In vitro Activities of Antifungal Drugs Against Yeasts Isolated from Blood Cultures and Moulds Isolated from Various Clinically Significant Sites in Singapore

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Abstract

Introduction: Fungaemia carries with it high mortality rates and appropriate as well as timely antifungal therapy has been shown to be life saving. Materials and Methods: We studied the in-vitro activities of antifungal agents using the Etest method, against 100 Candida isolates from blood cultures, 10 Cryptococcus isolates from blood or cerebrospinal fluid and 50 mould isolates from various clinically significant sites of patients in Singapore General Hospital, from June 2004 to December 2006. Results: Overall, the yeasts appeared to have low minimum inhibitory concentrations (MICs) for all the 5 antifungal drugs tested except for fluconazole. The overall high MIC90 values of the moulds against the azoles were largely attributed to the non-Aspergillus moulds. Posaconazole, itraconazole, voriconazole and caspofungin appear effective against local strains of Aspergillus species, although there are no interpretive breakpoints. Conclusions: The results show that the local fungal strains studied appear to be susceptible to the usual antifungal drugs recommended in the literature.

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Key words: Antifungal susceptibility, Aspergillus, Candida, Etest, Singapore

Introduction

Fungaemia carries with it high mortality rates and appropriate as well as timely antifungal therapy has been shown to be life saving.1 Amphotericin B has the broadest coverage amongst the antifungal drugs against fungal infection, and was regarded the gold standard treatment for severe fungal infection. However, because of toxicity of amphotericin, and with the introduction of less toxic antifungal drugs, the azoles and echinocandins (e.g. caspofungin), the latter are now alternative treatment options. Candida remains the most common systemic fungal infection worldwide, followed by Aspergillus. High-risk patients for invasive fungal infections include solid-organ and haematopoietic stem cell transplantation, cancer, receipt of immunosuppressive therapy, AIDS, premature birth, advanced age and major surgery.2 Unlike bacterial infections, antifungal susceptibility testing is not routinely done, because they are more costly, requires better standardisation, and often lack interpretative criteria.3,4 Treatment has therefore largely been guided by results of published literature. There are limited antifungal susceptibility studies done in Singapore.5,6 To the best of our knowledge, no studies have been carried out in Singapore, on the newer antifungal drugs such as posaconazole and caspofungin, which may used in certain difficult to manage cases. In addition, no local antifungal susceptibility studies have been done on moulds. Information on local strains will help clinicians in management of cases in Singapore.

The objective of this study was to look at the in vitro activities of antifungal drugs against selected yeast and mould strains isolated from blood cultures and other clinically significant sites of patients in the Singapore General Hospital from June 2004 to December 2006.

Materials and Methods

Archived yeast and moulds from patients in Singapore General Hospital were used in this study. One hundred Candida isolates from blood were selected, with roughly equal numbers from each of the 3 years. There were 24 C. albicans, 28 C. tropicalis, 27 C. glabrata, 12 C. parapsilosis, 7 C. dubliniensis, 1 C. krusei and 1 C. famata isolates. The isolates were identified using germ tube test, yeast morphology on Tween agar, and API 20 C AUX. In addition, 10 isolates of Cryptococcus species from blood or cerebrospinal fluid (CSF) were studied. Fifty mould isolates were studied, and were isolated from various sites and specimens. These were 34 Aspergillus species (12 A. fumigatus, 12 A. niger, 7 A. flavus, 3 A. clavatus/A. nidulans), 10 Fusarium solani, 2 Syncephalastrum species, 1 each of

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Rhizopus, Absidia, Acremonium and Paecilomyces species. The source or site of isolation of the moulds is summarised in Table 1.

The minimum inhibitory concentrations (MICs) were performed and determined using Etest on RPMI agar (with 2% glucose and MOPS) in accordance to the instructions from the Etest manufacturer.7 For the yeasts, the drugs tested were posaconazole, fluconazole, voriconazole, amphotericin B and caspofungin, and the plates were incubated at 35°C in a moist incubator until good growth was observed. For most Candida species, the readings were taken at 24 hours of incubation. For Candida tropicalis and Candida glabrata, however, the readings were taken at 48 hours, as instructed in the Etest manufacturer’s instructions.8 For any of the Candida species not showing sufficient growth at 24 hours, notably Candida parapsilosis, the readings were taken at 48 hours of incubation. Cryptococcus neoformans Etests were read at 48 hours of incubation. For moulds, the drugs tested were posaconazole, itraconazole, voriconazole, amphotericin B and caspofungin. The inoculum was standardised using spectrophotometer at 530 nm wavelength to obtain counts of approximately 10⁶ cfu/mL.9,10 The RPMI plates (with 2% glucose and MOPS) were incubated at 35°C for 1 to 3 days. Most moulds showed good growth and were read at 24 hours, while Fusarium species were read at 48 hours, and Acremonium and Paecilomyces at 72 hours.

