University Journal of Pre and Para Clinical Sciences

ISSN 2455–2879 2020, Vol.6(6)

Comparison of E-test and Microbroth dilution Methods for Determining Voriconazole, Itraconazole and Amphotericin B MICs for Aspergillus fumigatus and Aspergillus flavus

VINOTHA S
Department of Microbiology, MADRAS MEDICAL COLLEGE AND GOVERNMENT GENERAL HOSPITAL

Abstract : Background of the study Infections caused by filamentous fungi, especially Aspergillus fumigatus and Aspergillus flavus are responsible for majority of infections in immunocompromised hosts. The Clinical Laboratory Standards Institute Subcommittee on Antifungal Susceptibility Tests has proposed a standard procedure for antifungal susceptibility testing of molds (CLSI document M38-A2) . Although the reference procedure for molds is essential for standardization of results, it is cumbersome and time-consuming other methods have been evaluated . Among these methods, Etest has been suggested as an alternative procedure for antifungal susceptibility testing of molds. Hence the determination of invitro susceptibilities of Aspergillus isolates to the established and investigational methods are warranted .MaterialsMethods 64 isolates of clinically significant Aspergillus flavus(29) and Aspergillus fumigatus (35) obtained from various clinical samples were taken up for the study . Minimal inhibitory concentrations(MICs) for these isolates were determined by microbroth dilution method as per CLSI document M38-A2 and E-Test. The percentage of agreement between the E test and the reference microbroth dilution methods were calculated. Results In this study we evaluated the in vitro susceptibilities of 29 Aspergillus flavus isolates and 35 Aspergillus fumigatus isolates, to voriconazole, itraconazole, and amphotericin B by the E-test and CLSI M38-A2 microbrothdilution methods. An overall 100 percent agreement for voriconazole and itraconazole and 86 percent for amphotericin B at 24 hrs and 97,93and 73 percentages respectively at 48hrs was seen for Aspergillus flavus isolates. For Aspergillus fumigatus an overall agreement of voriconazole, itraconazole and amphotericin B was 97,91 and 97percentages respectively at 24 hrs and at 48hrs lower agreement of 71,77and 69 percentages were noted respectively. Greater discrepencies in MICs were noted at 48hours of incubation of E-Test suggesting the importance of incubation time for the Etest. Conclusion The results of this investigation showed a good level of overall agreement between the E-test and microbroth dilution methods . Our results suggest that the E test is suitable for routine use in susceptibility testing of Aspergillus spp. against amphotericin B and Triazoles.

Keyword :E-Test, Microbrothdilution method, Minimal inhibitory concentration(MIC).

INTRODUCTION:

Since the 1980s, a higher incidence of infections caused by filamentous fungi has been documented, especially *Aspergillus fumigatus and Aspergillus flavus* are responsible for the majority of infections (85 to 90%) in immunocompromised hosts[2,3,] . During this period, the number of immunocompromised patients has markedly increased. Many factors have contributed to this increase and include the use of new and more aggressive therapies to treat solid tumors, myelomas, lymphomas, and leukemia, the chronic use of corticosteroids, the increasing number of patients who undergo organ transplant and nally the spread of AIDS [1,3,8,10].These infections are associated with signicant morbidity and mortality of different clinical manifestations of severe mold infections. Paralleling the increasing incidence of fungal infection has been the development of new triazoles [3,9,10]. Because the number of serious infections caused by *Aspergillus spp.* has increased and resistance to established agents has been documented ,determination of the in vitro susceptibilities of *Aspergillus isolates* to the established and investigational methods are warranted . The need for reproducible, clinically relevant antifungal susceptibility testing has been prompted by the increasing number of invasive fungal infections, the expanding use of new and established antifungal agents, and recognition of antifungal resistance as an important clinical problem. The Clinical Laboratory Standards Institute (CLSI) Subcommittee on Antifungal Susceptibility Tests has proposed a standard procedure for antifungal susceptibility testing of molds (CLSI document M38-A2 [12]). Although development of reference procedure for molds (12) is essential for standardization of results, the method is cumbersome and time-consuming; other approaches have been evaluated for fungal testing in recent years. Among these approaches, E-test has been suggested as an alternative procedure for antifungal susceptibility testing of molds [4,7,14]. The collaborative efforts of numerous investigators and the Clinical and Laboratory Standards Institute (CLSI), Subcommittee on Antifungal Susceptibility Testing have generated consensus documents describing standardized methods for broth and agar-based antifungal susceptibility testing. As a result, in vitro antifungal susceptibility testing plays an increasingly important role in guiding therapeutic decision making, as an aid in drug

development studies, and as a means of tracking the development of antifungal resistance in epidemiologic studies.

