Comparative Evaluation of Disc Diffusion and E-test with Broth Micro-dilution in Susceptibility testing of Amphotericin B, Voriconazole and Caspofungin against Clinical Aspergillus isolates

PRASHANT GUPTA1, VINEETA KHARE2, DEEPAK KUMAR3, ABRAR AHMAD4, GOPA BANERJEE5, MASTAN SINGH6

ABSTRACT

Background: Clinical importance of Aspergillus has increased over the past few decades because of rise in immunosuppressive drugs and immune-modulating diseases. Antifungal susceptibility of Aspergillus is rarely performed by clinical laboratories because of lack of easier method. This study has investigated and compared susceptibility pattern of Aspergillus isolates by disc diffusion, E-test and broth micro-dilution for amphotericin B, voriconazole and caspofungin.

Materials and Methods: Disk diffusion (DD) method of antifungal susceptibility (AFS) was evaluated for three different classes of antifungals: amphotericin B (AMB), voriconazole (VCZ) and caspofungin (CAS). Forty four clinical isolates of Aspergillus were selected; these included 34 A. fumigatus, 8 A. flavus and 2 A. terreus. AFS by DD and E-test was done on non-supplemented Mueller Hinton Agar (MHA) and was compared to Clinical Laboratory Standard Institute (CLSI) broth micro-dilution (BMD) method of AFS.

Results: Disk diffusion method for amphotericin B showed 87.5% agreement while E-test showed 93.8% agreement with broth micro-dilution. The agreement with broth micro-dilution was similar for both disk diffusion and E-test in case of voriconazole (93.8%) and caspofungin (100%). 31.8% and 9.1% Aspergillus isolates were found to have amphotericin B and voriconazole MIC values above epidemiological cut off value (ECV) respectively. All isolates were within ECV for caspofungin.

Conclusion: CLSI method of DD promises to be easier, reproducible and cost effective method of susceptibility testing, but this method must be interpreted with caution in case of amphotericin B susceptibility testing. E-test correlates better than DD with BMD.

Keywords: Amphotericin B, Antifungal susceptibility, Aspergillus, Broth-micro-dilution, Caspofungin, Disk diffusion, E-test, Voriconazole

INTRODUCTION

Fungal infections especially by filamentous fungi are on rise due to increase in the risk factors like use of drugs to suppress immune system causing neutropenia (steroids, anticancer chemotherapy, and anti-rejection drugs) and disorders like AIDS, diabetes, immunological diseases, COPD etc. The incidence of invasive aspergillosis is highly variable depending on the patient population. In solid organ transplant the incidence of invasive aspergillosis may be up to 3.5% depending on the type of transplant recipients [1]. Xessal et al., from New Delhi, India had reported an incidence of 2.43 % for Aspergillosis [2]. Among Aspergillus spp., A. fumigatus is responsible for majority of infections, although other molds have also emerged as an important aetiological agent causing invasive fungal infections [3]. Amphotericin B is the most common antifungal agent being used worldwide for the treatment of filamentous fungal infections. Over the last few years voriconazole and caspofungin have also been approved for its treatment. Rise in MIC values to various antifungals in A. fumigates and non-fumigatus are increasingly being reported. A study done by Shivprakash et al., in India had reported 3 strains of A. flavus with high MIC values of 2 mg/l for AMB and 4 mg/l for VCZ [4].