The MIC readings were taken at the point where the ellipse of growth intersected the scale on the strip. For amphotericin B, the MIC was read at the point of complete (100%) inhibition. For the azoles and caspofungin, the MIC was read at the first point of significant inhibition/
marked decrease in growth intensity, using the principle of 80% inhibition to visually select the end point. For each isolate, the MIC readings were taken by at least 2 trained individuals where final consensus readings were obtained. The results were tabulated and analysed with the WHONET software, which is available from http://www.who.int/drugresistance/whonetsoftware/en. Interpretative breakpoints are available only for fluconazole, voriconazole and amphotericin B against Candida species.

**Quality Control**

Quality control strains were used during each run. For runs involving yeasts, 2 control strains, Candida parapsilosis ATCC 22019 and Candida albicans ATCC 90028 were used, and their MICs were all within the range for amphotericin B, fluconazole, voriconazole, posaconazole and caspofungin. For runs involving moulds, the control strains used were Aspergillus fumigatus ATCC 204305 and Candida parapsilosis ATCC 22019, and MICs were within range.

**Results**

The MIC<sub>50</sub> and MIC<sub>90</sub> for the various antifungal agents are shown in Tables 2 to 4. The MIC distributions for the various fungi are presented in Figures 1 to 4.

**Discussion**

Interpretive breakpoints are available only for amphotericin B, fluconazole and voriconazole against Candida species, but not for posaconazole and caspofungin. There are no known breakpoints for antifungal drugs against moulds. Where there are no breakpoints available, only MIC<sub>50</sub> and MIC<sub>90</sub> data are presented.

The yeasts appear in general to have low MICs for the 5 antifungal drugs tested, except for fluconazole. For antifungals with available breakpoints, the MIC<sub>90</sub> values of voriconazole and amphotericin B against Candida species are within susceptible values. The value for fluconazole, however, is 16 μg/mL just beyond the susceptible value of 8 and this is contributed by Candida glabrata (MIC<sub>90</sub> of 48 μg/mL) and Candida krusei which is innately resistant. The proportion of Candida species selected for this study may not be representative of the distribution in the clinical setting, hence the data in Table 3 should be used with this limitation in mind. Although there are no breakpoints for Cryptococcus species, amongst the azoles, posaconazole and voriconazole have low MICs, but fluconazole has the highest MIC values. The MIC results seem to indicate that Cryptococcus species are susceptible to amphotericin B and uniformly resistant to caspofungin, which is not unexpected as caspofungin is not active against Cryptococcus species. This is because caspofungin inhibits fungal cell wall synthesis of β-1-glucan, but the cell wall of Cryptococcus is β-4-glucan.

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**Table 3. In vitro Activities of Antifungals Against 100 Strains of Candida sp. with Interpretive Breakpoints**

<table>
<thead>
<tr>
<th>Antifungal drugs</th>
<th>Breakpoints</th>
<th>No.</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>MIC mean</th>
<th>MIC range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posaconazole</td>
<td>Unknown</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.064</td>
<td>1.5</td>
<td>0.098</td>
<td>0.002-64</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>S&lt;=8 R=&gt;64</td>
<td>100</td>
<td>3</td>
<td>19</td>
<td>78</td>
<td>0.38</td>
<td>16</td>
<td>0.794</td>
<td>0.032-512</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>S&lt;=1 R=&gt;4</td>
<td>100</td>
<td>1</td>
<td>0</td>
<td>99</td>
<td>0.032</td>
<td>0.38</td>
<td>0.031</td>
<td>0.002-6</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>S&lt;=1</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0.38</td>
<td>0.75</td>
<td>0.197</td>
<td>0.064-1</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>Unknown</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.094</td>
<td>0.25</td>
<td>0.085</td>
<td>0.002-1</td>
</tr>
</tbody>
</table>

NA: not applicable, as no breakpoints are available.

