Amphotericin B resistance testing of Candida spp.: a comparison of methods

Derek Law†, Caroline B. Moore and David W. Denning

Department of Microbiology and Department of Medicine (University of Manchester), Hope Hospital, Salford M 6 8 HD; Department of Infectious Diseases & Tropical Medicine (Monsall Unit), North Manchester General Hospital, Delaunays Road, Crumpsall, Manchester M 8 6 RB, UK

Several methods of susceptibility testing were compared for their ability to detect amphotericin B resistance among yeast isolates, including isolates known to be amphotericin-B-resistant in an animal model. A microtitre MIC method using antibiotic medium no. 3 and Etest strips were able to discriminate susceptible and resistant isolates. A disc susceptibility test was also able to identify resistant isolates and this method may be suitable for routine use as a screening test. A survey of resistance in 644 clinical isolates showed that the incidence of elevated MICs (≥0.25 mg/L) was only 1.2%.

Introduction

Amphotericin B is a polyene antibiotic, active against most species of pathogenic fungi. It is one of the drugs of choice for the treatment of systemic candidiasis. However, isolates resistant to this drug have been reported both in vivo and in vitro. A although the incidence of amphotericin B resistance is rare, it is thought to be relatively common among isolates of Candida lusitaniae.

The NCCLS M 27P MIC method, although useful for susceptibility testing of other antifungal drugs (notably the azoles), appears to be unable to detect resistance to amphotericin B. However, a modified MIC method using antibiotic medium no. 3 (AM 3) rather than RPM I was able to accurately detect resistance in isolates resistant to amphotericin B in an animal model. The Etest, a commercially available drug-containing strip that allows MIC determinations to be made, is also useful for detecting amphotericin B resistance.

In this study the modified amphotericin B susceptibility testing method was compared with the E test and disc diffusion methods, in order to identify methods capable of detecting resistance in the laboratory. As there is little data on the incidence of amphotericin B resistance in clinical isolates, large numbers of clinical isolates of yeasts were also tested to determine the distribution of MICs of amphotericin B.

Materials and methods

Organisms

Forty-nine isolates of Candida and one Saccharomyces species were tested in this study. Forty of these were clinical isolates, chosen to represent a range of species and MIC values. Eight isolates were kindly provided by John Rex, six of which had been shown to be amphotericin-B-resistant in an animal model. Two control isolates were also tested, an isolate of Candida albicans and an isolate of Candida kefyr. All organisms were stored frozen at −70°C in glycerol/nutrient broth. Organisms were subcultured on to Sabouraud agar and incubated at 37°C for 48 h before testing.

Antibiotic

Amphotericin B was obtained from Squibb (Hounslow, U K) as Fungizone powder; this was dissolved in water at a concentration of 1.28 mg/L, and this stock solution was stored at −20°C until use. E test amphotericin B strips were obtained from Difco (West Molesey, U K), and amphotericin B discs (20 μg per disc) were obtained from Mast (Bootle, U K).

*Corresponding author. Tel: +44-161-7202734; Fax: +44-161-7202732. †Present address: Hyder Environmental, Manor Park, Runcorn, Cheshire, WA7 1 SJ, U K.
MIC method

MICs were carried out in AM3 supplemented with glucose to a final concentration of 2% and the pH was adjusted to pH 7.0. A microtitre modification of the NCCLS M 27P MIC method was used for testing. Drug concentrations ranged from 32 to 0.03 mg/L and the final concentration of organism was $1.0-2.5 \times 10^3$/mL. Plates were incubated at 37°C for 48 h in a humid atmosphere. The MIC was defined as the lowest drug concentration that caused complete inhibition of growth.

E test

E test MIC determinations were made on AM3 solidified with 1.4% Lab M agar no. 1 (Lab M, Bury, U K). Each yeast was suspended in saline to produce a turbidity equivalent to a 0.5 McFarland standard. The inoculum was swabbed on to the agar plate and allowed to dry for 10-15 min before the amphotericin B E test strip was applied. Plates were incubated at 37°C for 48 h. Amphotericin B MICs were taken as the lowest concentration on the E test strip that gave complete inhibition of growth.

