Increasing in vitro resistance to fluconazole in Cryptococcus neoformans Cambodian isolates: April 2000 to March 2002

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Objectives: Cryptococcal meningitis is the third-most-common opportunistic infection in HIV patients in Cambodia. Hospitalized patients were given amphotericin B for initial therapy followed by fluconazole for maintenance therapy. The antifungal drug susceptibility of Cryptococcus neoformans isolated from cerebrospinal fluid (CSF) was determined.

Methods: Isolates of C. neoformans were collected during active laboratory-based surveillance, the first batch from April 2000 to March 2001 (134 new cases), the second batch from April 2001 to March 2002 (268 new cases). Etest strips were used to determine the MICs of amphotericin B and fluconazole. The antigenic agglutination slide test was used for serotyping.

Results: The MIC50s and MIC90s of fluconazole changed significantly from year 2000 to 2002; the MIC50s increased from 4 to 12 mg/L, and the MIC90s from 12 to 96 mg/L. For amphotericin B, the MIC50s and MIC90s remained stable. Moreover, in the second batch, fluconazole MICs were >256 mg/L for 20 isolates. By serotyping, it was found that 98.5% of the isolates were serotype A.

Conclusions: C. neoformans strains isolated from CSF of AIDS patients in Cambodia remain susceptible in vitro to amphotericin B. These strains are less susceptible in vitro to fluconazole, 2.5% being resistant in the first year and 14% in the second year of study. Nevertheless, in vitro resistance of C. neoformans to fluconazole appeared to be linked to extended maintenance treatments.

Keywords: cryptococcal meningitis, antifungals, Etest

Introduction

Cryptococcosis is an infectious disease caused by Cryptococcus neoformans, an encapsulated yeast-like fungus. Most patients with AIDS who are infected with C. neoformans develop meningitis. Since the start of the Cambodian AIDS pandemic, cryptococcal meningitis is the third-most-common opportunistic infection.1 HAART is still not available to treat patients in Cambodia. Amphotericin B remains the agent of choice for induction treatment but the oral triazole fluconazole has proven to be superior for long-term maintenance treatment. In most reported instances, factors other than drug resistance have accounted for failure or relapse during long-termazole maintenance treatment of cryptococcosis. To date, few reports of resistance to fluconazole or amphotericin B have been published concerning C. neoformans. However, it has been reported that the widespread use fluconazole can lead to emergence of less-susceptible strains of C. neoformans. For these reasons, it is necessary to develop routine surveillance of susceptibility to antifungal treatment in vitro. To investigate this further, we determined in vitro susceptibilities to fluconazole and amphotericin B of C. neoformans isolates collected in Cambodia from new cases of meningitis in AIDS patients, from April 2000 to March 2002.

Materials and methods

Origin of isolates

Four hundred and two clinical C. neoformans isolates collected by cerebrospinal fluid (CSF) sample culture from 402 new cases of
meningitis in AIDS patients were tested in this study. In total, 90% of CSF samples were from the patients in the Department of Infectious Disease of Preah Bat Norodom Sihanouk hospital. Of these, a first batch consisted of 134 isolates collected from April 2000 to March 2001, and a second batch of 268 isolates collected from April 2001 to March 2002. All of these isolates were stored and frozen at −20°C in 20% glycerol until the study was carried out. The isolates were identified as *C. neoformans* by standard methods, observation of capsulated yeast-like fungus by China-Ink examination, yeast culture with underlining of urease production.

### Etest method

The Etest was carried out according to the manufacturer’s instructions (AB Biodisk). Inocula were prepared from 24 h culture of *C. neoformans*. Cell suspensions were made in sterile 0.85% saline solution and adjusted to a concentration corresponding to a 1.0 McFarland unit. Agar formulations used for the Etest were RPMI 1640 (Gibco, Invitrogen Corporation, UK) supplemented with 1.5% agar and 2% glucose. Cell suspensions were inoculated onto RPMI agar, and then plates were dried at room temperature for 15 min before Etest strips were applied. The plates were incubated at 35°C for 48 h. The minimum inhibitory concentrations (MICs) were read at the intersection (at the point of approximately 80% growth inhibition for fluconazole) of the zone edge and the Etest strip.

### Broth microdilution method

The MIC of fluconazole was determined by the NCCLS broth microdilution method for 40 isolates of *C. neoformans*. Fluniconazole (Pfizer Laboratory, Porce sur Cisse, France) was diluted in RPMI medium with the final concentrations ranging from 0.125 to 256 mg/L. The yeast inoculum was adjusted to a concentration of 2.5 × 10^3 cells/mL in RPMI medium. The microtitre culture plates were incubated at 35°C. The MIC endpoints were read visually following 48 h of incubation and were defined as the lowest concentration that produced an 80% reduction in growth compared with that of the drug-free growth control.

### Serotyping

*C. neoformans* isolates were serotyped by a slide agglutination test with sera of 1, 5, 6, 7 and 8 types (Iatron, Tokyo, Japan). On the basis of the patterns of agglutination, 402 isolates of *C. neoformans* from new cases were classified as follows: serotypes A, B, C, D and AD.

### Quality control

*C. neoformans* isolates from the National Collection of Microorganisms Culture (Institut Pasteur, Paris, France), IP 960.67, IP 961.67, IP 962.67 and IP 1210.79 (serotypes A, B, C, D, respectively), were included for repeat testing to check the reproducibility of the results.

### Results

Out of 402 CSF samples from AIDS patients suffering meningitis, 134 had been sampled in the first period and 268 in the second period. China-Ink microscopic examinations of CSF were negative in 6% and 7% in the first and second groups, respectively.

