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ORIGINAL ARTICLE

In Vitro Susceptibilities of *Aspergillus* Species to Triazoles and Amphotericin B

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ABSTRACT

Objective: to identify the efficacy of certain antifungal agents against different *Aspergillus* species and the development of acquired resistance.
Methods: A total of 177 clinical isolates of different *Aspergillus* spp. were obtained from different medical centers in Baghdad. In vitro susceptibility tests are based on the measurement of the fungal growth in the presence of different drug concentrations (Posaconazole, voriconazole, itraconazole and amphotericin B) so as to determine the minimum inhibitory concentration (MIC) of antifungals. The broth microdilution (BMD) method was performed according to the CLSI M38-A2 standard.
Results: MIC(µg/ml) distribution for azoles and amphotericin B with different *Aspergillus* species were as follows: *A. fumigatus*, amohptericin B, 1 ; itraconazole, 0.5 ; voriconazole, 0.25 ; posaconazole, 0.06 ; *A.flavus*, amohptericin B, 1 ; itraconazole, 0.5 ; voriconazole, 0.5; posaconazole, 0.125 ; *A. niger*, amohptericin B, 1 ; itraconazole, 1 ; voriconazole, 0.5 ; posaconazole, 0.25 ; *A. terreus*, amohptericin B, 2 ; itraconazole, 0.5 ; voriconazole, 0.5 ; posaconazole, 0.25 and *A. versicolor*, amohptericin B, 2 ; itraconazole, 1 ; voriconazole, 0.5 ; posaconazole, 0.5. 7.8% of *A. fumigatus*, 17.4% of *A. flavus* and 26.6% of *A. terreus* showed in vitro resistance to amphotericin B. 4.3% of *A. fumigatus* had resistance to itraconazole, with cross resistance to posaconazole observed in 80%.
Conclusion: Triazoles and amphotericin B have good in vitro activities against most *Aspergillus* species in the following order: posaconazole, voriconazole, itraconazole and amphotericin B. Majority of *Aspergillus* species exhibited in vitro resistance to amphotericin B followed by itraconazole and there was cross resistance between itraconazole and posaconazole, but not between itraconazole and voriconazole.

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INTRODUCTION

Although *Candida* spp. and *Cryptococcus neoformans* remain the most common causes of invasive opportunistic mycotic infection, serious infections due to *Aspergillus* spp. and other filamentous fungi are emerging as prominent causes of infectious morbidity and mortality worldwide[1]. Aspergillosis is a large spectrum of fungal diseases, which primarily affect the lungs and are caused by members of the genus aspergillus[2]. Mortality rates associated with systemic mycoses, particularly those involving members of the zygomycetes, remain unacceptably high. Effective treatment requires both an early diagnosis, to facilitate prompt initiation of therapy, and broad spectrum therapeutic agents with activity against both common and "emerging" pathogens. Until recently, the drugs available to treat invasive fungal infections were limited by their spectrum of activity, the development of resistance, and less than optimal tolerability and drug interaction profiles[3]. Amphotericin B in its various formulations and triazole (itraconazole, voriconazole, and posaconazole) have a broad spectrum of in vitro activity against molds and are important therapeutic

agents for the systemic treatment and prevention of severe mold infections, including aspergillosis[4]. In vitro susceptibility tests are based on the measurement of the fungal growth in the presence of different drug concentrations so as to determine the minimum inhibitory concentration (MIC) of antifungals[5]. Epidemiologic cut-off values (ECVs) of the MIC were established for different aspergillus species and different antifungal agents, in order to assess the emergence of strains with decreased susceptibility[6]. There are two independent standards for broth microdilution (BMD) antifungal susceptibility testing of triazole activity against aspergillus species: the Clinical and Laboratory Standards Institute (CLSI) method and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method[7]. The two methods are similar in that both use BMD, RPMI 1640 broth, incubation at 35 to 37°C for 48 h, and a complete (100%) inhibition visual MIC endpoint. They differ in their values for inoculum density (0.4 to 5 X 10^4 CFU/ml [CLSI] versus 2 to 5 X 10^5 CFU/ml [EUCAST]) and glucose content of the medium (0.2% [CLSI] and 2.0% EUCAST]) and in the use of round-bottom (CLSI) versus flat-bottom (EUCAST) microdilution wells[8].