An ideal method of susceptibility testing must be easy, reproducible, accurate and cost-effective. But antifungal susceptibility (AFS) testing for filamentous fungi is a labor intensive method and therefore most of the laboratories do not perform it. Hence, the data on local MIC values are scant. Various AFS testing methods have been proposed, including broth micro-dilution (CLSI M38-A2), disk diffusion (DD), E-test (Biomerieux, USA) and other commercial tests. Among these the gold standard is CLSI broth micro-dilution (M38-A2) [5]. The BMD of AFS is time consuming and labor intensive. E-test is one of the alternatives but requires a special media (RPMI 1640 or Casitone agar) also sometimes it shows higher MIC values [6]. CLSI in year 2010 published a reference method (M51-A) for disk diffusion antifungal susceptibility testing of non-dermatophyte filamentous fungi [7]. This document describes guidelines for testing the susceptibility of opportunistic molds to triazoles, amphotericin B, and caspofungin. Different authors have evaluated different media for disk diffusion and E-test. Some of these media are antibiotic medium 3, Mueller-Hinton with 2% glucose (MGM), Roswell Park Memorial Institute (RPMI) agar or non-supplemented MHA [8,9]. Espinell-Ingroff et al., first time demonstrated that non-supplemented MHA has better correlation with broth micro-dilution than any other media being used for disk diffusion method of antifungal susceptibility [8]. The present study was done to compare Disk Diffusion method of CLSI (M51-A), E-test and the reference gold standard CLSI (M38-A2) broth micro-dilution on Indian isolates of Aspergillus spp.

MATERIALS AND METHODS

Panel of 44 Aspergillus isolates from clinical samples like sputum, nasal mass, endotracheal aspirate, blood cultures, deep seated pus, brain tissue, pleural and peritoneal fluid, were tested over a period of two years (2011-2013) in the Department of Microbiology, King George’s Medical University, Lucknow, U.P, India. These isolates comprised of 34 A. fumigatus, 8 A. flavus, 2 A. terreus. Two reference
Antifungal Susceptibility of Aspergillus against Amphotericin B, Voriconazole and Caspofungin

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
<th>Range</th>
<th>Geometric mean</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AMB</td>
<td>VCZ</td>
<td>CAS*</td>
</tr>
<tr>
<td>Aspergillus fumigatus (34)</td>
<td>M38-A2</td>
<td>1-16</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>E test</td>
<td>1.5-2</td>
<td>0.012-0.125</td>
</tr>
<tr>
<td></td>
<td>M51-A</td>
<td>11-26 mm</td>
<td>13-70 mm</td>
</tr>
<tr>
<td>Aspergillus flavus (8)</td>
<td>M38-A2</td>
<td>2-4</td>
<td>1-8</td>
</tr>
<tr>
<td></td>
<td>E test</td>
<td>1.5-12</td>
<td>0.006-0.047</td>
</tr>
<tr>
<td></td>
<td>M51-A</td>
<td>14-18 mm</td>
<td>13-52 mm</td>
</tr>
<tr>
<td>Aspergillus terreus (2)</td>
<td>M38-A2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E test</td>
<td>4</td>
<td>0.094</td>
</tr>
<tr>
<td></td>
<td>M51-A</td>
<td>12 mm</td>
<td>55 mm</td>
</tr>
</tbody>
</table>

**Table/Fig-1**: Susceptibility of 44 filamentous fungi to amphotericin B (AMB), Voriconazole (VCZ), Caspofungin (CAS)

- For caspofungin minimum effective concentration (MEC) was used in place of MIC. In disk diffusion micro colonies or trailing growth within a well-defined zone of inhibition was ignored when testing caspofungin.

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Antifungals</th>
<th>A. fumigatus (n=34)</th>
<th>A. flavus (n=8)</th>
<th>A. terreus (n=2)</th>
<th>Total (n=44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AMB</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>VCZ</td>
<td>75</td>
<td>75</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3.</td>
<td>CAS</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table/Fig-2**: Distribution of Non Wild type (non-WT) strains among Aspergillus isolates

- All clinical isolates grew well in the RPMI media used for BMD and E-test and disk diffusion method (CLSI M51-A) when broth micro-dilution was interpreted at 48 hours.

- Overall agreement (%) of reference broth micro-dilution (CLSI M38-A2) with E-test and disk diffusion method (CLSI M51-A) when broth micro-dilution was interpreted at 48 hours.

- Overall agreement (%) of reference broth micro-dilution (CLSI M38-A2) with E-test and disk diffusion method (CLSI M51-A) when broth micro-dilution was interpreted at 24 hours.

- Data Analysis: For comparative evaluation of the disk diffusion, E-test and broth micro-dilution methods, the geometric mean (GM) and range of the MICs and MECs and the range of the inhibition zone diameters were calculated for each genus-species combination. The percentage of agreement between all the methods was calculated for each genus-species and antifungal drug.

