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COMPARATIVE EVALUATION OF VORICONAZOLE SUSCEPTIBILITY TESTING BY DISK DIFFUSION, E-TEST AND MICROBROTH DILUTION METHOD AGAINST CLINICAL AND ENVIRONMENTAL ISOLATES OF ASPERGILLUS SPECIES

Clinical Microbiology

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ABSTRACT

Aspergillus is ubiquitously present in the environment of air, soil and water. Aspergillosis spectrum in human varies from allergic bronchopulmonary aspergillosis to invasive aspergillosis. Owing to widespread use of azoles in agricultural field there are chances of acquiring azole resistance infections from the environmental sources. This study was conducted to detect easy, rapid, reproducible and cost effective method for the susceptibility testing which aids in phenotypic identification of azole resistance in *Aspergillus* isolates. A total of 162 *Aspergillus* isolates were tested (clinical isolates n=32 and environmental isolates n =130).Clinical isolates were collected from patients admitted to Respiratory medicine and Chest Disease (RMCD) ward. Environmental air samples were collected from RMCD wards (n=100) and control samples (n=30) from community by using air sampler (Hi media model no.LA881) by collecting 1000 L/plate on czapek dox and sabouraud dextrose agar with chloramphenicol. All cultures isolates were identified using standard mycological protocol. Antifungal susceptibility testing for voriconazole was done by using three different methods Disk diffusion (DD),E-test and broth micro dilution (BMD)according to standard CLSI guideline M51-A and M38-A2 respectively. All isolates were categorized as wild type (WT) of zone size >17mm by DD method. Single clinical isolates of *A. fumigatus* was categorized as non-WT (MIC>1µg/ml) by both E-test and BMD. The percentage agreement of DD and E-test with the gold standard BMD for voriconazole ranged from 99.38% to 100 % respectively. The overall agreement of E-test with BMD was better than that of DD. The study concluded that E-test should be a better alternative method to reference broth microdilution methods which helps in early detection of susceptibility pattern of isolates. Hence help in prompt treatment and improving the prognosis of aspergillosis patient.

KEYWORDS

Aspergillus fumigatus, Aspergillosis, Voriconazole resistance, E-test

INTRODUCTION

Aspergillus is ubiquitously present in the environment of air, soil and water. Aspergillosis spectrum in human varies from allergic bronchopulmonary aspergillosis to invasive aspergillosis. Pulmonary aspergillosis occurs in various clinical forms such as invasive pulmonary aspergillosis (IPA), chronic pulmonary aspergillosis, bronchial colonization, allergic broncho- pulmonary aspergillosis, and severe asthma [1]. The global burden of aspergillosis is rising posing a threat to healthcare systems worldwide. The prevalence of aspergillosis in India is poorly documented with only few studies conducted in specific areas. A study in 2017 reported global estimates of 3,000,000 cases of chronic pulmonary aspergillosis and ~250,000 cases of invasive aspergillosis [2].The immunocompromised hosts, patients with chronic lung diseases, and those exposed to environmental triggers such as dust and mould are disproportionately affected by aspergillosis [3].

Aspergillus fumigatus is a frequently observed species that causes approximately 70–80% of human aspergillosis cases. Infections with Non *Aspergillus fumigatus* are also becoming more common particularly in immunocompromised hosts [4].There are chances of acquiring azole resistance from two routes firstly due to long-term azoles treatment of aspergillosis patients in human & veterinary hospital and secondly from the environmental route by intensive use of azoles fungicides in agricultural field. The environmental route of azole resistance has been reported since late 2000s and is largely responsible for the global emergence of resistance [5-8]. Acquired resistance to azole has been emerging in *Aspergillus fumigatus* along with Non *Aspergillus fumigatus.* The prevalence of such a resistant species varies in the world, and several studies have shown high rates, particularly in the Netherlands (0.8–29%), the United Kingdom (6.6–27.8%), and Germany (3.2–30%) [9-10].

The antifungal susceptibility testing (AFST) has been increased in recent years, especially in cases of invasive infections, acquired drug resistance and failing therapy of patients. For each of these scenarios, knowing the in vitro antifungal susceptibility pattern with broth micro dilution method (BMD) have become essential tool to guide patient therapy which would help the clinicians in making therapeutic choices or changes as this method of AFST provides MIC values[11]. Microbroth dilution is the standard method of antifungal testing for moulds but being complicated, labour intensive and costly this method is not performed in routine practices. Voriconazole(VCZ)is the first line drug of choice for aspergillosis infection. Hence this study was conducted to detect feasible, rapid, reproducible and cost effective method for the susceptibility testing of *Aspergillus* to voriconazole by using different antifungal susceptibility testing methods, which may aid in early phenotypic identification of azole resistance.

