



***In vitro* Activities of Amphotericin B, Itraconazole and Voriconazole against Isolates of *Aspergillus* spp.**

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ABSTRACT

This study was aimed at the drug susceptibility testing of *Aspergillus fumigatus* and *Aspergillus flavus* against amphotericin B, itraconazole and voriconazole using microdilution method proposed by National Committee for Clinical Laboratory Standards (NCCLS M38-P document).

50 isolates of *Aspergillus* spp. supplied from environment of Emam Khomeini hospital and minimum inhibitory concentrations (MIC) were determined after 48 h against mentioned drugs in accordance with NCCLS M38-P document. MICs results showed that voriconazole was more active drug than itraconazole with MICs between 0.5 to 1 µg/ml. The MICs ranges for itraconazole and amphotericin B were between 1-8 µg/ml and 0.5-2 µg/ml, respectively. Significant decrease in MICs was seen for voriconazole with respect to itraconazole ($P < 0.05$). Significant difference was not seen for each of drug in both *A.fumigatus* and *A.flavus* strains ($P < 0.05$). None of isolates showed in-vitro resistance to used drugs but for better judgment of drug resistance, clinical trials abreast of in-vitro results need to be established.

Keywords: Drug susceptibility test, *Aspergillus* spp., Amphotericin B, Azoles drugs

INTRODUCTION

In recent years, incidence of fungal infections, because of increase in number of immunocompromised patients, has been raised [1]. The majority of clinical mold infections (more than 85%) are caused by *Aspergillus* spp. [2]. The genus *Aspergillus* spp. are ubiquitous fungi which most of their species are capable of sporulation. Inhalation of conidia, found in environment, can cause infection in susceptible hosts [3]. Among *Aspergillus* species, *Aspergillus fumigatus* is responsible for majority of chronic pulmonary aspergillosis [4]. However, other species of *Aspergillus* spp. such as *Aspergillus flavus* are responsible for invasive and non-invasive diseases in humans.

A.flavus also is potentially capable of aflatoxin production, considered to be human liver carcinogens. Azoles and polyenes drugs such as itraconazole and amphotericin B are respectively recommended first-line drugs in treatment of aspergillosis. However, because of much exposure to mentioned drugs, resistance has been documented [3]. *A.fumigatus* normally shows high susceptibility to all three antifungal drug classes, but reports based on resistance to itraconazole are various, also multiple triazole resistance (MTR) has reported in *A.fumigatus* [5, 6]. *A.fumigatus* intrinsically shows resistance to fluconazole, in contrast to new triazoles such as posaconazole and voriconazole which show potent activity against *Aspergillus* spp. [7].

In this study, we compared the susceptibility testing of traditional and new drugs used in treatment of invasive and non-invasive aspergillosis in Iran against isolates of *A.fumigatus* and *A.flavus* isolated from environment of Emam Khomeini hospital during April 2010 to September 2011.

MATERIALS AND METHODS

25 isolates of each of *A.flavus* and *A.fumigatus* were supplied from environment of Emam Khomeini hospital (corridors, examination rooms, and patient's room). The isolates were grown on sabouraud dextrose agar (SDA) (Himedia, India), for 7 days at 35°C. Identification of strains was based on macroscopic and microscopic features. *Candida kruseii* (ATCC 6258) and *A.flavus* (ATCC 204304) were considered as quality control (QC).

Preparation of antifungal drugs and drug dilutions:

The antifungal reference powder of voriconazole (Pfizer, Sandwich, UK), itraconazole (Roos Daru, Tehran, Iran) and amphotericin B (Cipla, India) were obtained from their manufacturers. Voriconazole, itraconazole and amphotericin B were dissolved in 100% dimethyl sulfoxide (DMSO) (Daejung, Korea) and stock solutions were prepared as two fold serial dilution from each drug with RPMI 1640 (Merck, Germany) with L- glutamine but without bicarbonate buffered to pH 7.0 with 3-N – morpholinopropene sulfonic acid (MOPS) which achieved to final concentration of 0.0165 M, as diluent described in NCCLS broth microdilution method (M38-P document), and final concentration were supplied between 0.03125 to 16 $\mu\text{g}/\text{ml}$ for itraconazole and voriconazole and 0.007812 to 4 $\mu\text{g}/\text{ml}$ for amphotericin B.

Inoculate preparation:

Inocula suspensions were prepared from 7 day-old cultures grown on SDA. Briefly, colonies were covered with 3 ml of sterile saline 85% and gently probed with the tip of Pasteur pipette. After transfer of resulting mixture of conidia and hyphae to sterile tubes and setting the heavy particles of the suspensions for 3 to 5 min, final inoculum size was adjusted by spectrophotometer at the wavelength of 530 nm. The goal percentages of transmittance for *Aspergillus spp.* were 80 to 82 % (optical density (OD) 0.09 to 0.11).

