Rates of antifungal resistance among Spanish clinical isolates of *Cryptococcus neoformans* var. *neoformans*

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**Objectives:** Activities in vitro of six antifungal agents were tested against a collection of 317 *Cryptococcus neoformans* var. *neoformans* clinical isolates.

**Methods:** The procedure described in document 7.1 by the European Committee on Antibiotic Susceptibility Testing with minor modifications was employed.

**Results:** Amphotericin B, itraconazole, voriconazole and ravuconazole exhibited a potent activity with geometric mean (GM) MICs under 0.26 mg/L. The GM MIC of flucytosine was 7.33 mg/L and that of fluconazole was 4.16 mg/L. The rates of antifungal resistance were 5.3% for amphotericin B, 0.9% for voriconazole and 3.1% for ravuconazole. Fifteen point eight per cent of strains had itraconazole MICs ≤1 mg/L, and 46% of strains had flucytosine MICs ≥8 mg/L. Fluconazole susceptibility (MIC ≤8 mg/L) stood at 53.4%.

**Conclusions:** The percentage of fluconazole susceptibility was significantly lower than that in other surveys. Cross-resistance to itraconazole was common (33.8%) but almost the whole collection was susceptible to voriconazole and ravuconazole. These results should be confirmed with prospective and population-based surveillance programmes.

Keywords: fluconazole resistance, EUCAST, surveillance programmes

**Introduction**

*Cryptococcus neoformans* causes infection mainly in immuno-compromised hosts. Current treatment regimens for cryptococcal meningitis are based on amphotericin B, with or without flucytosine. This treatment is followed by maintenance therapy with fluconazole which has been related to the development of antifungal resistance. The mechanisms of azole resistance described in *C. neoformans* include enhanced energy-dependent drug efflux and point mutations in the *ERG11* gene which are responsible for the amino acid substitution glycine 484 for serine (G484S). Resistance in *C. neoformans* clinical isolates remains uncommon and has not increased in the last decade in the United States and the UK, but local increases in resistance have been reported in other areas such as Cambodia. These studies have shown geographic differences in species distribution and antifungal resistance. Recently, Pfaffer et al. reported a 15 year survey including susceptibility data on 1811 clinical isolates of *C. neoformans* obtained from five geographic regions. Although resistance was reported, uncommonly some geographical differences were found. We describe the susceptibility of 317 clinical isolates of *C. neoformans* var. *neoformans* isolated in Spain during a period of 10 years (1995–2004).

**Materials and methods**

A collection of 317 isolates was included in the study. All strains were recovered from different Spanish hospitals over a period of 10 years, from 1995 to 2004. The origin of isolates was as follows: 207 strains (65.3%) from CSF, 68 (21.5%) from blood culture, 12 (3.8%) from respiratory tract samples, 9 (2.8%) from biopsies, 8 (2.5%) from skin and 13 (4.1%) from other samples. Isolates were identified by routine physiological and morphological tests. The variety *neoformans* was distinguished from the variety *bacillispora* by incubating cultures on canavanine–glycine–Bromothymol Blue agar. Strains were stored at –70°C.

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C. neoformans and antifungal resistance

Table 1. Susceptibility data (in mg/L) of 317 clinical isolates of C. neoformans var. neoformans

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>Range</th>
<th>Mode</th>
<th>Geometric mean</th>
<th>Percentage of strains with antifungal resistance&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.25</td>
<td>1.00</td>
<td>0.031–2</td>
<td>0.50</td>
<td>0.26</td>
<td>5.3</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>4</td>
<td>16</td>
<td>0.125–128</td>
<td>8</td>
<td>4.16</td>
<td>46</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>4</td>
<td>16</td>
<td>0.125–32</td>
<td>16</td>
<td>7.33</td>
<td>46.6</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.25</td>
<td>1</td>
<td>0.015–2</td>
<td>0.25</td>
<td>0.26</td>
<td>15.8</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.12</td>
<td>0.50</td>
<td>0.015–4</td>
<td>0.25</td>
<td>0.12</td>
<td>0.94</td>
</tr>
<tr>
<td>Ravuconazole</td>
<td>0.12</td>
<td>1</td>
<td>0.015–4</td>
<td>0.25</td>
<td>0.16</td>
<td>3.1</td>
</tr>
</tbody>
</table>