Disc susceptibility testing

Each yeast was suspended in saline to produce a turbidity equivalent to a 0.5 McFarland standard. The suspension was inoculated on to one half of an AM3 plate using a cotton-tipped swab. The opposite half of the plate was inoculated with a suspension of a control organism, C. kefyr (SA isolate). A 20 μg amphotericin B disc was placed on the centre of the plate. Plates were incubated at 37°C for 48 h and then the inhibition zone radius of the test and control organisms was measured using dial calipers; measurements were taken from the centre of the disc to the zone edge. The zone radius of the test organism was expressed as a percentage of the zone radius of the control organism to give the inhibition zone ratio (IZR). Examination of the results for the resistant isolates and the clinical isolates showed that all the resistant isolates had an IZR of <85% relative to the control whilst the majority of the clinical isolates had values of >85%. Consequently, IZR values of <85% were considered resistant and values of >85% were considered susceptible.

Survey of amphotericin B resistance amongst clinical isolates

Isolates referred to the laboratory for susceptibility testing had MIC determinations performed by broth microdilution using AM3. In total, 644 isolates of numerous Candida spp. were tested. These were isolated from a variety of body sites of AIDS patients, ICU patients, neonates and immunosuppressed individuals.

Results

The MICs obtained in AM3 and by E test for the six resistant isolates and the remaining isolates are shown in Table I. The resistant isolates can be clearly differentiated from the majority of the clinical isolates. However, as judged by MIC testing in AM3 and by E test, several clinical isolates had MICs similar to those of the resistant isolates (Table II).

Disc susceptibility tests gave clear, well-defined zone edges. The C. kefyr isolate used as the control had an MIC of 0.12 mg/L by broth microdilution in AM3, and an IZR of approximately 11 mm. Using the 85% threshold previously described, all of the resistant isolates gave an IZR of lower than the cut-off and 35 of 40 of the clinical isolates gave an IZR greater than the cut-off. Thus five clinical isolates were categorized as resistant by this method.

Each of the three testing methods identified several clinical isolates as having MICs of IZR similar to those of the known amphotericin-B-resistant isolates. The MICs for these isolates are shown in Table II. Two isolates, one Candida glabrata and one Saccharomyces sp., were resistant by all three methods and these isolates may show true resistance. The remaining four isolates were all C. krusei and these showed resistance in only one or two tests and the degree of resistance was low. A comparison of MICs obtained in AM3 and by E test with the IZR is shown in Figure 1. There is an excellent correlation between the IZR obtained by disc test and MICs obtained by both methods.

MIC results for 644 clinical isolates of Candida are shown in Figure 2. Using a microtitre broth method with AM3, very low MICs ($\leq 0.06$ mg/L) were obtained for 86% of isolates. Only eight (1.2%) isolates showed reduced susceptibility, with MICs of $\geq 0.25$ mg/L.

Table I. Comparison of MICs and disc results between resistant isolates and other isolates

<table>
<thead>
<tr>
<th></th>
<th>Microtitre MIC in AM3 (mg/L)</th>
<th>E test MIC on AM3 (mg/L)</th>
<th>Disc test (IZR)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GM range mean</td>
<td>median</td>
<td>MIC$_{90}$</td>
</tr>
<tr>
<td>Resistant isolates n = 6</td>
<td>1.0 0.25–16</td>
<td>0.75</td>
<td>1.0</td>
</tr>
<tr>
<td>Clinical isolates n = 44</td>
<td>0.048 0.015–0.5</td>
<td>0.045</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Amphotericin B resistance testing of Candida spp.

Discussion

Although the apparent incidence of resistance among Candida to amphotericin B is low, laboratories require a technically simple, reproducible and accurate method for detecting resistance in yeasts isolated from serious infections. In this study microtitre MICs performed in AM3 and the Etest were capable of detecting amphotericin B resistance. In addition, resistant isolates could be detected using a disc susceptibility test. IZR determinations obtained by the disc tests showed excellent correlation with MICs obtained in AM3 and by Etest (Figure 1).

Amongst a group of clinical isolates, two were detected that had raised amphotericin B MICs in AM3 and by Etest. Both isolates were also classified as resistant by disc test. Four isolates of C. krusei had higher MICs than the other species tested and this species may have an intrinsically reduced susceptibility to amphotericin B in vitro. This is consistent with earlier in-vivo data.

The disc method is relatively simple to perform and inexpensive, and so is highly suited to routine testing. Disc testing could be used as a screening test for detecting amphotericin B resistance amongst clinical isolates. Those isolates showing reduced IZRs could then be tested by MIC using AM3 or Etest to give a definitive MIC value. MIC testing of 644 clinical yeast isolates in AM3 showed that 1.2% had MICs indicative of reduced susceptibility. In critically ill patients with candidaemia the use of amphotericin B may be unsuccessful, although clinically validated breakpoints are lacking at present.

References


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