**RESULTS**

All clinical isolates grew well in the RPMI media used for BMD and non-supplemented MHA media used for DD and E-test, giving definitive endpoints. The MIC and zone diameter ranges and geometric means obtained by BMD, E-test and DD are summarized in [Table/Fig-1].

**Antifungal Susceptibility**

Based on ECVs, Aspergillus isolates were classified into WT and non-WT for all three antifungals tested [Table/Fig-2].
Comparison of Amphotericin B BMD, DD and E-test
Eight WT isolates of A. fumigatus by BMD were categorized as non-WT by DD. Also, 6 non-WT isolates by BMD were categorized as WT by DD. Among these 6, 4 were A. fumigatus and 2 were A. flavus isolates. In comparison to BMD lower MIC values by E-test were seen in 4/44 (9.1%) Aspergillus isolates (2 A. flavus and 2 A. fumigatus).

Comparison of Voriconazole BMD, DD and E-test
Four isolates categorized as non-WT (MIC’s > 1 µg/ml) by BMD were categorized as WT by DD and E-test. Among these 2 were A. fumigatus, 2 were A. flavus. MIC values by E-test for these 4 isolates were <1 µg/ml.

Agreement between CLSI broth-micro dilution (M38-A2), E-test and CLSI Disk diffusion (M51-A)
The percentage of agreement of E-test and DD with the gold standard BMD in different species for the three antifungals is summarized in Table/Fig-3. Percent agreement of susceptibility testing by DD and E-test ranged from 75% to 100%, being lowest for A. flavus. When testing for AMB and VCZ, the overall agreement of E-test with BMD was better than that of DD [Table/Fig-4]. 100% agreement was seen between all the three methods for CAS.

Interpretation of results at 48h vs 24 h
For 20 isolates BMD MIC values obtained were different at 24h vs 48h. Of these, 14 isolates whose MIC were within ECV, the interpretation at 24h gave still lower MIC’s. For 6 isolates, which were non-WT at 48h, the interpretation at 24h gave MIC within ECV (WT). There was no difference in the results of either DD or E-test at 24h or 48h.

When the BMD results were interpreted at 24h, the agreement of both E-test and DD decreased for AMB, increased for VCZ while no change was seen for CAS [Table/Fig-4,5].

DISCUSSION
CLSI in year 2010 published disk diffusion method of antifungal susceptibility of non-dermatophyte filamentous fungi (CLSI M51-A). This method promises to be convenient for use in routine diagnostic laboratories. Though the fungal infections are on rise in India, data on antifungal susceptibility especially for filamentous fungi are lacking. In this study high MIC value of ≥ 4 µg/ml for amphotericin B was found in 31.8% isolates of Aspergillus spp. High MIC values ≥ 2 µg/ml had also been reported by Shivprakash et al., in 3 isolates of A. flavus [14]. The trend in increase in non-WT [probable resistant] strains of Aspergillus in India is now on rise and this trend could be because of rampant use of amphotericin B in the patients suspected of fungal infections. There is a possibility of increase in MIC values during therapy, as has been noted by Espinell-Ingroff et al., for a single A. fumigatus isolate [15].

The mechanism of resistance in A. flavus has been reported to be due to efflux of drug or increased transcription of AtfMdr1 gene, alteration of cell wall composition [16]. The mechanism of the intrinsic resistance in A. terreus to amphotericin B is not clear and it has been postulated that much less ergosterol content in the cell membrane of A. terreus partially accounts for the poor activity of amphotericin B against this fungus [17]. One of the study also reported that A. terreus produces significantly more catalase than A. fumigatus which may play an important role in the amphotericin B resistance [18].