MATERIALS AND METHODS

A total of 162 *Aspergillus* isolates were tested (clinical isolates n=32 and environmental isolates from air n=130) over a period of one years (2022-23) in the Department of Microbiology. Clinical isolates were collected from proven/probable invasive aspergillosis patients admitted to Respiratory Medicine and Chest Disease (RMCD) ward. Environmental air samples were collected from RMCD wards (n= 100) and control samples (n=30) from community by using air sampler (Hi media model no.LA881), collecting 1000 L/plate on czapek dox and sabouraud dextrose agar with chloramphenicol. All cultures isolates were identified using standard mycological protocol. *Aspergillus* isolates included in this study were *As.fumigatus* (n=19), *As.flavus* (n=45) and *As.niger* (n=98). Antifungal susceptibility testing for voriconazole (disk 1µg/ml and MIC strip range 0.002-32 µg/ml) was done by Disk diffusion (DD) and E-test on non-supplemented Mueller Hinton Agar (MHA) according to standard CLSI guideline M51-A. Micro broth dilution method was done using RPMI1640 media according to the standard CLSI guideline M38-A2.For VCZ testing the isolates were considered as wild type (WT) at zone size ≥ 17mm by DD. The VCZ MIC was determined from the inhibition ellipse that intersected the scale on the strip by E-test. The MIC was read at the point of significant inhibition in fungal growth. The isolate was considered as WT for VCZ when MIC ≤ 1µg/ml for *As.fumigatus* & *As.flavus* and ≤ 2 µg/ml for *As.niger* by BMD [12-13].Two reference strains, *Candida krusei* ATCC 6258 and *As.fumigatus* ATCC 203408 were included in all the tests as references for quality controls and were tested by all the methods in each series of assays.

RESULTS

The MIC values of 35.8% of isolates were different at 24h and 48h.The interpretation at 24h have lower MIC's as compared to 48hr.For 99.38% of isolates MIC were within epidemiological cutoff value (ECV). A total of 0.62% of isolates showed MIC more than ECV (non WT).There was no significant difference in the results of either DD or E-test at 24h or 48h.The MIC using E-test ranges from 0.012-1.5µg/ml

for clinical isolates and 0.016-0.75 µg/ml for environmental isolates. While by micro broth dilution method MIC ranges from 0.0313-2µg/ml for clinical isolates and 0.0313-1µg/ml for environmental isolates (Table 1). All isolates were categorized WT (zone size ≥ 17 mm) by DD method. Single clinical isolates of *As.fumigatus* was categorized as non-WT (MIC >1µg/ml) by both E-test and BMD. Percent agreement of susceptibility testing by DD and E-test was 99.38%.The percentage agreement of DD and E test with the gold standard BMD for voriconazole ranged from 99.38% to 100 % respectively. The overall agreement of E test with BMD was better than that of DD.

Table1. In vitro susceptibility profile of *Aspergillus* isolates for voriconazole using E-test and micro broth dilution method

Clinical Isolate(32)	MIC by E test Method	Range	GM	MIC by MBD Method	Range	GM
As.fumigatus (13)	24 hr	0.016-1	0.12	24 hr	0.0313-1	0.176
	48 hr	0.016-1.5	0.1464	48 hr	0.0625-2	0.353
As.niger(14)	24 hr	0.012-0.094	0.0337	24 hr	0.0313-0.0625	0.044
	48 hr	0.016-0.125	0.0424	48 hr	0.313-0.125	0.062
As.flavus(5)	24 hr	0.016-0.125	0.0424	24 hr	0.0313-0.125	0.062
	48 hr	0.016-0.19	0.0523	48 hr	0.0625-0.125	0.088
Environment Isolate(130)	**MIC by E test Method**	**Range**	**GM**	**MIC by MBD Method**	**Range**	**GM**
As.fumigatus (6)	24 hr	0.012-0.125	0.0384	24 hr	0.0313-0.125	0.062
	48 hr	0.016-0.125	0.0424	48hr	0.0625-1	0.25
As.niger(84)	24 hr	0.012-0.125	0.0384	24 hr	0.0313-0.125	0.062
	48 hr	0.016-0.25	0.06	48 hr	0.0313-1	0.176
As.flavus (40)	24 hr	0.012-0.19	0.0474	24 hr	0.0313-0.25	0.088
	48 hr	0.016-0.75	0.1039	48 hr	0.0625-1	0.25