**Table 4. Comparative In vitro Activities (MICs in μg/mL) of Various Antifungal Drugs against Moulds**

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>No.</th>
<th>POS</th>
<th>ITR</th>
<th>VOR</th>
<th>AMB</th>
<th>CAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>All moulds</td>
<td>50</td>
<td>0.047</td>
<td>&gt;32</td>
<td>0.19</td>
<td>&gt;32</td>
<td>0.094</td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>34</td>
<td>0.023</td>
<td>0.125</td>
<td>0.094</td>
<td>0.25</td>
<td>0.094</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>12</td>
<td>0.032</td>
<td>0.047</td>
<td>0.094</td>
<td>0.19</td>
<td>0.094</td>
</tr>
<tr>
<td>A. niger</td>
<td>12</td>
<td>0.012</td>
<td>0.032</td>
<td>0.064</td>
<td>0.19</td>
<td>0.032</td>
</tr>
<tr>
<td>A. flavus</td>
<td>7</td>
<td>0.094</td>
<td>0.19</td>
<td>0.19</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td>A. clavatus/A. nidulans</td>
<td>3</td>
<td>0.016</td>
<td>1</td>
<td>0.25</td>
<td>0.38</td>
<td>0.094</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>10</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>2</td>
</tr>
<tr>
<td>Others</td>
<td>6</td>
<td>0.38</td>
<td>&gt;32</td>
<td>1</td>
<td>&gt;32</td>
<td>6</td>
</tr>
</tbody>
</table>
Figs. 1a-1e. Antifungal MIC distribution for *Candida* sp. (n = 100).

Figs. 2a-2e. Antifungal MIC distribution for all moulds (n = 50).
Figs. 3a-3e. Antifungal MIC distribution for *Aspergillus* sp. (n = 34).

Figs. 4a-4e. Antifungal MIC distribution for *Cryptococcus* sp. (n = 10).
The moulds in general have high MIC$_{90}$ values, although their MIC$_{50}$ appear low. The MIC$_{90}$ value for amphotericin B for all moulds is $3 \mu g/mL$ and is close to the susceptible breakpoint, which is generally taken to be $1 \mu g/mL$.$^7$ However for Aspergillus, the most common and significant mould isolate, the MICs are low for all the 5 antifungal drugs tested, which indicates that besides amphotericin B, posaconazole, itraconazole, voriconazole and caspofungin may be useful in treating Aspergillus. Note that Aspergillus flavus has high MIC value for amphotericin B. The overall high MIC$_{90}$ values for moulds are therefore due to non-Aspergillus moulds consisting mainly of Fusarium species in this series.

Although there are no known breakpoints for posaconazole, the low MIC results suggest that posaconazole could be effective against Candida, Cryptococcus and Aspergillus species. Voriconazole, in our study, also appeared to have low MIC$_{90}$ values against the same fungi, with an added advantage of relatively lower MIC$_{90}$ values compared to posaconazole when tested against Fusarium solani. Caspofungin in this study appears to be effective against Candida species (except for Candida parapsilosis which corresponds to expected result), and the Aspergillus species. Nonetheless, the efficacy of these drugs would have to take into account their achievable serum levels (peak and sustained), other pharmacokinetic parameters, and would ultimately be determined by accumulation of clinical data.

One limitation of this study was the limited number of moulds available for testing. Although posaconazole has been demonstrated in vitro by Pfaffer et al$^{12}$ to be more active against some of the zygomycetes compared to voriconazole, we were not able to validate this finding due to the limited number of zygomycetes in our study, as these are uncommonly isolated from our clinical specimens.

MIC testing per se is an in vitro test, while the MIC breakpoints for interpretative criteria have to take into account the pharmacokinetic and pharmacodynamic studies and clinical data. MICs may be used as a guide to treatment. Nonetheless, the efficacy of these drugs depends also on their achievable levels (peak and sustained) in serum or at site of infection, and other pharmacokinetic parameters.$^{13}$

More clinical data are needed to correlate MIC results with clinical outcome studies, in order to determine usefulness of the antifungal drug against specific fungi. Hence, interpretive criteria are lacking in many moulds and for the newer agents against Candida species.

**Conclusion**

*Candida* species in Singapore appear susceptible to the usual antifungal drugs recommended in the literature, that is amphotericin B, fluconazole (except for *Candida glabrata* and *Candida krusei*) and voriconazole. The other newer drugs, posaconazole and caspofungin (except for *Candida parapsilosis*), also appear to be suitable although there are no interpretive breakpoints.

Amphotericin B appears suitable against Aspergillus species, with perhaps the exception of *Aspergillus flavus*, which has a higher MIC$_{90}$ value. Posaconazole, itraconazole, voriconazole and caspofungin also appear effective against local strains of *Aspergillus* species, although there are no interpretive breakpoints.

There are too few strains studied for the other fungal species, hence, no conclusion could be made.

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**REFERENCES**