We also found voriconazole MIC of ≥ 2 µg/ml in 9.1% Aspergillus isolates. Voriconazole MIC of ≥ 4 µg/ml had also been reported by Shivprakash et al., in 4.9% of A. flavus [14]. Espinell-Ingroff et al., also reported voriconazole MIC exceeding 4 µg/ml in 5.8% of the isolates, including one A. fumigatus, 7.7% F. solani and all zygomycetes [15]. A study from Spain found voriconazole MIC of > 4 µg/ml in 10 A. fumigatus clinical isolates collected from patients with hematological malignancies who had long-term exposure to either voriconazole or itraconazole [19]. Resistance of A. fumigatus to the azoles has been reported to vary from a high of 52% and 38% with itraconazole and ravuconazole, respectively, to a low of 11% with voriconazole [20]. The studies on the resistant mechanisms have shown that amino acid residues substitution derived from mutations in the azole-target-enzyme gene cyp51A, overexpression of this gene and drug efflux genes, and up regulation of homeostatic stress-response pathways contribute to azole-resistance in A. fumigates [21]. Liu et al., identified that T788G missense mutation in cyp51C gene was responsible for voriconazole resistance in A. flavus [22].

None of the Aspergillus isolate was found to have MEC values above ECV’s for caspofungin. These findings are similar to the findings of Shiv prakash et al., who also could not find high MEC values in any of the A. flavus [14]. Resistance to caspofungin in Aspergillus had not been reported from India. This could be because caspofungin is still the least used antifungal in India. Arendrup et al., had reported a clinical isolate of A. fumigatus to be resistant to caspofungin, confirmed by E-test with an MIC >32 µg/ml. This patient also failed to respond to caspofungin [23]. Laboratory-selected strains with varying degree of caspofungin resistance have been described. Some of these laboratory-manipulated strains have been found to have mutations in the ECM33 gene (afuEcm33), a gene which encodes cell wall protein important for fungal cell wall organization [24].

E-test has been evaluated by various authors and most of the authors have used RPMI 1640 agar with 2% glucose and 0.165 M morpholine propane sulfonic acid (MOPS) buffer. We evaluated E-test on non-supplemented MHA as is being used for DD method of CLSI. Espinell Ingroff et al., used RPMI 1640 agar and found agreement between E-test and BMD ranging from 64.5% to 96.3% for voriconazole, posaconazole, itraconazole and amphotericin B [13]. Since agreement in our study for E-test ranged from 75 to 100% for Aspergillus spp. and also since there was no difference in MIC or MEC values at 24h and 48h, therefore we suggest E-test to be done on non-supplemented MHA in place of RPMI 1640 agar with 2% glucose and MOPS, which is also the common media available in most of the laboratories.

CLSI M38-A2 guidelines recommend BMD interpretation at 48 hours of incubation for amphotericin B and voriconazole and at 24h of incubation for caspofungin. In this study comparison between methods for assessing the susceptibilities of Aspergillus spp. against amphotericin B, voriconazole and caspofungin showed that overall agreement after 48 h of incubation was highest for voriconazole (93.8% by both E-test and DD) and caspofungin (100% by both E-test and DD). Agreement was lowest for amphotericin B (93.8% by E-test and 87.5% by DD) at 48 h of incubation. But if the BMD interpretation is done at 24h the agreement between all the methods increases specially for voriconazole (95% by both E-test and DD) but decreases considerably for amphotericin B [Table/Fig-5]. We therefore suggest BMD interpretation of voriconazole also at 24h in place of 48h as suggested by Espinell-Ingroff et al., [10].

Among Aspergillus spp. least agreement was found in susceptibility testing of A. flavus for amphotericin B and voriconazole by both E-test and DD [Table/Fig-3]. DD method of susceptibility testing of A. fumigatus for amphotericin B also showed lower agreement when compared to BMD [Table/Fig-3]. All MEC values by BMD for caspofungin were same, both at 24h and 48h. This finding is similar to the findings of Espinell-Ingroff et al., [11].

CONCLUSION
Global trend of increasing antifungal resistance in Aspergillus spp. is also being seen in India. CLSI method of disk diffusion must be interpreted with caution in case of amphotericin B susceptibility
testing. MIC or MEC values do not change significantly for VCZ when BMD is interpreted at 24h in place of 48h as suggested by CLSI, indirect BMD MIC values at 24h have better correlation with DD and E-test. We also propose E-test to be done on non-supplemented MHA media in place of RPMI 1640 agar. To increase the reproducibility of data large sample size and multi-centric studies are required.

REFERENCES