Aim of the study:

To compare the E-Test and Microbroth dilution methods for determining the MIC's of voriconazole, itraconazole and amphotericin B for the *Aspergillus fumigatus* and *Aspergillus flavus* isolates from various clinical samples.

Study design and period :

The study was a Cross sectional study conducted at the Institute of Microbiology, RGGGH, Madras Medical College Chennai for a period of 6 months from July 2014 to December 2014.

Materials &Methods:

64 isolates of clinically significant *Aspergillus flavus and Aspergillus fumigatus species* obtained from various clinical samples such as sputum, BAL, tissue biopsy were taken up for the study . 1.Identification of *Aspergillus flavus and Aspergillus fumigatus* isolates done by,

- Direct microscopic examination by KOH mount.

-Culture on SDA at 37°C and identification of the growth by LPCB mount

2.Microbroth dilution method:[12]

A. Preparation of antifungal drugs and drug dilutions: The antifungal reference powder of voriconazole , itraconazole and amphotericin B were obtained from their manufacturers. Stock solution of voriconazole, itraconazole and amphotericin B were prepared by dissolving the drug in 100% dimethyl sulfoxide (DMSO) . Then two fold serial dilution for each drug were prepared with RPMI 1640 with L- glutamine but without bicarbonate buffered to pH 7.0 with 3-(N– morpholino)propane sulfonic acid (MOPS). The final concentration of drugs used were between 0.03125 to 16µg/ml for itraconazole , voriconazole and amphotericin B as described in CLSI microbroth dilution method (M38-A2 document).

B. Inoculum preparation: [12] Inoculum suspensions were prepared from 3-7 day-old cultures grown on Potato dextrose agar. Colonies were covered with 3 ml of sterile distilled water and gently probed with the tip of Pasteur pipette and allowed to stand for 3 to 5 minutes for the large particles to settle down. Then without disturbing, the suspension of conidia was gently transferred to sterile tubes. The final inoculum was then adjusted by spectrophotometer at the wavelength of 530 nm to the optical density (OD) range 0.45 to 0.55 ,which corresponds to 0.4 to 5.0×104CFU/ml .

C. Susceptibility testing method:[12] As per the CLSI micro broth dilution method (document M38-A2), MICs were determined in 96-well round-bottom microtitre plates. 100 µl of the (twofold) diluted drug concentration was taken in corresponding labeled wells and each well was inoculated with 100 µl of the diluted conidial inoculum suspension (final volume in each well, 200 µl).Growth control and drug control was set up in separate wells. Microtitre trays were incubated at 35°C and examined at 48 h for MIC determination. MICs were determined by visual inspection as described in CLSI document M38-A2 for complete, or 100%, growth inhibition . *A. flavus* ATCC 204304 was tested each time.

3. E-Test:

The E test was performed in accordance with the manufacturer's instructions. Spore suspensions were prepared in sterile saline and adjusted to a concentration of 106 spores/ml with the spectrophotometer set at 530 nm(78 to 82% transmission). The medium used was RPMI 1640 agar (1.5%), supplemented with 2% glucose and buffered to pH 7.0 with MOPS .The molten medium was dispensed in 20-ml amounts into 90-mm-diameter petri dishes, giving an agar depth of 4 mm. Plates were incubated at 35°C, and MICs were determined following incubation times of 24 and 48 h. The E-test MIC was the lowest drug concentration at which the border of the elliptical inhibition intercepted the scale on the antifungal strip (Fig. 1).

Clear E-test ellipses were taken, since reference MIC end points for all triazoles and amphotericin B corresponds to 100% growth inhibition [7]. *A. flavus* ATCC 204304 was tested each time.