The final inoculum suspensions ranging from 0.5 to 5 $\times 10^5$ CFU/ml were supplied, and confirmed by quantitative colony counts on SDA.

Susceptibility testing method:

Susceptibility testing was done using the BMD assay method (NCCLS M38-P) (8). The 10 \times drug dilutions were dispensed into the sterile wells with 0.1 ml volumes, then each well inoculated by 0.1 ml volumes of inoculum suspension and all tubes inoculated at 35°C and minimum inhibitory concentration (MIC) were determined after 48h by visual inspection for 100% growth inhibition of fungi.

Candida kruseii (ATCC 6258) and *A.flavus* (ATCC 204304) were tested by each run for QC. The growth control well contained 0.1 ml of the adjusted inoculum suspension with 0.1 ml of the drug diluent without antifungal agent.

Date analysis:

ANOVA besides *t- test* was used to analyze the MICs by SPSS 17. ANOVA was used for comparison of MICs of each drug between isolates and *t- test* was used to comparison the drugs and ($P < 0.05$) was statistically considered significant.

RESULTS AND DISCUSSION

MICs obtained for 25 isolates of *A.flavus* and 25 of *A.fumigatus* isolates are summarized in table 1. The MIC ranges of *A.flavus* for amphotericin B were between 0.5 to 2 $\mu\text{g}/\text{ml}$.

Of 25 isolates of *A.flavus*, 14 isolates showed MIC 0.5 $\mu\text{g}/\text{ml}$, 7 isolates with 1 $\mu\text{g}/\text{ml}$ and 4 isolates with 2 $\mu\text{g}/\text{ml}$, respectively for amphotericin B. Of 50 isolates of *Aspergillus spp.* 22 isolates of *A.flavus* and 16 isolate of *A.fumigatus* showed MIC with 0.5 $\mu\text{g}/\text{ml}$ and 3 isolates of *A.flavus* and 9 isolates of *A.fumigatus* showed 1 $\mu\text{g}/\text{ml}$ for voriconazole, respectively. The MIC ranges of itraconazole were between 1 to 8 $\mu\text{g}/\text{ml}$ for all isolates. The MICs of 12 isolates of *A.fumigatus* was 1 $\mu\text{g}/\text{ml}$ and 13 other isolates were 2, 4 and 8 $\mu\text{g}/\text{ml}$, respectively. The MICs of 8 isolates of *A.flavus* were 1 $\mu\text{g}/\text{ml}$ and followed by 7, 6 and 4 isolates were 2, 4, and 8 $\mu\text{g}/\text{ml}$, respectively. The comparison of the MIC in mentioned drugs is shown in Fig 1.

The MICs of voriconazole significantly decreased with respect to amphotericin B and itraconazole against both *A.fumigatus* and *A.flavus* ($P < 0.05$). Significant decrease in MICs was seen for amphotericin B with respect to itraconazole ($P < 0.05$). Significant difference was not seen for each of drug in both *A.fumigatus* and *A.flavus* ($P < 0.05$).

Voriconazole was more potent drug against environmental *A.flavus* and *A.fumigatus* than other drugs used in this study. The MICs ranges of amphotericin B were between 0.5 to 2 $\mu\text{g}/\text{ml}$ against *A.fumigatus* and *A.flavus* which was in accordance with studies of Arikan et al, Espinel- Ingroff et al and Pugol et al. with ranges 0.5 to 2 $\mu\text{g}/\text{ml}$ [9, 10, 11]. It seems *in-vitro* susceptibility of *Aspergillus spp.* to amphotericin B mimics from a pattern with low variation of MICs. Although, the MICs ranges of amphotericin B were between 0.5 to 2 $\mu\text{g}/\text{ml}$ for both *A.fumigatus* and *A.flavus* but uniformity was not seen in MICs ranges. The MIC of 14 (56 %) isolates of *A.flavus* versus 9 (36 %) isolates of *A.fumigatus* was 0.5 $\mu\text{g}/\text{ml}$ also, (7 vs. 13) and (4 vs. 3) isolates of *A.flavus* and *A.fumigatus* showed MICs 1 $\mu\text{g}/\text{ml}$ and 2 $\mu\text{g}/\text{ml}$, respectively. MICs obtained for itraconazole were between 1-8 $\mu\text{g}/\text{ml}$ which in agreement with other studies [3, 11, 12]. Arikan et al. have reported itraconazole MICs between 0.03 to 2 $\mu\text{g}/\text{ml}$ for susceptible isolates of *A.flavus* and *A.fumigatus* and >16 $\mu\text{g}/\text{ml}$ for itraconazole resistant isolates [8]. In this study, itraconazole showed superior MICs to amphotericin B and voriconazole which opposite from the results of Espinel-Ingroff et al. for *A.flavus* [13]. In present study, resistant isolate was not seen but in study of Badiiee et al.