DISCUSSION

Fungal infections are increasing since two decades, still AFST data especially for filamentous fungi is lacking. Due to increasing trend of azole resistance it is essential to check mould antifungal susceptibility testing. The main goal of AFST is to guide the clinician in making therapeutic choices, to select the dose of antifungal and also to help in the prognosis of patients. There are two standardized guidelines by CLSI and EUCAST for AFST of mould using microbroth dilution method. Though these standard methods are albeit accurate but they are time consuming, labour intensive, costly and required experts for correlation of result due to which these tests were less common in routine laboratories. This study was done to provide an alternative method for reference method of AFST for mould so that it can be started in routine labs and rapid detection of azole resistance can be done.

In the present study azole resistance was reported in single *As.fumigatus* isolate from clinical origin. While all other isolates were found to be non-wild type. The azole resistance rate in clinical *As.fumigatus* was found to be 0.62% which is very low in comparison to other studies. Sung.C et al reported 5.3% of azole resistance rate in clinical *As.fumigatus* isolates from hematologic malignancy patients with IA and 6.5% in environmental isolates. There was no significant difference among azole resistance rate in *As.fumigatus* isolated from clinical and environmental source [14]. Tsitsopoulou et al reported azole resistance rate more than 10% in Dutch regions and 6% in UK in environmental samples.[15] Prashant et al reported voriconazole resistance (MIC of ≥ 2 µg/ml) in 9.1% *Aspergillus* isolates[16]. Shivprakash et al reported voriconazole resistance in 4.9% of *As.flavus* with MIC ≥ 4 µg/ml [17] while in the present all *As.flavus* isolates were susceptible to voriconazole. Espinell-Ingroff et al., also reported voriconazole MIC ≥ 4 µg/ml in 5.8% of the isolates, including one *As.fumigatus*[18].Berkow et al, from Spain reported voriconazole resistance(MIC > 4 µg/ml) in ten *As.fumigatus* clinical isolates collected from patients with hematological malignancies [19].

Many authors have been evaluated E-test by using different media. Espinell Ingroff et al., have used RPMI 1640 agar with 2% glucose and 0.165 M morpholine propane sulfonic acid (MOPS) buffer and found agreement between E-test and BMD ranging from 64.5% to 96.3% for voriconazole, posaconazole, itraconazole and amphotericin B[18]. In the present study E-test was evaluated on non-supplemented MHA as is being used for DD method of CLSI. Since agreement in our study for DD and E-test was found to be 99.38% for *Aspergillus* spp. and also there was no significant difference in MIC values at 24h and 48 h. Therefore non-supplemented MHA is better media for evaluation of E-test as it is easily available in most of the laboratories.

This study demonstrated that E-test for *Aspergillus* spp. provides lower MIC than standard microbroth methods (CLSI) which is consistent with a previous study. The agreement between E-test and BMD methods were found to be comparable in the present study. So we can use E-test as an alternative method for BMD but this study recommends caution regarding utilization of E-test for the AFST against *Aspergillus* spp. since it is unclear whether the lower MIC determined with E-test might affect the interpretation and choice of treatment and, the in vivo outcome.

CONCLUSION

In this study voriconazole resistance was observed in 0.64% of *As.fumigatus* isolate. So resistance due to voriconazole in *Aspergillus* isolates is not a critical problem in our setting which may be attributed to restricted use of this antifungal. Voriconazole should be used cautiously to prevent development of resistance in the future. E-test using Mueller-Hinton agar was found to be easy, rapid, reproducible and cost effective for the susceptibility testing of *Aspergillus* species. This study reveals that *Aspergillus* infections especially systemic infections where MIC play a very important role E-test should be a better alternative method for BDM which helps in early detection of susceptibility pattern of isolates. This shall aid the clinician in selecting appropriate antifungal therapy at earliest, which will help in prompt treatment and improving the prognosis of aspergillosis patient.

Future Prospect

Extensive study should be done on large number of clinical and environmental isolates by incorporating more no. of antifungals, which will help us to establish antibiogram for antifungal stewardship.

