Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.17.011

THE EMERGENCE OF DECREASED ACTIVITY OF AMPHOTERICIN B AND VORICONAZOLE AGAINST CLINICAL ISOLATES OF ASPERGILLUS SPECIES: A SUBTROPICAL REGION OF THE MIDDLE EAST

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Abstract:

Introduction: *Aspergillus* species are a group of opportunistic molds that can cause various types of aspergillosis in individuals, particularly those with weakened immune systems. In recent years, there have been increasing reports of antifungal resistance among clinical isolates of *Aspergillus*.

Objective: This study aims to determine the *in vitro* antifungal susceptibility patterns of *Aspergillus* clinical isolates causing human infections in a subtropical region of the Middle East.

Methods: In this study, the minimum inhibitory/effective concentrations (MICs/MECs) of four antifungal agents (amphotericin B, itraconazole, voriconazole, and caspofungin) were determined using the CLSI M38-A2 broth microdilution method for 60 clinical *Aspergillus* isolates, including *A. flavus* (n=39), *A. niger* (n=9), *A. fumigatus* (n=6), *A. tubingensis* (n=5), and *A. oryzae* (n=1). Statistical analysis was performed using the Chi-square test in SPSS 20 software.

Results: The MEC range for caspofungin was 0.007-4 µg/ml, with a geometric mean (GM) of 0.45 µg/ml. For itraconazole, voriconazole, and amphotericin B, the MIC ranges were 0.031-4 µg/ml, 0.5-4 µg/ml, and 1-4 µg/ml, respectively, with GMs of 1.21 µg/ml, 2.51 µg/ml, and 3.43 µg/ml, respectively.

Conclusion: Among the antifungal agents tested, caspofungin was the most effective against all *Aspergillus* clinical isolates, while amphotericin B and voriconazole were the least effective. *A. flavus*, *A. niger*, and *A. tubingensis* were more susceptible to caspofungin, whereas *A. fumigatus* was more susceptible to itraconazole.

Keywords: Aspergillosis, *Aspergillus*, MIC, Antifungal, Middle East

Introduction:

Over the past decade, the incidence of aspergillosis, an opportunistic fungal infection, has increased (Erjavec, Kluin-Nelemans and Verweij 2009). Invasive aspergillosis (IA) is a significant complication in highly immunosuppressed patients, especially those with prolonged neutropenia, and has been observed among COVID-19 patients as well (Kousha, Tadi and Soubani 2011, Salehi and Ahmadikia 2020). Despite the approval of various antifungal drugs for the treatment of aspergillosis, their efficacy is limited due to challenges associated with diagnosing invasive forms, the severity of underlying diseases, and the limited number of therapeutic options (Ahangarkani, Puts and Nabili 2020, Erjavec, Kluin-Nelemans and Verweij 2009). Antifungal resistance has also been increasingly reported among *Aspergillus* spp., with both primary and secondary resistance observed (Pfaller et al. 2008). While the *A. fumigatus* complex has been reported as the main cause of invasive pulmonary aspergillosis (IPA) in many parts of the world, non-*fumigatus Aspergillus* and rare species are on the rise (Dagenais and Keller 2009, Najafzadeh et al. 2021a, Zanganeh et al. 2018). Additionally, the worrying increase in resistance among community-recovered isolates, especially to azoles, is expected to rise among clinical isolates as well (Abastabar et al. 2016, Pfaller 2012). Despite the development of antifungal drugs, the mortality rate of infections caused by *Aspergillus* species has not decreased (Pagano et al. 2006). Therefore, determining the antifungal susceptibility of *Aspergillus* clinical isolates is necessary to guide treatment protocols and detect antifungal resistance (Pagano et al. 2006). However, there are challenges associated with effective antifungal treatment and their pharmacokinetics/pharmacodynamics, and some fungi may exhibit both *in vitro* and clinical in vivo resistance (2008, Beardsley et al. 2018, Pfaller et al. 2008). Nonetheless, antifungal susceptibility testing can aid in detecting drugs for appropriate antifungal therapy (Nabili et al. 2016). Limited information is available on the antifungal susceptibility patterns of clinical *Aspergillus* species, especially in the Middle East (Hosseinikargar et al. 2021, Kashefi et al. 2021, Nabili et al. 2016). This study was conducted to investigate the *in vitro* antifungal susceptibility of amphotericin B, itraconazole, voriconazole, and caspofungin against clinical *Aspergillus* isolates obtained from patients with aspergillosis in Mashhad, Iran, a subtropical region of the Middle East.