MIC<sub>50</sub>, MIC value causing inhibition of 50% of isolates; MIC<sub>90</sub>, MIC value causing inhibition of 90% of isolates.
<sup>a</sup>Antifungal resistance was defined as: MICs of amphotericin B ≥2 mg/L; MICs of flucytosine ≥8 mg/L; MICs of fluconazole ≥16 mg/L; MICs of itraconazole ≥1 mg/L; MICs of voriconazole ≥2 mg/L; MICs of ravuconazole ≥2 mg/L.

The susceptibility testing strictly followed the recommendations proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing for fermentative yeast (AFST-EUCAST, document 7.1).<sup>6</sup> These recommendations are based on the Clinical Laboratory Standards Institute (CLSI) reference procedure described in document M27-A2,<sup>7</sup> but include some modifications to allow for automation of the method and to permit the incubation period to be shortened from 72 or 48 h to 24 h. In addition, in order to improve the growth of some organisms, another minor modification was included.<sup>6</sup> This modification was that the microplates were wrapped with film sealer to prevent the medium from evaporating, attached to an electrically driven wheel inside the incubator, agitated at 350 rpm and incubated at 30°C for 48 h. Candida parapsilosis ATCC 22019, Candida krusei ATCC 6258 and Cryptococcus neoformans ATCC 90112 were used as quality control strains.

The antifungal agents used in the study were amphotericin B (Sigma-Aldrich Química S.A., Madrid, Spain), flucytosine (Sigma-Aldrich Química S.A., Madrid, Spain), fluconazole (Pfizer S.A., Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer Ltd, Sandwich, UK) and ravuconazole (Bristol-Myers Squibb, Princeton, NJ, USA). For amphotericin B, the MIC end points were defined as the lowest drug concentration exhibiting reduction in growth of 90% or more compared with that of the control growth. For flucytosine and azole drugs the MIC endpoint was defined as 50% inhibition.

Antifungal resistance in vitro was defined as follows: MICs of amphotericin B ≥2 mg/L; MICs of flucytosine ≥8 mg/L; MICs of fluconazole ≥16 mg/L; MICs of itraconazole ≥1 mg/L; MICs of voriconazole ≥2 mg/L; and MICs of ravuconazole ≥2 mg/L. EUCAST has not still defined interpretive breakpoints, and the breakpoints proposed by the CLSI for flucytosine, fluconazole and itraconazole were not used owing to the fact that substantial discrepancies between methodologies have been observed for some fluconazole-resistant isolates.<sup>7</sup> Antifungal resistance was defined based on wild-type distribution of MICs determined by the EUCAST method, on preliminary studies of correlation in vitro/vivo with strains causing oropharyngeal candidosis in AIDS patients, and on PK/PD bibliographic data.<sup>10,11</sup> It should be emphasized that these breakpoints are tentative and could be changed when AFST-EUCAST report new susceptibility data.

All statistical analysis was done with the Statistical Package for the Social Sciences (SPSS, version 13.0) (SPSS S.L., Madrid, Spain). The Student’s t-test, Fisher’s exact test or $\chi^2$ analysis were used. $P<0.01$ was considered significant.