*In vitro* susceptibility of the 402 *C. neoformans* isolates to amphotericin B and fluconazole is reported as MICs inhibiting 50% (MIC50) and 90% (MIC90) of the isolates (Table 1). Amphotericin B MIC50s were 0.19 mg/L in the first batch of isolates and 0.09 mg/L in the second batch. Amphotericin B MIC90s were 0.5 mg/L for the first batch and 0.50 mg/L in the second batch. Fluconazole MIC50s were 4 and 12 mg/L for the first and second batches, respectively, whereas MIC90s were 12 and 96 mg/L. In a series of tests, MICs for the quality control strains were within the expected limits for both antifungal agents.

Of 402 recent isolates of *C. neoformans* from new cases, 396 (98.5%) displayed agglutination patterns consistent with serotype A *C. neoformans* var. *neoformans*. The other six isolates showed positive reactions with characteristics of serotype B, *C. neoformans* var. *gattii*.

The Student’s *t*-test for paired data was used for statistical analysis. Statistical significance was set at *P* < 0.05.

### Discussion

Antifungal susceptibility testing has proven notoriously difficult to introduce into the routine clinical laboratory. Although the NCCLS M27-A broth dilution method remains the gold standard, it is not convenient to carry out on a routine basis. Alternative methods have been developed over the past years, including the broth colorimetric microdilution technique, flow cytometry, and MIC diffusion strips, the so-called Etest. Maxwell *et al.* and Aller *et al.* suggested that the Etest is a useful alternative to the microdilution method for use in the clinical laboratory for the determination of susceptibility of *C. neoformans* to amphotericin B and fluconazole. The Etest seems to be easier for routine use, and, in several reports, as reliable as the microdilution method.

This method enabled us to monitor the susceptibility of 402 *C. neoformans* isolates to amphotericin B and fluconazole over a 2 year period. For these two antifungals, MIC50s and MIC90s increased from April 2000–March 2001 to April 2001–March 2002, especially for fluconazole, *P* < 0.005. The exact agreement

<table>
<thead>
<tr>
<th>Period</th>
<th>No. tested</th>
<th>CD4* mean (cells/mm³)</th>
<th>Amphotericin B MIC (mg/L) range</th>
<th>MIC50</th>
<th>MIC90</th>
<th>Fluconazole MIC (mg/L) range</th>
<th>MIC50</th>
<th>MIC90</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 2000–March 2001</td>
<td>134</td>
<td>47</td>
<td>0.002–1</td>
<td>0.19</td>
<td>0.50</td>
<td>0.50–&gt;256</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>April 2001–March 2002</td>
<td>268</td>
<td>45</td>
<td>0.002–1</td>
<td>0.09</td>
<td>0.50</td>
<td>0.50–&gt;256</td>
<td>12</td>
<td>96</td>
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between the Etest method and the reference broth microdilution method for 40 isolates of *C. neoformans* was defined as the proportion of Etest results which fell within the reference test MIC range for fluconazole (data not shown).

In fact, fluconazole MICs were ≥32 mg/L for five of 134 isolates (3.7%) in the first batch and for 55 of 268 isolates (20.5%) in second batch. In contrast, such an upward shift was not observed for amphotericin B between 2000 and 2002. While close correlations between *in vitro* susceptibilities to antifungals and clinical outcome have not been significantly established, Aller et al.\(^4\) demonstrated that a positive clinical response to fluconazole maintenance therapy could be better expected when the fluconazole MIC of the infecting strain of *C. neoformans* was <16 mg/L.

Although other factors, such as the CD4\(^+\) T lymphocyte level and the fungal load in the CSF, are important in determining the clinical outcome in patients with cryptococcal meningitis, there is some evidence that high fluconazole MICs are often predictive of treatment failure.

It has been suggested that the widespread use of fluconazole could bring about selective pressure leading to the emergence of less-susceptible strains of *C. neoformans*. Koletar et al.\(^1\) reported an upward shift of fluconazole MICs in *C. neoformans* strains isolated from blood and CSF between 1991 and 1994. In 1991, none of 11 isolates tested had fluconazole MICs ≥32 mg/L, compared with 11 of 20 isolates tested in 1994. In contrast, Davey et al.\(^1\) did not detect any change in fluconazole MICs in *C. neoformans* strains isolated between the 1970s and 1990s. The case–control study conducted in the Centers for Disease Control and Prevention (CDC) from 1992 to 1998 for a total of 732 isolates showed that the majority of isolates of *C. neoformans* tested appeared to be susceptible to fluconazole.\(^6\)

Moreover, such an increase in fluconazole MICs seems to be linked to clinical failure of cryptococcal meningitis in AIDS patients.\(^5\)

*C. neoformans* serotype A appears to be the predominant serotype involved in AIDS-related opportunistic infections in Cambodia. These serotyping data corroborate those reported by Pienthaweechai et al.\(^10\) from AIDS patients in Khon Kean, Thailand.

This study reveals a worrying increase in fluconazole MICs for *C. neoformans* over a short 2 year period, probably linked to the widespread prescription of this antifungal agent for AIDS patients in Cambodia. Although correlations between *in vitro* resistance to fluconazole and clinical efficacy of the antifungal therapy are difficult to establish, this trend leads to worries of an increase in clinical failure among cryptococcal meningitis patients receiving the standard treatment. More studies are warranted to further determine the relationships between acquired fluconazole resistance in *C. neoformans* and the consumption of this antifungal agent in hospitals and in the community. Extended maintenance treatments have to be strictly controlled, especially after discharge. Above all, the management of cryptococcal meningitis due to a fluconazole-resistant strain remains to be clarified.

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### References