30.6 % isolates were resistant to itraconazole which didn't agreed with our study [14]. Voriconazole showed MICs between 0.5 to 1 $\mu\text{g}/\text{ml}$, which in agreement of Oakley et al. results [15]. The MICs of 22 isolates of *A.flavus* were 0.5 $\mu\text{g}/\text{ml}$ vs. 16 isolates of *A.fumigatus* for voriconazole. 3 isolates MICs of *A.flavus* and 9 isolates of *A.fumigatus* were 1 $\mu\text{g}/\text{ml}$. Voriconazole is a triazole agent which its MICs results were better versus itraconazole and amphotericin B in various studies [8, 14].

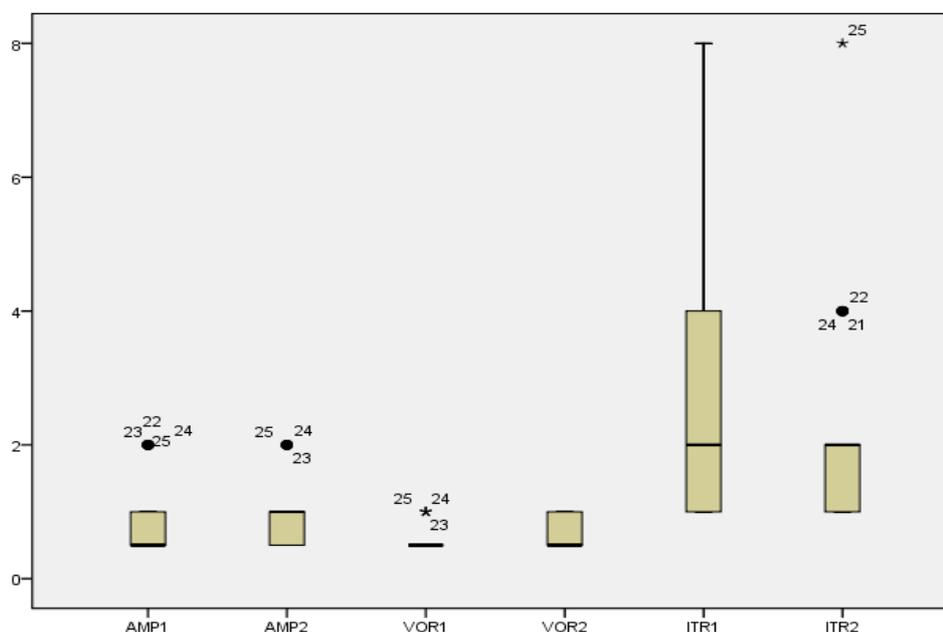
Conclusion: Voriconazole is commercially not in Iran and with regard to reports based on presence of itraconazole and amphotericin B-resistant isolates, the presence of voriconazole is essential in Iran. It seems voriconazole to be a potent and promising alternative drug for azole-resistant isolates specially for aspergillosis. With regard to results obtained in this study further investigations abreast of *in-vivo* studies are recommended.

Table 1- MICs obtained by NCCLS broth microdilution (M38 – P document) for 50 isolates.

Fungus (No. of isolates)	Antifungal agent ^a	MIC ranges ($\mu\text{g}/\text{ml}$)	Mean \pm S.E.M
<i>A.flavus</i> (25)	VOR	0.5- 1	0.56 \pm 0.03
	AMP	0.5- 2	0.9 \pm 0.1
	ITR	1- 8	3.1 \pm 0.49
<i>A.fumigatus</i> (25)	VOR	0.5 - 1	0.68 \pm 0.04
	AMP	0.5- 2	0.94 \pm 0.09
	ITR	1- 8	2.1 \pm 0.33

a, VOR: Voriconazole, AMP: Amphotericin B, ITR: Itraconazole

Fig 1- Comparison of drugs activity against *A. flavus* and *A. fumigates*



AMP 1: Drug susceptibility of amphotericin B against *A.flavus*, AMP 2: Drug susceptibility of amphotericin B against *A.fumigatus*, VOR 1: Drug susceptibility of voriconazole against *A.flavus*, VOR 2: Drug susceptibility of voriconazole against *A.fumigatus*, ITR 1: Drug susceptibility of itraconazole against *A.flavus*, ITR 2: Drug susceptibility of itraconazole against *A.fumigatus*

ACKNOWLEDGMENT

This research has been supported by Tehran University of Medical Sciences and health Services grant 90-03-31-14542.