MATERIALS AND METHDS

A total of 177 clinical isolates of different *Aspergillus* spp. were obtained from different medical centers in Baghdad between January 2015 and December 2016. The isolates were obtained from a variety of sources, including sputum, bronchoscopy and tissue biopsy specimens. The isolates were collected at individual sites and sent to the Central Health Laboratory in Baghdad for identification and susceptibility testing. The collection included the following isolates: 115 of *Aspergillus fumigatus*, 23 of *Aspergillus flavus*, 18 of *Aspergillus niger*, 15 of *Aspergillus terreus*, and 6 of *Aspergillus versicolor*. All isolates were identified by standard microscopic morphology and were stored as spore suspensions in sterile distilled water at room temperature until used in the study. Before testing, each isolate was subcultured at least twice on potato dextrose agar to ensure viability and purity.

Posaconazole, voriconazole, itraconazole and amphotericin B were all obtained as reagent-grade powders from the manufacturers. Stock solutions were prepared in polyethylene glycol (posaconazole and itraconazole) and dimethyl sulfoxide (voriconazole and amphotericin B). The BMD method was performed according to the CLSI M38-A2 standard[9]. Trays containing a 0.1-ml aliquot of the appropriate drug solution (2 ×the final drug concentration) in each well were sealed and stored at -70 °C until used in the study. The stock conidial suspension (10^6 spores/ml) was diluted to a final inoculum concentration of 0.4 × $10^4$to 5×10^4CFU/ml and dispensed into the microdilution wells. The final concentrations of drugs in the wells ranged from 0.008 to 8 µg/ml. The inoculated microdilution trays were incubated at 35°C and read at 48 h. The MIC end point for the azoles and amphotericin B was defined as the lowest concentration that produced complete inhibition of growth.

Table 1. **Epidemiological cut-off values (µg/ml) for amphotericin, itraconazole, voriconazole and posaconazole according to the Aspergillus species** [6, 10]**.**

Species	Amphotericin B	Itraconazole	Posaconazole	Voriconazole
A.fumigatus	4	1	0.5	1
A.flavus	4	1	0.5	1
A.niger	4	2	0.5	2
A.terreus	4	1	0.5	1
A.versicolor	4	2	1	2

RESULTS

The minimal inhibitory concentrations (MICs) of triazoles and amphotericin B against different aspergillus species are shown in details in Table 2. Posaconazole showed the minimal MIC compared with the other 3 drugs and hence a greatest activity against most of aspergillus species. At MIC 0.5 µg/ml, posaconazole inhibited 96.5% of *A. fumigatus*, 95.6% of *A. flavus*, 100% of *A. niger* and *A. terreus* and 83.3% of *A. versicolor*. These results are the highest compared to all the other medications used in this study, followed by voriconazole, itraconazole and amphotericin B in order of decreasing activity. At same concentration (0.5 µg/ml), amphotericin B inhibited only 17.3% of *A. fumigatus*, 21.7% of *A. flavus*, 33.3% of *A. niger*, 6.6% of *A. terreus* and zero% of *A. versicolor*.

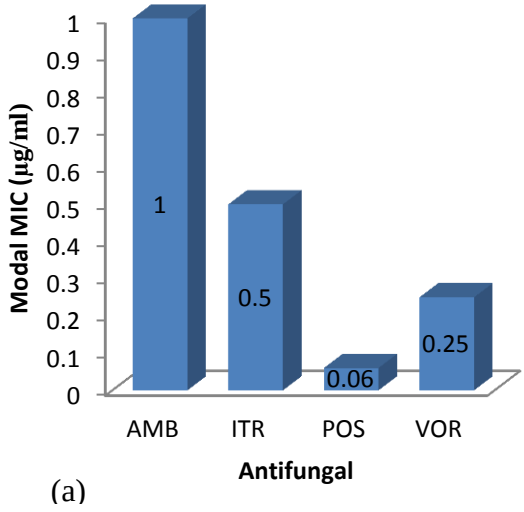
The modal MICs (the most frequent MIC: highlighted by red color in Table 1) of each antifungal agent are shown in Table 3. Again posaconazole presented with the lowest and amphotericin B with the highest modal MIC (Figure 1). The percentages of isolates at modal MIC (specific value for each antifungal agent) were as follows: posaconazole: 41.7% of *A. fumigatus*, 34.7% of *A. flavus*, 38.9% of *A. niger*, 33.3% of *A. terreus* and 50% of *A. versicolor*; voriconazole: 43.5% of *A. fumigatus*, 39.1% of *A. flavus*, 33.1% of *A. niger*, 40% of *A. terreus* and 50% of *A. versicolor*; itraconazole: 42.6% of *A. fumigatus*, 43.4% of *A. flavus*, 33.3% of *A. niger*, 40% of *A. terreus* and 50% of *A. versicolor*; amphotericin B: 39.1% of *A. fumigatus*, 39.1% of *A. flavus*, 44.4% of *A. niger*, 40% of *A. terreus* and 50% of *A. versicolor*. The modal MIC ±1 twofold dilution represented the following percentages of isolates: posaconazole: 80.8% of *A. fumigatus*, 78.2% of *A. flavus*, 72.2% of *A. niger*, 80% of *A. terreus* and 83.3% of *A. versicolor*; voriconazole: 82.6% of *A. fumigatus*, 78.2% of *A. flavus*, 66.6% of *A. niger*, 80% of *A. terreus* and 83.3% of *A. versicolor*; itraconazole: 83.4% of *A. fumigatus*, 78.2% of *A. flavus*, 61.1% of *A. niger*, 73.3% of *A. terreus* and 83.3% of *A. versicolor*; amphotericin B: 69.5% of *A. fumigatus*, 73.9% of *A. flavus*, 88.8% of *A. niger*,66.6% of *A. terreus* and 100% of *A. versicolor*.