REFERENCES

1. Vuong MF, Hollingshead CM and Waymack JR (2022) Aspergillosis. In StatPearls (Internet). Treasure Island, FL: StatPearls Publishing. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482241/.
2. Bongomin F, Gago S, Oladele RO and Denning DW (2017) Global and multi-national prevalence of fungal diseases-estimate precision. J Fungi (Basel) 3(4), 57.
3. Otu A, Kosmidis C, Mathioudakis AG, Ibe C and Denning DW (2023) The clinical spectrum of aspergillosis in chronic obstructive pulmonary disease. Infection 51, 813–829.
4. Yang X, Chen W, Liang T, Tan J, Liu W, Sun Y, Wang Q, Xu H, Li L and Zhou Y (2021) A 20-year antifungal susceptibility surveillance (from 1999 to 2019) for *Aspergillus* spp. and proposed epidemiological cutoff values for *Aspergillus fumigatus* and *Aspergillus flavus*: A study in a tertiary hospital in China. Front. Microbiol 12, 680884.
5. Verweij, P.E.; Chowdhary, A.; Melchers, W.J.G.; Meis, J.F. Azole Resistance in *Aspergillus fumigatus*: Can we retain the clinical use of mold-active antifungal azoles? Clin. Infect. Dis. 2016, 62, 362–368.
6. Berger, S.; El Chazli, Y.; Babu, A.F.; Coste, A.T. Azole resistance in *Aspergillus fumigatus*: A consequence of antifungal use in agriculture? Front. Microbiol. 2017, 8, 1024.
7. Snelders, E.; Camps, S.M.T.; Karawajczyk, A.; Schaftenaar, G.; Kema, G.H.J.; Lee, H.A.; Klaassen, C.H.; Melchers, W.J.G.; Verweij, P.E. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. PLOS ONE 2012, 7, e31801.
8. Snelders, E.; Huis In 't Veld, R.A.G.; Rijs, A.J.M.M.; Kema, G.H.J.; Melchers, W.J.G.; Verweij, P.E. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. Appl. Environ. Microbiol. 2009, 75, 4053–4057.
9. Van der Linden, J.W.M.; Arendrup, M.C.; Warris, A.; Lagrou, K.; Pelloux, H.; Hauser, P.M.; Chryssanthou, E.; Mellado, E.; Kidd, S.E.; Tortorano, A.M.; et al. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg. Infect. Dis. 2015, 21, 1041–1044.
10. Resendiz Sharpe, A.; Lagrou, K.; Meis, J.F.; Chowdhary, A.; Lockhart, S.R.; Verweij, P.E. ISHAM/ECMM *Aspergillus* resistance surveillance working group Triazole resistance surveillance in *Aspergillus fumigatus*. Med. Mycol. 2018, 56, 83–92.
11. Otto WR, Arendrup MC and Fisher BT (2023) A practical guide to antifungal susceptibility testing. J. Pedia. Infect. Dis. Society 12, 214–221.
12. Clinical and Laboratory Standards Institute; USA 2010.)Reference Method for Antifungal disk diffusion Susceptibility Testing of Nondermatophytes Filamentous Fungi; Approved Standard—Second Edition. CLSI document M51-A. Wayne, PA:
13. Clinical and Laboratory Standards Institute; USA 2008.)Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard—Second Edition. CLSI document M38-A2. Wayne, PA:
14. Sung.C ,Dong L,Won K,Hye C, Chulmin P, Jun M et al Epidemiology and Antifungal Susceptibility Profile of *Aspergillus* Species: Comparison between Environmental and

Clinical Isolates from Patients with Hematologic Malignancies J.Clin. Microbiol 2019, 57, Issue 7, 1-13
15. Tsitsopoulou A, Posso R, Vale L, Bebb S, Johnson E, White PL. 2018. Determination of the prevalence of triazole resistance in environmental *Aspergillus fumigatus* strains isolated in South Wales, UK. Front. Microbiol 9:1395.
16. Prashant G, Vineeta K, Deepak K , Abrar A, Gopa B, Mastan S. 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 J. Clin. Diag. Resea. 2015, Vol-9(1)
17. Shivaprakash MR, Chakrabarti A, Geertsen E, Mouton JW, Meis JF. In vitro activity of isavuconazole against 208 *Aspergillus flavus* isolates in comparison with 7 other antifungal agents: assessment according to the methodology of the European Committee on Antimicrobial Susceptibility Testing. Diag. Microbiol. Infect. Dis. 2011;
18. Espinel-Ingroff A, Johnson E, Hockey H, Troke P. Activities of voriconazole, itraconazole and amphotericin B in vitro against 590 moulds from 323 patients in the voriconazole Phase III clinical studies. J Antimicrob Chemother. 2008; 61:616- 20.
19. Berkow, E.L.; Lockhart, S.R.; Ostrosky-Zeichner, L. Antifungal susceptibility testing: Current approaches. Clin. Microbiol. Rev. 2020, 33, e00069-19.