Kazemi et al

REFERENCES

1. Pfaller, J.B., Messer, S.A., Hoillis, R.J., Diekema, D.J. & Pfaller, M.A. (2003). In vitro susceptibility testing of *Aspergillus* spp.: comparison of E-test and reference microdilution methods for determining the voriconazole and itraconazole MICs. *J. Clin. Microbiol.*, 41(3):1126-1129.
2. Espinel-Ingroff, A. & Rezusta, A. (2002). 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. *J.Clin. Microbiol.*, 40(6): 2101-2107.
3. Mortensen, K.L., Mellado, E., Lass-Flörl, C., Radriguez-Tudela, J.L., Johansen, H.K. & Arendrup, M.C. (2010). Environmental study of azole - resistant *Aspergillus fumigatus* and other Aspergilli in Austria, Denmark, and Spain. *Antimicrob. Agents .Chemother.*, 54(11): 4545-4549.
4. Sabatelli, F., Patel, R., Mann, P.A., Mendrick, C.A., Norris, C.C. Hare, R., Loebenberg, D., Black T.A. & McNicholas, P.M.(2006). In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole and amphotericin B against a large collection of clinically important mold and yeasts. *Antimicrob. Agent. Chemother.*, 50(6): 2009-2015.
5. Moore C.B., Sayers, N., Mosquera, J., Salven J. & Denning, D.W. (2000). Antifungal drug resistance in *Aspergillus*. *J. Infect.*, 41(3): 203-220.
6. Diaz-Guerra, E., Vernal, R., Cantero, W., Müllner E.W. & Garcia-Sanz, J.A. (2008). Translation controlled mRNAs: new drug targets in infectious disease? *Infect. Disord. Drug .Targets.*, 8(4): 252-61.
7. Verweij, P.E. & Brandt, M.E. (2007). *Aspergillus*, *Fusarium* and other opportunistic moniliaceous fungi. In Manual of clinical microbiology (Eds, American Society for Microbiology; Pfaller M.A.) Washington DC, p.1802 -38
8. National Committee for Clinical Laboratory standards. Reference method for broth dilution antifungal susceptibility testing of conidium- forming filamentous fungi. 1998. Proposed standards M38-P. National Committee for Clinical Laboratory Standards, Wayne, Pa 1998.
9. Arikan, S., Lozano-Chiu, M., Paetzink, V., Nangia, S. & Rex, H.J.(1999). Microdilution susceptibility testing amphotericin B, itraconazole and voriconazole against clinical isolates of *Aspergillus* and *Fusarium* species. *J. Clin. Microbiol.*, 37(12): 3946-3951.
10. Espinel- Ingroff, A., Dawson, K., Pfaller, M.A., Anaissie, E., Breslin, B., Dixon, D., Fothergill, A., Paetznick, V., Peter, J. & Rinaldi, M. (1995). Comparative and collaborative evaluation of standardization of antifungal susceptibility testing for filamentous fungi. *Antimic Agent Chemother.*, 39(2):314-319.
11. Pugol, I., Guarro, J., Liop, C., Soler L. & Fernandez - Ballart, J. (1996). Comparison study of broth macrodilution and microdilution antifungal susceptibility testing for filamentous fungi. *Antimicrob. Agents. Chemother.*, 40(9): 2106-2110.
12. Graybill, J.R., Najvar, L.K., Holmberg, J.D., Correa, A. & Luther, M.F. (1995). Fluconazole treatment of *Candida albicans* infection in mice: does in vitro susceptibility predict in vivo response? *Antimicrob. Agents. Chemother.*, 39(10) :2197-200.
13. Espinel-Ingroff, A., Canton, E., Fothergill, A., Ghannoum, M., Johnson, E., Jones, R.N., Ostrosky-Zeichner, L., Schell, W., Gibbs, D. L., Wang, A. & Turnidge, J. (2011). Quality Control Guidelines for amphotericin B, itraconazole, posaconazole, and voriconazole disk diffusion susceptibility tests with non supplemented Mueller-Hinton Agar (CLSI M51-A Document) for non dermatophyte filamentous fungi. *J.Clin. Microbiol.*, 49(7): 2568-71.
14. Badiie, P., Alborzi, A., Moeini, M., Haddadi, P., Farshad, S., Japoni, A. & Ziyaeyan, M.(2012). Antifungal susceptibility of the aspergillus species by Etest and CLSI reference methods. *Arch Iran Med.*, 15(7):429-32
15. Oakely, K.L., Moore C.B. & Denning, D.W. (1998). In vitro activity of the echinocandin antifungal agent LY303, 366 and comparison with itraconazole and amphotericin B against *Aspergillus* spp. *Antimicrob .Agents .Chemother.*, 42(10): 2726-30.