Methods:

This study was approved by the Ethics Committee of Mashhad University of Medical Sciences (MUMS) with ethics committee code of IR.MUMS.MEDICAL.REC.1397.406.

Specimen types, isolation and identification

This study involved three main stages: sampling of *Aspergillus* infections, isolation of the causative agents and their identification, and *in vitro* antifungal susceptibility testing of clinical isolates of *Aspergillus* species. A total of 60 clinical isolates were obtained from patients with aspergillosis (39 proven and 21 probable cases) at two university hospitals (Imam Reza and Ghaem) in Mashhad, Northeastern Iran. *Aspergillus* species included *A. flavus* (n=39), *A. niger* (n=9), *A. fumigatus* (n=6), *A. tubingensis* (n=5), and *A. oryzae* (n=1), which were identified using morphological characteristics, PCR sequencing (using partial calmodulin (CaM) and β-tubulin (BenA) gene sequences and the primers CMD5 and CMD6), and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), as previously described (Hedayati et al. 2019, Najafzadeh et al. 2021b, Zanganeh et al. 2018). During the MALDI-TOF MS protocol (Bruker Daltonik GmbH, Bremen, Germany), the *Aspergillus* isolates were sub-cultured on Sabouraud dextrose agar, and the

resulting colonies were inoculated into brain heart infusion broth centrifuge tubes until sufficient fungal growth was observed. The cultivation tubes were then placed on the bench to sediment filamentous fungi, which were harvested by centrifugation. After adding deionized water and ethanol and vortexing, the cells were centrifuged and air-dried. Pellets were re-suspended in formic acid, vortexed, mixed with acetonitrile, and centrifuged, and the supernatant was placed into a MALDI steel plate and dried. Bruker matrix α-cyano-4-hydroxycinnamic acid was spotted over the samples and allowed to dry. Finally, each spectrum was achieved using positive linear mode at 60 Hz laser frequency in the mass range of 2000-20,000 Da.

In vitro antifungal susceptibility testing

The *in vitro* antifungal susceptibility testing was conducted according to the reference method provided by the Clinical and Laboratory Standards Institute (CLSI) M38-A2 (2008). The following antifungal drugs were tested: itraconazole (Hakim Drugs Fanavaran), voriconazole, amphotericin B, and caspofungin (Sigma-Aldrich, USA). The concentration ranges for antifungal drugs were 0.016–16 μg/mL for itraconazole, voriconazole, and amphotericin B, and 0.008–8 μg/mL for caspofungin. To ensure adequate sporulation, the isolates were cultured on potato dextrose agar (PDA) (Liofilchem, Italy) and incubated at 28°C for 24-48 hours. To prepare inoculum suspensions, the surface of mature colonies was gently scraped with a loop or sterile swab, and the cells were suspended in a 3-mL tube containing sterile saline solution (0.85%) with tween 20 (1%) under biosafety level 2. The tubes were vortexed for 30 seconds, and the cell suspensions were allowed to settle for 10 minutes at room temperature. The inoculum suspension was adjusted via a spectrophotometer at a 530 nm wavelength to optical densities (ODs) that ranged from 80% to 82% transmission. The final concentration of the stock inoculum ranged from 0.5–5 × 10^3 colony-forming units per milliliter (CFU/mL). The 96-well microplates were inoculated with 100 μL of the diluted conidial inoculum suspension and then incubated at 35°C for 48 hours. The susceptibility results were visually read using an inverted mirror. The minimum inhibitory concentration (MIC) values were determined for azoles as the lowest concentration of the antifungal drug that yielded complete growth inhibition. The minimum effective concentration (MEC) for caspofungin was defined as the lowest concentration that led to rounded compact hyphal growth compared to unchanged growth in the control well. Finally, the MIC/MEC range, geometric mean (GM), MIC50/MEC50, and MIC90/MEC90 were calculated. All the tests were performed in duplicates, and the mean values were determined using ANOVA and Multiple Comparisons test with the statistical SPSS package (version 20). P values of <0.05 were considered statistically significant.

