Cape Town hospital in 2007-2008 when amphotericin B induction-phase treatment and antiretroviral treatment were the standard of care (24). Of the 69 relapse episodes that were detected over this 2-year period, most were due to IRIS (45%) or nonadherence to or nonprescription of fluconazole maintenance treatment (43%) (24). In contrast to the earlier Cape Town study, very few isolates with elevated fluconazole MICs (MIC ≥ 16 µg/ml, determined by the Etest) were detected from this group of patients.

In our study, there were some cases where the MIC value of the second isolate compared to that of the incident isolate dropped when both were tested in parallel using the same broth microdilution method, a phenomenon that has been documented previously (10). Although we do not currently understand this mechanism, recent work by Desnos-Ollivier and colleagues indicates that at least 20% of C. neoformans infections may be comprised of multiple strains and genotypes (16). By testing only selected subpopulations cultured from the original clinical specimen, other strains contributing to disease in a given patient may be missed (16).

Major challenges to improving management of patients with cryptococcosis include (i) preventing cryptococcosis by early diagnosis of HIV infection and timely initiation of cART well before the CD4+ T-cell count falls below 200 cells/ml, (ii) diagnosing cryptococcal meningitis early using strategies such as screening high-risk patients (with CD4+ T-cell counts below 100 cells/ml) with the cryptococcal antigen test (21), (iii) improving access to first-line antifungal drugs such as amphotericin B and fluconazole, (iv) improving management of raised intracranial pressure (5), (v) facilitating access to cART soon after diagnosis of cryptococcosis, and (vi) reducing the high mortality rate by optimal management of patients during and after hospital admission. cART improves long-term survival if patients survive the first episode of cryptococcal meningitis (27). Recognizing the need to optimize management of the first episode and improve survival rates, South African clinicians have treated an increasing proportion of patients with amphotericin B deoxycholate since 2005 (19).

Although the cryptococcal isolates in this study were obtained through active population-based surveillance, this study has several limitations. First, cases of incident cryptococcosis were drawn from a relatively small geographic area (four hospitals in Gauteng Province). However, we selected these sites because long-term trends could be examined and because we expected that fluconazole nonsusceptibility was more likely to emerge in settings where patients were likely to have previously received fluconazole for other indications. Second, the sample may have been underpowered to detect a small change in MIC for both MIC50g and MIC90g between the two surveillance periods. Third, patient follow-up was limited to the duration of hospital admission; hence, any association between MIC and outcome was unlikely to be meaningful. Fourth, we lacked sufficient clinical and laboratory data to make the distinction between persistence and relapse among cases with serially collected isolates.

In conclusion, we have found no evidence for the emergence of resistance to fluconazole among incident cryptococcal isolates in South Africa. However, fungicidal agents should still be preferentially selected for induction treatment when they are available. Similarly, only one case with serially collected isolates was associated with significantly changed fluconazole MIC values, suggesting that clinical attention needs to be focused on other more common causes of recurrence.

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