Original Article

DOI: 10.7860/JCDR/2015/10467.5395

Microbiology Section

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

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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]. Xessl 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 (Biomeriux, 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

MICor MEC (µg/ml)/ Zone size (mm)							
Species	Method	Range			Geometric mean		
		AMB	VCZ	CAS*	AMB	VCZ	CAS
Aspergillus fumigatus (34)	M38-A2	1-16	0.5-2	0.06	2.4	0.6	0.06
	E test	1.5-2	0.012-0.125	0.004-0.016	1.6	0.03	0.009
	M51-A	11-26 mm	13-70 mm	18-24 mm	17 mm	54 mm	21 mm
Aspergillus flavus (8)	M38-A2	2-4	1-8	0.06	2.8	1.7	0.06
	E test	1.5-12	0.064-32	0.008- 0.047	2.7	0.2	0.02
	M51-A	14-18 mm	13-52 mm	20-24 mm	16 mm	36 mm	22 mm
Aspergillus terreus (2)	M38-A2	4	1	0.06	8	1	0.06
	E test	4	0.094	0.047	4	0.094	0.047
	M51-A	12mm	55 mm	20 mm	12 mm	55 mm	20 mm

[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

S.no.	Antifungals	*A.fumigatus* (n=34)	*A.flavus* (n=8)	*A.terreus* (n=2)	Total (n= 44)
1.	AMB	8	4	2	14
2.	VCZ	2	2	0	4
3.	CAS	0	0	0	0

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

Isolates	AMB		VCZ		CAS		All drugs	All drugs
	E test	DD	E test	DD	E test	DD		
A.fumigatus (n=34)	100	75	100	100	100	100	100	91.7
A.flavus (n=8)	75	75	75	75	100	100	83.3	83.3
A.terreus (n=2)	100	100	100	100	100	100	100	100

[Table/Fig-3]: Percentage 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 48hours

strains, *Candida krusei* ATCC 6258 and *A. flavus* ATCC MYA 3631 were included in all the tests as references for quality controls and were tested by all the methods in each series of assays.

Inoculum Preparation: Inoculum was prepared according to M51-A and M38-A2 CLSI guidelines. In brief to induce conidium the isolates were grown on potato dextrose agar (PDA) slants at 35°C for 2–7 days before testing. The suspensions of conidial inocula were prepared from 2-7 days old cultures grown on PDA slants. The turbidity of the cell suspension was adjusted by spectrophotometry to an optical density of 0.09 to 0.13 for *Aspergillus* spp.

Broth Micro-dilution (BMD): Voriconazole (Cayman, USA), Amphotericin B (BioVision, USA) and Caspofungin (Cayman, USA) were obtained as powders. Stock inoculum and working suspensions for all the drugs were prepared and BMD was done according to the method described in CLSI M38-A2 document [4]. All BMD plates were incubated at 35°C for 48 h. MIC for AMB and VCZ and MEC for CAS were interpreted in accordance with ECV values suggested by CLSIM38-A2. Results were read at 48 h for VCZ and AMB and at 24 h for CAS. In brief, isolate was considered as wild type (WT) when MIC was ≤ 1µg/ml for VCZ (≤ 2 µg/ml for *A.flavus*), ≤ 2 µg/ml for AMB (for *A. fumigatus*, *A. flavus*, A.niger and ≤ 4 µg/ml for *A. terreus*) and MEC was ≤ 0.06 µg/ml for CAS [10-13].

Disk diffusion (DD): Antifungal susceptibility by disk diffusion was done on non-supplemented MHA according to the method described in CLSI M 51-A document [7]. In brief entire surface of MHA was inoculated with non-toxic cotton swab with the undiluted mold stock inoculum suspension. Disks of AMB (10µg, prepared Inhouse); VCZ (1 µg, Himedia); CAS (5µg, In house) were placed

AMB		VCZ*		CAS	
E test	DD	E test	DD	E test	DD
93.8	87.5	93.8	93.8	100	100

[Table/Fig-4]: 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

AMB		VCZ*		CAS	
E test	DD	E test	DD	E test	DD
75	70	95	95	100	100

[Table/Fig-5]: 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

onto the surface of each of the inoculated MHA plate. The plates were incubated at 35°C and were read after 24h and 48h. Slight trailing around the zone edge or hyphal element extending into the inhibition zone was ignored when testing triazoles, but not for AMB. Microcolonies or trailing growth within a well-defined zone of inhibition was ignored when testing CAS.Zone diameter were interpreted in accordance with CLSI M51-A. In brief for AMB isolates were considered as WT at zone size ≥ 15 mm. For VCZ and CAS, isolates were considered as WT at zone size ≥ 17 mm [7].

E-test: The E-test strips (Biomeriux, USA) of AMB, VCZ and CAS contained concentration gradients of 32–0.002 µg/ml of the respective drug. The entire surface of non-supplemented MHA was inoculated by the same method as used for DD. E strips were placed on MHA and the plates were incubated at 35°C and read after 24h and 48h. The MIC was determined from the inhibition ellipse that intersected the scale on the strip. The MIC was read at the point of significant inhibition in fungal growth. Micro colonies or trailing growth within a well-defined zone of inhibition ellipse was ignored when testing CAS [13].

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. fumigates* 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 24 h 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 48 h.

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 AflMDR1 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 W 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 48 h, 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 24 h 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 voriconazolealso 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, infact 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.

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FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Jun 30, 2014**
Date of Peer Review: **Sep 28, 2014**
Date of Acceptance: **Oct 31, 2014**
Date of Publishing: **Jan 01, 2015**