Results:

The *Aspergillus* isolates were obtained from various clinical specimens, including 27 ear canal masses, 21 bronchoalveolar lavages, 11 sinonasal and lung tissue biopsies, and 1 nail (Table 1). All clinical specimens showed positive direct examination (with branched septate hyphae) and culture, except specimen MG387183, which had a negative direct examination and grew *A. fumigatus*. Furthermore, all patients exhibited general clinical signs and symptoms associated with different types of aspergillosis.

The results of *in vitro* antifungal susceptibility testing of four antifungal agents against *Aspergillus* species are summarized in Table 2. Overall, amphotericin B was the least active drug, with a range of 1-4 μg/ml, and MIC50, MIC90, and GM values of 4, 4, and 3.43 μg/ml, respectively. Caspofungin was the most active drug, with a range of 0.007–4 μg/ml, and MEC50, MEC90, and GM values of 0.5, 4, and 0.45 μg/ml, respectively. Among the azoles, itraconazole was more effective against *Aspergillus* isolates than voriconazole, with a GM of 1.21 μg/ml compared to 2.51 μg/ml (Table 2).

Statistical Analysis

The GM of the MICs for the different antifungal agents and the differences between mean values were calculated using SPSS software (version 16).

Discussion:

The emergence of antifungal drug resistance is a crucial issue in clinical mycology, making standardized treatment strategies for aspergillosis crucial (Nasrolahiomran 2018). Assessing antifungal susceptibility profiles can provide helpful information about resistance patterns and possible treatment difficulties (Pfaller 2012). Combination antifungal therapy may be a suitable option, particularly for resistance cases (Beardsley et al. 2018). In this study, we determined the MIC/MEC values of four antifungal agents against various *Aspergillus* clinical isolates obtained from Mashhad, Iran, a subtropical region of the Middle East. According to our previous studies on clinical specimens, *Aspergillus* section *Flavi* and section *Nigri* identified more than other species were the most isolated species, followed by *A. fumigatus* (Najafzadeh et al. 2021b, Zanganeh et al. 2018). This could be due to the emergence of non-*fumigatus Aspergillus* species as human pathogens, climatic factors, geographical location, and the use of new laboratory diagnostic methods compared to traditional methods (Zanganeh et al. 2018).

This is the first study to investigate the *in vitro* activity of various antifungal drugs against *Aspergillus* clinical isolates obtained from patients affected by aspergillosis in Mashhad. Antifungals have various mechanisms to eliminate fungal pathogens from hosts. Polyenes such as amphotericin interact with sterols in the cell membrane to form channels. Azoles such as itraconazole and voriconazole inhibit cytochrome P450-dependent enzymes involved in the biosynthesis of ergosterol. Echinocandins such as caspofungin act by non-competitively inhibiting β-1,3-D-glucan synthase (Pfaller 2012).

Our study showed that caspofungin had the highest activity against all *Aspergillus* isolates, which is consistent with previous studies (Badali et al. 2016, Oakley, Moore and Denning 1998, Öz et al. 2016). Faten Al-Wathiqi reported in 2013 that echinocandins had the highest fungistatic activity against *A. flavus*, with the MIC90/MEC90 for amphotericin B, voriconazole, and caspofungin being 3, 0.25, and 0.032 μg/mL, respectively (Al-Wathiqi, Ahmad and Khan 2013). This suggests that echinocandins can be more effective against *Aspergillus* species and can serve as an alternative in case of treatment failure with other recommended drugs. However, we obtained a higher MIC90/MEC90 for amphotericin B, voriconazole, and caspofungin (4 μg/mL) for *A. flavus* in our study. Amphotericin B resistance in *A. flavus* is a major concern because amphotericin B deoxycholate is the first-line treatment of aspergillosis in our hospitals. Karen *et al*. (Oakley, Moore and Denning 1998) also reported an MIC50 and MIC90 for amphotericin B and itraconazole of 4 μg/mL for *A. flavus*. Yasemin *et al*. and Yali *et al*. showed that the susceptibility of caspofungin was higher than that of amphotericin B against non-*fumigatus Aspergillus* isolates (Li et al. 2015, Öz et al. 2016). In their study of 448 isolates of *Aspergillus* spp., Diekema *et al.* showed that caspofungin and voriconazole were more effective than other antifungals (Diekema et al. 2003).