Results

The geometric mean (GM) MICs, modes, ranges, MICs including 50% of isolates (MIC<sub>50</sub>) and MICs including 90% of isolates (MIC<sub>90</sub>) of all antifungal agents tested are shown in Table 1. The GM MIC of amphotericin B was 0.26 mg/L. Fluconazole exhibited less potent activity in vitro, with MICs consistently ≥4 mg/L (GM 7.33 mg/L) and MIC<sub>90</sub> ≥16 mg/L. Overall, the otherazole compounds exhibited higher activity than that of flucytosine. The GMs of itraconazole, voriconazole and ravuconazole were 0.26, 0.12 and 0.16 mg/L, respectively. Voriconazole and ravuconazole appeared to be the most active drugs in vitro of theazole group and their activity was more potent than that of amphotericin B. Most of the isolates were inhibited by MICs ~4 mg/L of flucytosine (GM 4.16 mg/L).

No statistically significant differences were found when MIC values were analysed per isolation site and per year of study. The GM values of MICs of antifungal agents remained almost unchanged and did not exhibit any sign of an upward shift during the decade of study (1995–2004).

With regard to strains exhibiting high MIC values, a total of 17 strains (5.3%) had MICs of amphotericin B ≥2 mg/L, 131 organisms (41.3%) exhibited MICs of flucytosine ≥8–16 mg/L and 15 (4.7%) ≥32 mg/L. Meanwhile, 148 strains (46.6%) had fluconazole MICs ≥16–32 mg/L but none had MICs ≥64 mg/L. In addition, 50 strains were found with MICs ≥1 mg/L of itraconazole, 3 with MICs of voriconazole ≥2 mg/L and 10 with MICs of ravuconazole ≥2 mg/L.

It should be stressed that isolates exhibiting high MICs of itraconazole, voriconazole and ravuconazole had fluconazole MIC values >16 mg/L. In addition, when strains which showed MICs of fluconazole ≥16 mg/L were studied further, itraconazole, voriconazole and ravuconazole MICs were significantly higher ($P<0.01$ by Student’s t-test) than those observed for strains exhibiting fluconazole MICs <16 mg/L. However, on the whole, the MICs of voriconazole and ravuconazole for fluconazole-resistant strains were <0.25 mg/L, with GMs of 0.23 and 0.21 mg/L, respectively.

The GM MICs of voriconazole and ravuconazole for strains with itraconazole MIC ≥1 mg/L were 0.19 and 0.24 mg/L, respectively. Meanwhile, strains with itraconazole MICs <1 mg/L showed GM MICs of voriconazole and ravuconazole of 0.11 and 0.15 mg/L. The GM MIC of ravuconazole for strains with
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Table 2. MIC ranges (mg/L) for control strains by the M27-A2 reference procedure and by the modified method

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Candida parapsilosis ATCC 22019</th>
<th>Candida krusei ATCC 6258</th>
<th>Cryptococcus neoformans ATCC 90112</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MICs obtained by modified method</td>
<td>MICs obtained by M27-A2 procedure</td>
<td>MICs obtained by modified method</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.12–0.50</td>
<td>0.25–1.0</td>
<td>0.12–0.50</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>0.06–0.25</td>
<td>0.06–0.50</td>
<td>0.25–1.0</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1.0–4.0</td>
<td>10.0–16.0</td>
<td>8.0–16.0</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.03–0.12</td>
<td>0.015–0.12</td>
<td>0.03–0.12</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.03–0.12</td>
<td>0.015–0.12</td>
<td>0.03–0.12</td>
</tr>
<tr>
<td>Ravuconazole</td>
<td>0.015–0.06</td>
<td>0.015–0.12</td>
<td>0.015–0.06</td>
</tr>
</tbody>
</table>

Table shows the MICs after 30 repetitions on different days. *References 6 and 8.

MICs ≥2 mg/L of voriconazole was 1.09 mg/L and the GM MIC of ravuconazole for strains with MICs <2 mg/L of voriconazole was 1.55 mg/L. These differences in GM MIC values did not have statistical significance.