Analysis of results. Since the E-test scale has a continuous gradient of concentrations, the MIC's between twofold dilutions were rounded to the next twofold level of the reference method scheme to facilitate comparison of the results. The percentage of agreement between the E test and the reference microbroth dilution method was defined as the proportion of E-test results which fell within ±1 or± 2 log2 dilutions of the standard MIC results.

Result:

This study included 64 filamentous fungal isolates of which 29 were Aspergillus flavus and 35 were Aspergillus fumigatus. MIC's were obtained for all the isolates by microbroth dilution method and Etest.

Table 1: In vitro susceptibilities of 64 isolates to three antifungal agents as determined by E-test and microbrothdilution methods

Organism (no: tested)	Antifungal agent	Incubation time in hrs	E-Test (MIC range) µg/ml	Mean	Micro broth dilution(MIC range) µg/ml	Mean
A. flavus (29)	Amphotericin B	24	0.25–8	2.2		
		48	0.5–8	3.4	0.5-8	2.8
	Itraconazole	24	0.12- 0.25	0.22		
		48	0.12-1	0.34	0.0625-0.0.5	0.24
	Voriconazole	24	0.12–0.25	0.18		
		48	0.25-0.5	0.28	0.12-0.5	0.34
A.fumigatus(35)	Amphotericin B	24	0.25-8	2.8		
		48	0.25-8	3.2	0.25-8	2.4
	Itraconazole	24	0.06-16	2.4		
		48	0.12-16	2.8	0.06-16	2.2
	Voriconazole	24	0.03-4	0.26		
		48	0.06-4	0.52	.06-4	0.58

Table 2: Distribution of differences in MICs for 64 *Aspergillus* isolates within ±log2 dilutions for the E-test and M38-A2 method

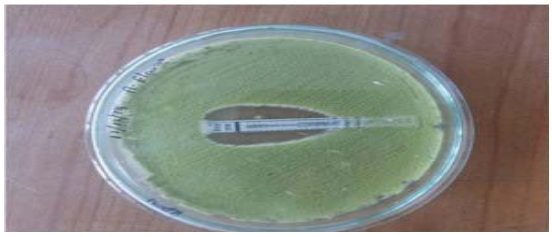
Organism (no. tested)	Antifungal agent	Incubation time (hrs)	No. of isolates with E-test MIC's different from the CLSI M38-A2 method MIC's						
			>+2 log2 dilution	+2 log2 dilution	+1 log2 dilution	0	-1 log2 dilution	-2 log2 dilution	>-2 log2 dilution
A. flavus (29)	Amphotericin B	24	1	6	5	6	3	5	3
		48	6	6	8	2	4	2	2
	Itraconazole	24		8	6	7	5	3	
		48	2	9	8	4	5	1	
	Voriconazole	24			8	11	6	4	
		48	1	5	7	8	5	3	
A.fumigatus(35)	Amphotericin B	24		3	7	7	11	6	1
		48	11	8	4	4	8		
	Itraconazole	24	2	7	8	11	5	1	1
		48	8	7	9	8	2	1	
	Voriconazole	24		2	16	13	2	1	1
		48	10	9	12	2	2		

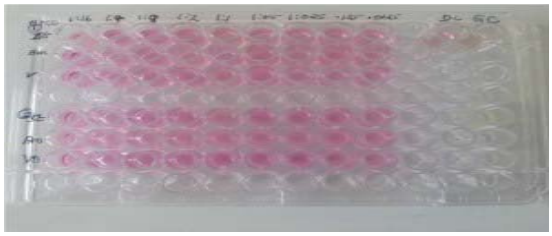
Table 3: Percentage of agreement between E-test and micro broth dilution methods for 64 isolates

Organism (no. tested)	Antifungal agent	Incubation time hrs	% Agreement	
			±1 log2 dilution	±2 log2dilution
A. flavus (29)	Amphotericin B	24	48.27	86.20
		48	48.27	72.41
	Itraconazole	24	62.06	100
		48	58.62	93.10
	Voriconazole	24	86.20	100
		48	68.96	96.55
A.fumigatus(35)	Amphotericin B	24	71.42	97.14
		48	45.71	68.57
	Itraconazole	24	54.28	91.42
		48	54.28	77.14
	Voriconazole	24	88.57	97.14
		48	45.71	71.42

Percentage of agreement between the results is defined as proportion of E-test MIC results that were within ±1 or± 2 log2 dilutions of the broth microdilutioMIC results.