The susceptibility of *A. fumigatus* isolates to voriconazole varied in our study compared to a study outside of Iran (Beardsley et al. 2018) and other studies (Meletiadis et al. 2012, Ziółkowska, Tokarzewski and Nowakiewicz 2014), indicating that susceptibility patterns vary among *A. fumigatus* isolates from different regions. Itraconazole and voriconazole have demonstrated good activity against *A. flavus* isolates in different regions, including India (Shobana et al. 2015), Tunisia (Gheith et al. 2014), Portugal (Araujo, Pina-Vaz and Rodrigues 2007), and Greece (Arabatzis et al. 2011). In one study by Hoseinnejad (Hoseinnejad et al. 2016), *A. flavus* showed high susceptibility to itraconazole (97.7% MIC ≤ 1) and voriconazole (100% MIC ≤ 1), whereas in our study, lower susceptibility patterns were observed for voriconazole and amphotericin B. Previous studies have reported itraconazole and voriconazole resistance (Hsueh et al. 2005, Lionakis et al. 2005, Shivaprakash et al. 2011), which could be a result of excessive use of azole antifungals either for patients or in agriculture. In 2013, Goncalves *et al.* reported higher echinocandin activity against *A. flavus* compared to other drugs (Gonçalves et al. 2013), which is consistent with our findings. However, there have been limited attempts to evaluate the *in vitro* activities of various antifungal drugs against *A. flavus* in Qatar, the Middle East (Salah et al. 2019). The MIC ranges of antifungal drugs were variable for different species of *Aspergillus* in various studies, and *A. fumigatus* showed higher susceptibilities to antifungal drugs than other *Aspergillus* species (Alborzi, Moeini and

Haddadi 2012, Silvanose, Bailey and Di Somma 2011). In other studies from Iran, *A. fumigatus* has been resistant to most azoles (Mohammadi et al. 2018, Nabili et al. 2016), and triazole-resistant *A. fumigatus* has been reported in other countries (Verweij et al. 2015). However, *in vitro* antifungal susceptibility results do not always correlate with *in vivo* activities of the antifungal drugs (Kashefi et al. 2021), so it is necessary to evaluate the therapeutic outcomes for patients with aspergillosis. Triazole resistance for *Aspergillus* spp. can be acquired through extensive exposure of the fungus to azole fungicides used in agriculture (Verweij et al. 2015), and the use of azoles as empirical therapy may contribute to the widespread azole resistance among *A. fumigatus* isolates (Nabili et al. 2016).
Information about the antifungal susceptibility of *A. tubingensis* and *A. oryzae* is limited (Salah et al. 2019, Xu et al. 2021), but resistance to antifungal drugs has not been reported in either species. This study is the first to investigate the *in vitro* activity of *A. tubingensis* and *A. oryzae* clinical isolates in Iran, a subtropical region of the Middle East. In our study, *A. tubingensis* and *A. oryzae* exhibited low MICs for amphotericin B, itraconazole, voriconazole, and caspofungin. Among these antifungals, caspofungin showed the best *in vitro* activity. No reports of antifungal resistance have been found for *A. tubingensis* and *A. oryzae* in clinical settings. Voriconazole had a lower MIC than itraconazole for *A. tubingensis* isolates among the azoles. Colozza *et al.* (Colozza et al. 2012) reported a lower MIC/MEC value (0.5-1 μg/ml) for *A. tubingensis* compared to our study. One of the most significant findings of this study was that caspofungin, an echinocandin, generally exhibited higher *in vitro* activity than triazoles and amphotericin B against all *Aspergillus* species. Our set of *Aspergillus* strains did not exhibit azole resistance, and *A. fumigatus* was more susceptible to all antifungals than other species. Some new antifungal drugs have been investigated, but their significant effects have not yet been observed in some species (Taheri Rizi et al. 2020).
The present study has some limitations, including a relatively small number of *Aspergillus* isolates and species, particularly *A. fumigatus*. Additionally, the limited number of antifungal drugs tested may affect reproducibility. However, due to current limitations in facilities, it was not possible to increase the number of isolates or antifungal drugs tested.