Table 2. **MIC distributions of triazoles and amphotericin B for different *Aspergillus* spp.**

Species	Antifungal agents	No. of isolates MIC (µg/ml)									
		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8
	Amphotericin B	0	0	0	0	8	12	**45**	23	18	9
A. fumigatus	Itraconazole	0	3	5	6	28	**49**	19	2	2	1
(n = 115)	Posaconazole	8	27	**48**	18	6	4	3	1	0	0
	Voriconazole	0	0	8	13	**50**	32	11	1	0	0
	Amphotericin B	0	0	0	0	0	5	**9**	3	2	4
A. flavus	Itraconazole	0	1	2	2	5	**10**	3	0	0	0
(n = 23)	Posaconazole	0	2	6	**8**	4	2	1	0	0	0
	Voriconazole	0	0	2	3	7	**9**	2	0	0	0
	Amphotericin B	0	0	0	0	1	5	**8**	3	1	0
A.niger	Itraconazole	0	1	1	2	3	3	**6**	2	0	0
(n = 18)	Posaconazole	1	2	2	4	**7**	2	0	0	0	0
	Voriconazole	0	1	2	2	4	**6**	2	1	0	0
	Amphotericin B	0	0	0	0	0	1	3	**6**	1	4
A.terreus	Itraconazole	0	1	1	2	3	**6**	2	0	0	0
(n = 15)	Posaconazole	0	1	2	4	**5**	3	0	0	0	0
	Voriconazole	0	1	1	1	4	**6**	2	0	0	0
	Amphotericin B	0	0	0	0	0	0	1	**3**	2	0
A.versicolor	Itraconazole	0	0	0	0	1	1	**3**	1	0	0
(n = 6)	Posaconazole	0	0	0	1	1	**3**	1	0	0	0
	Voriconazole	0	0	0	0	1	**3**	1	1	0	0

Table 3. **Modal MIC and ECVs of triazoles and amphotericin B for different *Aspergillus* spp.**

Species	Antifungal agents	Modal MIC (µg/ml)	ECV (µg/ml)	% of isolates ≤ ECV
	Amphotericin B	1	4	92.2
A.fumigatus	Itraconazole	0.5	1	95.2
(n = 115)	Posaconazole	0.06	0.5	96.5
	Voriconazole	0.25	1	99.1
	Amphotericin B	1	4	82.6
A.flavus	Itraconazole	0.5	1	100
(n = 23)	Posaconazole	0.125	0.5	95.6
	Voriconazole	0.5	1	100
	Amphotericin B	1	4	100
A.niger	Itraconazole	1	2	100
(n = 18)	Posaconazole	0.25	0.5	100
	Voriconazole	0.5	2	100
	Amphotericin B	2	4	73.3
A.terreus	Itraconazole	0.5	1	100
(n = 15)	Posaconazole	0.25	0.5	100
	Voriconazole	0.5	1	100
	Amphotericin B	2	4	100
A.versicolor	Itraconazole	1	2	100
(n = 6)	Posaconazole	0.5	1	100
	Voriconazole	0.5	2	100