Finally, 17 strains that showed MICs of amphotericin B ≥2 mg/L had GM MICs of flucytosine, fluconazole, itraconazole, voriconazole and ravuconazole of 4.33, 6.52, 0.44, 0.11 and 0.07 mg/L, respectively. Strains that had an MIC of amphotericin B <2 mg/L displayed MICs of flucytosine, fluconazole, itraconazole, voriconazole and ravuconazole of 4.16, 7.37, 0.25, 0.12 and 0.16 mg/L, respectively.

The MIC values for the three quality control strains were consistent within two or three 2-fold dilutions (Table 2), values that agree with those in the discussion document 7.1 by EUCAST and the CLSI document M27-A2.6,7

Discussion

In our study, we had 53.4% of strains with MICs of fluconazole ≤8 mg/L and the percentage of strains susceptible to itraconazole was 84.2%. Resistance rates for other antifungal agents were similar to those reported by other studies.1,4 The percentage of amphotericin B resistance was ~5%, the rate of ravuconazole resistance was 3%, and the percentage of voriconazole resistance was <1%. Cross-resistance among azole agents was also analysed. A total of 50 out of 148 strains (33.8%) with fluconazole resistance were resistant in vitro to itraconazole. However, percentages of both voriconazole and ravuconazole resistance were very low.

Epidemiological studies have been shown to be effective for finding geographic differences in antifungal susceptibility patterns and the extent of antifungal resistance among C. neoformans. A 15 year study by Pfaller et al.4 observed that amphotericin B, flucytosine and fluconazole resistance was <1% overall. The study included a total of 1811 clinical isolates and analysed strains from Africa, Europe, Latin America, the Pacific region and North America. However, fluconazole resistance differed depending on the region studied; only 75% of isolates from North America were susceptible to fluconazole (MIC ≤8 mg/L), but percentages of susceptibility in other zones were much higher (94–100%). In addition, other studies have described percentages of susceptibility to fluconazole among clinical strains of C. neoformans from 65 to 90%,1,12 and a 3-fold rise in fluconazole MICs during the study period.5

We found a percentage of fluconazole decreased susceptibility (46.6%) that was significantly higher than those of other surveys and remarks about it should be made. The susceptibility testing method that was used is based on document M27-A2 by the CLSI but with minor modifications such as RPMI-2% as assay medium, an inoculum size of 10⁵ cfu/mL, and incubation under constant agitation. The modified technique used for testing our collection might yield MICs that are falsely elevated, overestimating the rates of fluconazole resistance. However, modifications of the reference procedure have been evaluated previously in several studies8,10,12 owing to the poor growth of C. neoformans and other non-fermentative yeasts with the medium recommended by the CLSI. Media such as buffered yeast nitrogen base or RPMI supplemented with 2% glucose, higher inoculum size and incubation under constant agitation have been employed, getting better rates of growth and MIC values comparable to those achieved by the reference method.13,14 In addition, MIC values for quality control strains included in the experiments agreed with those published in the CLSI document.7

Although it could be argued that differences in fluconazole susceptibility rates are due to methodological variations, it is also a fact that there is likely to be a survey bias in favour of resistance. Reference laboratories usually receive isolates for susceptibility testing collected from patients suffering from infections that are difficult to treat and recidivating mycoses. These patients have to undergo a prolonged course of treatment and there are opportunities to develop resistance to antifungal agents.

To sum up, the collection of C. neoformans analysed was highly susceptible to amphotericin B, voriconazole and ravuconazole. Fifteen per cent of the isolates were resistant in vitro to itraconazole and 46% were resistant to flucytosine. The most significant result was the rate of fluconazole susceptibility, 53.4%. Cross-resistance to itraconazole was common among fluconazole-resistant strains (33.8%), but almost the whole collection was susceptible to voriconazole and ravuconazole. Retrospective surveys can reach biased conclusions as an entire population is not analysed. These results should be confirmed with prospective and population-based surveillance programmes.
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Transparency declarations

We have no conflicts of interest to report.

References