Fig:1:E-test for Aspergillus flavus
Fig:2:E-test for Aspergillus fumigatus
Fig:3: Microbroth dilution test





Discussion:

In this study we evaluated the suitability of E-test against standard micobrothdilution method for determining the MIC's of *Aspergillus flavus and Aspergillus fumigatus* isolates to voriconazole , itraconazole and amphotericin B. Results for the reference isolate *A. flavus* ATCC 204304 were within acceptable ranges (e.g., 0.25 to 0.5 µg/ml for voriconazole , 0.25 to 1 µg/ml amphotericin B and 0.12 to 0.5 µg/ml for itraconazole) which is similar to the study by Ana Espinel-Ingroff et al and Barry A. L et al[15,2]. Also the isolates for which high MIC' s were obtained were tested again by both procedures and same values or values within 1 dilution were obtained. These preliminary results indicates the potential reproducibility of E-test for testing isolates of *Aspergillus* spp. Table 1 summarises the MIC range of 64 *Aspergillus flavus and Aspergillus fumigatus* isolates to voriconazole ,itraconazole and amphotericin B as determined at 48 h by the micro broth dilution method and the corresponding 24 and 48-h Etest results.Minimal inhibitory concentrations for the isolates were given as a range for each drug in µg/ml.MIC range for amphotericin B were 0.25-8 µg/ml for both isolates in both the methods whereas for itraconazole and voriconazole higher range of MIC's were noted for Aspergillus fumigatus than A.flavus by both the methods. It is also noted from the mean that, discrepancy between the methods was seen in each instance such that higher E-test MICs were obtained than the microbrothdilution method for *A. fumigatus* and *Aspergillus flavus* which was similar to the study by Pfaller et al al[14]. This favours the most important role of antifungal susceptibility testing to detect potential resistance. This is different from the study by Espinel-Ingroff, A et al in which voriconazole Etest MIC's were lower than microbrothdilution MIC's[7]. Number of isolates that fall within each log dilutions are listed in Table 2 and percentage of agreement between the E-test and microbrothdilution methods are shown in Table3.We observed an overall 100% agreement for Voriconazole and itraconazole at 24 hrs for Aspergillus flavus isolates and 86% for Amphotericin B whereas it is 97%,93% and 73% respectively at 48hrs similar to studies by Adrien szekely et al and Ana Espinel-Ingroff et al[2,4] . For Aspergillus fumigatus an overall agreement of Voriconazole, Itraconazole and Amphotericin B was 97%,91% and 97% repectively at 24 hrs and at 48hrs lower agreement of71%,77%and 69% were noted respectively. Percentage of agreement was comparatively lower for amphotericin B than for voriconazole and itraconazole at 48 hrs for both the isolates which is similar to studies by Szekely et al, Pfaller M. A et al [4,16].Greater discrepencies in MIC's were noted at 48hours of incubation of E-Test in this study which is similar to Pfaller et al. Ana Espinel-Ingroff et al and Szekely et al, [2,4,14] suggesting the importance of incubation time for the E-test.

C**onclusion**:

In conclusion, this investigation has demonstrated that the E-test method is a reproducible method of antifungal drug susceptibility testing with molds. It is less labor-intensive and much simpler to set up than the microbroth dilution test. The results of this investigation showed a good level of overall agreement between the E-test method and microbrothdilution method performed according to CLSI guidelines. Our results suggest that the E test is suitable for routine use in susceptibility testing of *Aspergillus* spp. against amphotericin B , itraconazole and voriconazole.