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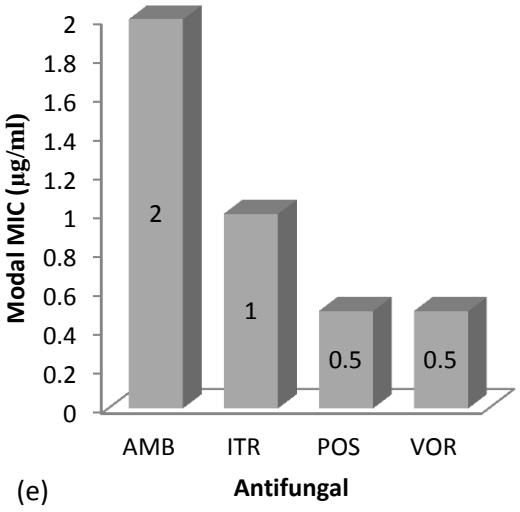

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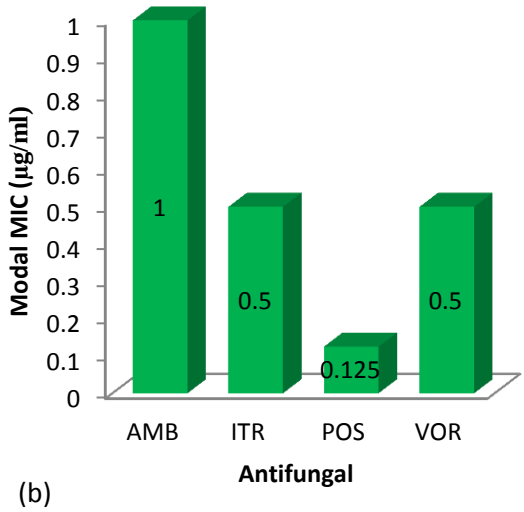

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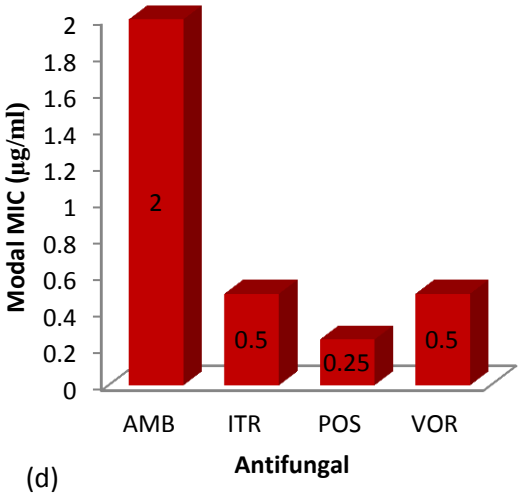

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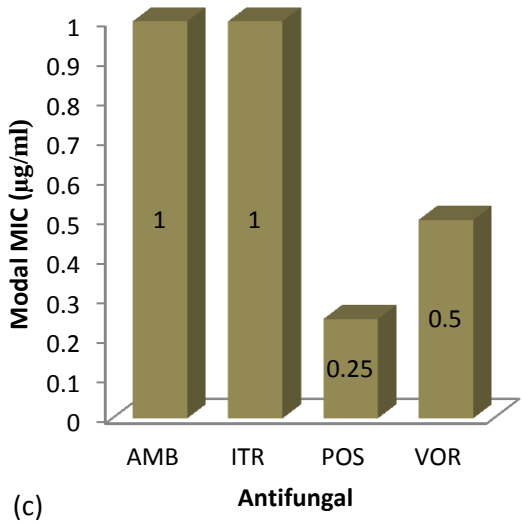

(c)

Fig. 1 **Modal MIC distribution of triazoles and amphotericin B for different *Aspergillus* spp.: (a) *A. fumigatus*, (b) *A. flavus*, (c) *A. niger*, (d) *A. terreus*, and (e) *A. versicolor*.**
AMP: amphotericin B ; ITR: itraconazole.
POS: posaconazole ; VOR: voriconazole.

The epidemiologic cut-off values (ECVs) of the MICs were obtained from other studies and considered in our study as a starting point of decreasing susceptibility and emergence of resistance to antifungal agents. Five isolates (4.3%) of *Aspergillus fumgiatus* showed MICs of itraconazole between 2 – 8 µg/ml and considered as harboring resistance to itraconazole. A high degree of cross-resistance is apparent between itraconazole and posaconazole but not between itraconazole and voriconazole (table 4). 9 isolates of *A. fumigatus* (7.8%), 4 isolates of *A. flavus* (17.4%) and 4 isolates of *A. terreus* (26.6%) showed in vitro resistance to amphotericin B.