Conclusion:

In summary, our study found no *in vitro* antifungal resistance among the clinical isolates of *Aspergillus* species (*A. flavus*, *A. niger*, *A. fumigatus*, *A. tubingensis*, and *A. oryzae*) collected in Mashhad, Iran, a subtropical region of the Middle East. Caspofungin was the most effective antifungal agent, while amphotericin B and voriconazole were the least effective against *Aspergillus* species. Among the species tested, *A. flavus*, *A. niger*, and *A. tubingensis* were more susceptible to caspofungin, whereas *A. fumigatus* showed more susceptibility to itraconazole. However, the limitations of the study include the relatively small number of isolates and species of *Aspergillus* tested, as well as the limited number of antifungal drugs screened, which could impact reproducibility. Future studies with larger sample sizes and a wider range of antifungal drugs could provide a more accurate understanding of antifungal resistance patterns in this region.

Acknowledgments

We appreciate the staff of Medical Mycology and Parasitology Laboratory in Imam Reza Teaching hospitals affiliated to Mashhad University of Medical Sciences.

Funding

This work was derived from a Master's thesis and financially supported by the Deputy of Research of MUMS (grant No. 960996).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Table 1: The characteristics and details of the clinical specimens, *Aspergillus* species, and GenBank accession numbers.

Clinical specimen (no.)	*Aspergillus* species (no.)	GenBank accession number
Ear canal masses (27)	*A. flavus* (13)	MT350619, MT350611, MT350620, MT350617, MT350618, MT350616, MT350615, MT350614, MT350608, MT350609, MT350612, MT350613 and MT350610
	A. niger (9)	MT350607, MT350599, MT350600, MT350601, MT350606, MT350602, MT350603, MT350604 and MT350605
	A. tubingensis (5)	MT350624, MT350625, MT350623, MT350622 and MT350621
Bronchoalveolar lavages (BAL) (21)	*A. flavus* (15)	MG387172, MG387173, MG387177, MG387178, MG387179, MG387180, MG387181, MG387182, MG387184, MG387185, MG387186, MG387187, MG387188, MG387189, MG387190
	A. fumigatus (5)	MG387176, MG387183, MG490538, MG490576 and MG490577
	A. oryzae (1)*	-
Sinonasal and lung tissue biopsies (11)	*A. flavus* (10)	MZ027901, MZ027902, MG490645, MG490646, MG490647, MG490648, MZ027903, MZ027904, MZ027905 and MZ027907
	A. fumigatus (1)	MZ027900
Nail (1)	*A. flavus* (1)	MG490590

* Identified by using MALDI-TOF MS

Table 2: Distribution of *Aspergillus* species and susceptibility profile of 4 antifungal drugs against them in Mashhad, Northeastern Iran

Aspergillus species	No. (%)	Antifungal drug	MIC / MEC* (µg/mL)			
			Range	MIC_{50}	MIC_{90}	GM
A. flavus	39 (65%)	Itraconazole	0.031-4	2	4	0.751
		Amphotericin B	1-4	4	4	3.063
		Voriconazole	1-4	4	4	3.231
		Caspofungin	0.007-4	0.5	4	0.249
A. niger	9 (15%)	Itraconazole	0.062-4	2	4	1.654
		Amphotericin B	2-4	4	4	3.526
		Voriconazole	1-4	2	4	2.573
		Caspofungin	0.007-4	0.5	4	0.462
A. fumigatus	6 (10%)	Itraconazole	0.031-4	0.5	-	0.444
		Amphotericin B	2-4	4	-	3.174
		Voriconazole	0.5-4	4	-	2.244
		Caspofungin	0.031-4	1	-	0.629
A. tubingensis	5 (8.3%)	Itraconazole	2	-	-	2
		Amphotericin B	4	-	-	4
		Voriconazole	2	-	-	2
		Caspofungin	0.062-4	0.5	-	0.498
A. oryzae	1 (1.7%)	Itraconazole	2	-	-	-
		Amphotericin B	4	-	-	-
		Voriconazole	4	-	-	-
		Caspofungin	0.031	-	-	-

MIC, Minimmum Inhibitory Concentration; GM, geometric mean; MEC, Minimum Effective Concentration (for caspofungin drug); -, means Not determined