REFERENCES

1**. M. C. Serrano1, M. Ramírez1, D. Morilla1, A. Valverde1, M. Chávez1, A. Espinel-Ingroff2, R. Claro,A. Fernández3, C. Almeida3 and E. Martín-Mazuelos1** A comparative study of the disc diffusion method with the brothmicrodilution and Etest methods for voriconazole susceptibility testing of *Aspergillus* spp.Journal of Antimicrobial Chemotherapy (2004) **53**, 739–742

2**. Ana Espinel-Ingroff1,2 and A. Rezusta** E-Test Method for Testing Susceptibilities of *Aspergillus* spp to the New Triazoles Voriconazole and Posaconazole and to Established Antifungal Agents: Comparison with NCCLS Broth Microdilution Method **.**Journal of clinical microbiology, June 2002, p. 2101–2107

3**. Iwen, P. C., M. E. Rupp, and S. H. Hinrichs.** 1997. Invasive mold sinusitis: 17 cases in immunocompromised patients and review of literature. Clin. Infect. Dis. **24:**1178–1184.

4. **Adrien szekely, Elizabeth m. Johnson, and David w. Warnock** Comparison of E-Test and Broth Microdilution Methods forAntifungal Drug Susceptibility Testing of Molds Journal of clinical microbiology,May 1999, p. 1480–1483

5.Denning, D. W. 1996. Therapeutic outcome in invasive aspergillosis. Clin. Infect. Dis. **23:** 608–615.

6. **Denning, D. W.** 1998. Invasive aspergillosis. Clin. Infect. Dis. **26:**781–805.

7.Espinel-Ingroff, A., M. Bartlett, V. Chaturvedi, M. A. Ghannoum, K. Hazen, M. A. Pfaller, M. G. Rinaldi, and T. J. Walsh. 2001. Optimal susceptibility testing conditions for detection of azole resistance in *Aspergillus* spp.: NCCLS collaborative evaluation. Antimicrob. Agents Chemother. **45:** 1828–1835.

8**.Verweij, P. E., M. F. Q. van den Bergh, P. M. Rath, B. E. de Pauw, A. Voss,and J. F. G. M. Meis.** 1999. Invasive aspergillosis caused by *Aspergillus ustus*: case report and review. J. Clin. Microbiol. **37:**1606–1609.

9**.Denning, D. W., K. Venkateswarlu, K. L. Oakley, M. J. Anderson, N. J. Manning, D. A. Stevens, D. W. Warnock, and S. L. Kelly.** 1997. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **41:**1364– 1368.

10.**Lortholary, O., M.-C. Meyohas, B. Dupont, J. Cadranel, D. Salmon-Ceron, D. Peyramond, D. Simonin, et al.** 1993. Invasive aspergillosis in patients with acquired immunodeficiency syndrome: report of 33 cases. Am. J. Med. **95:**177–187.

11. **Patterson, T. F., W. R. Kirkpatrick, M. White, J. W. Hiemenz, J. R. Wingard, B. Dupont, M. G. Rinaldi, D. A. Stevens, and J. R. Graybill.** 2000. Invasive aspergillosis: disease spectrum, treatment practices, and outcomes. Medicine **79:**250–260.

12.**CLSI (2008b)** Reference method for broth dilution antifungal susceptibility testing of fi lamentous fungi; approved standard CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne.

13.Anaissie, E. 1992. Opportunistic mycoses in the immunocompromised host:experience at a cancer center and review. Clin. Infect. Dis. **14**(Suppl. 1)**:**S43–S53.

14.**Pfaller, M. A., S. A. Messer, A. Houston, K. Mills, and A. Bolmstrom.** 2000. In vitro susceptibility testing of filamentous fungi: comparison of Etest and reference microdilution methods for determining itraconazole MICs. J. Clin.Microbiol. **38:**3359–3361.

15.**Barry A. L., M. A. Pfaller, S. D. Brown, A. Espinel-Ingroff, M. A. Ghannoum,C. Knapp, R. P. Rennie, J. H. Rex, and M. G. Rinaldi.** 2000. Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. J. Clin. Microbiol. **38:**3457–3459.

16. **Pfaller M. A., S. A. Messer, R. J. Hollis, R. N. Jones, and the Sentry Participants Group.** 2002 Antifungal activities of posaconazole, ravuconazole and voriconazole compared to those of itraconazole and amphotericin B against 239 clinical isolates of *Aspergillus* spp. and other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. Antimicrob. Chemother. **46:**1032–1037.