Table 4. **Cross-resistance between itraconazole, posaconazole, and voriconazole among 5 A. fumigatus with high itraconazole MICs (≥ 2 µg/ml)**

Species	Antifungal agents	Range (µg/ml)	Modal MIC (µg/ml)	% of isolates ≤ ECV
A.fumigatus (n = 5)	Itraconazole	2 - 8	4	0
	Posaconazole	0.5 - 2	1	20
	Voriconazole	0.25 - 2	1	80

DISCUSSION

The utility of susceptibility testing is achieved when the test result can define the susceptibility or resistance of the organism being tested against a specific agent. In our study the modal MICs of different antifungal agents against various *Aspergillus* spp. were obtained and compared to each other to identify the efficacy of these medications. Posaconazole had the lowest MICs compared to other medications and accordingly the greatest activity against different aspergillus species followed by voriconazole, itraconazole and amphotericin B in order of increasing MIC and decreasing activity. Some studies performed by Pfaller[1, 7, 11] and Espinel[10] showed that posaconazole had the greatest activity against majority of aspergillus species followed by voriconazole and itraconazole in order of decreasing activity. Espinel[12] also demonstrated a highest activity of posaconazole against all aspergillus species, followed by voriconazole with better activity against *A. fumigatus* and *A. niger* and itraconazole with better activity against *A. flavus* and *A. terreus*. While other studies performed by Arikan[13], García[14] and Abraham[15] showed that voriconazole had the greatest activity and amphotericin B had the least activity against various aspergillus species.

As mentioned previously, the modal MIC represented the most frequent MIC recorded for each specific medication against each aspergillus species. Modal MIC ±1 twofold dilution represented more than 70% of all values i.e. more than 70% of fungi were inhibited by these three consecutive MICs. By comparing our results with other studies results, we found that modal MICs obtained by other studies are either similar to ours or in the range of modal MIC ±1 twofold dilution. These results are found in studies done by Pfaller[1, 11, 20], Espinel[10, 12], Gheith[19] and Sabatelli[21].

Organisms with acquired resistance mechanisms may be identified as those with MIC higher than the ECV of the MICs for the relevant drug-organism combinations. These ECVs may aid in the evaluation of clinical isolates by identifying those strains with reduced triazole susceptibility due to *cyp51A* mutations and may serve as an early warning of emerging subtle changes in the susceptibility patterns of these organisms. Given these findings, isolates for which the itraconazole MICs are 2 µg/ml or greater may be considered as outliers with an increased probability of harboring an azole resistance mechanism. In our study, Five isolates of *A. fumigatus* (4.3%) showed resistance to itraconazole. Pfaller[11] and Abraham[15] demonstrated *A. fumigatus* and *A. flavus* resistance to itraconazole. In our study, we found cross resistance between itraconazole and posaconazole but not between itraconazole and voriconazole. Although the mechanisms of resistance were not determined in this study, the itraconazole-posaconazole cross-resistance phenotype observed here suggests a G54 substitution in *cyp51A*[17]. It has been postulated, based on molecular modeling studies, that a G54 substitution confers resistance to posaconazole and itraconazole by perturbing the binding of the long side chain in the hydrophobic channel of the enzyme. Substitutions at G54 would be predicted to have less effect on the binding of voriconazole, which lacks a long side chain[22]. Pfaller[20] agreed with this finding while Espinel[10] disagreed and found a cross resistance between itraconazole with both posaconazole and voriconazole. In our study, isolates of *A. fumigatus*, *A. flavus* and *A. terreus* showed in vitro resistance to amphotericin B. Meletiadis[4] and Espinel[12] demonstrated in vitro resistance of *A. terreus* to amphotericin B, Meletiadis[4] and Gheith[19] found *A. flavus* resistance against amphotericin B and Abraham[15] discovered resistance of *A. fumigatus* against amphotericin.

CONCLUSION

Triazoles and amphotericin B have good in vitro activities against most aspergillus species in the following order: posaconazole, voriconazole, itraconazole and amphotericin B. Majority of aspergillus species exhibited in vitro resistance to amphotericin B followed by itraconazole and there was cross resistance between itraconazole and posaconazole, but not between itraconazole and voriconazole.

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