In vitro activities of five antifungal agents against 199 clinical and environmental isolates of *Aspergillus flavus*, an opportunistic fungal pathogen

**Summary**  *Aspergillus flavus* is the second leading cause of invasive and non-invasive aspergillosis, as well as the most common cause of fungal sinusitis, cutaneous infections, and endophthalmitis in tropical countries. Since resistance to antifungal agents has been observed in patients, susceptibility testing is helpful in defining the activity spectrum of antifungals and

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* Corresponding author.  
E-mail address: srezaie@sina.tums.ac.ir (S. Rezaie).

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Antifungal drugs
determining the appropriate drug for treatment. A collection of 199 clinical and environmental strains of *Aspergillus flavus* consisted of clinical (*n* = 171) and environmental (*n* = 28) were verified by DNA sequencing of the partial b-tubulin gene. MICs of amphotericin B, itraconazole, voriconazole, posaconazole, and MEC of caspofungin were determined in accordance with the Clinical and Laboratory Standards Institute M38-A2 document. Caspofungin, followed by posaconazole, exhibited the lowest minimum inhibitory concentrations (MIC). All isolates had caspofungin MEC90 (0.063 µg/ml) lower than the epidemiologic cutoff values, and 3.5% of the isolates had amphotericin B MIC higher than the epidemiologic cutoff values. However, their clinical effectiveness in the treatment of *A. flavus* infection remains to be determined.

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### Introduction

The incidence of fungal infections has increased greatly over the past few decades [7]. *Aspergillus* species account for the highest rates of morbidity and mortality among severely immunocompromised patients [27]. Despite a better understanding of the epidemiology of *Aspergillus* infections, important diagnostic limitations persist [23]. However, most of the information available on *Aspergillus* infections has originated from the study of *Aspergillus fumigatus*. *Aspergillus flavus* is the second leading cause of invasive and non-invasive aspergillosis (approximately 15–20%), and a common cause of fungal nasal, sinus, and cutaneous infections [17,18]. Chronic conditions such as chronic cavity pulmonary aspergillosis and sinus fungal balls have rarely been associated with *A. flavus* [17]. The larger size of *A. flavus* spores, in comparison with those of *A. fumigatus* spores, may favor their deposition in the upper respiratory tract [23]. Studies have shown that *A. flavus* spores are particularly prevalent in the air of some tropical countries such as India, Pakistan, Qatar, Iran, Saudi Arabia, and Sudan [1,16,19,21]. The introduction of several new antifungals has expanded the therapeutic armamentarium and resulted in improved outcomes for *Aspergillus* therapies during the last decade [8]. Itraconazole, voriconazole, posaconazole, and echinocandins have been approved as antifungals, particularly for the treatment of infections due to *Aspergillus* spp. [22,25].

Recently, there have been sporadic reports of the emergence of azole resistance in *A. fumigatus*; resistance can develop during chronic azole therapy of patients with aspergillosis, as well as through exposure toazole fungicides used in the environment [5]. Since resistance to antifungal agents has been observed in patients, susceptibility testing can be helpful in defining the activity spectrum of an antifungal and determining the appropriate drug for treatment [9]. Nonetheless, limited information is available on the susceptibility profiles of currently available antifungal agents against *A. flavus* isolated from different sources in Iran; in most studies, only a small number of strains were used and novel agents were not tested. In the current study, we evaluated the *in vitro* antifungal susceptibilities of five antifungal agents, including amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin, against nearly 200 clinical and environmental strains of *A. flavus* isolated from Iran.

### Materials and methods

#### Fungal isolates

One hundred and ninety-nine clinical and environmental *A. flavus* isolates from Tehran (*n* = 184), Isfahan (*n* = 12), and Sari (*n* = 3) were tested. The collection consisted of 171 clinical isolates from a variety of specimens, comprising nose discharge (*n* = 19), sputum (*n* = 8), cutaneous samples (*n* = 40), bronchoalveolar lavage (*n* = 31), sinus (*n* = 61), lung biopsy (*n* = 8), and ear swabs (*n* = 4). In addition, 28 environmental isolates were collected from air (*n* = 11), surface...
Antifungal susceptibility testing

In vitro antifungal susceptibility testing, involving determination of minimum inhibitory concentrations (MIC) and minimum effective concentrations (MEC, for caspofungin only), of five antifungal agents was performed according to recommendations stated in the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document [6]. Amphotericin B (Bristol-Myers-Squibb, Woerden, The Netherlands), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer, New York, NY, USA), posaconazole (Schering-Plough, Kenilworth, NJ, USA), and caspofungin (Merck Sharp & Dohme, Haarlem, The Netherlands) were obtained as quality controls to assess every batch of MIC plates. All tests were performed in duplicate, and the significance of the differences in MICs between clinical and environmental isolates of A. flavus was determined by the Student’s t-test. P values < 0.05 were considered statistically significant.

Results

Table 1 and Fig. 1 summarize the results of the in vitro antifungal susceptibility testing of the antifungal agents. We observed the widest range and the highest MICs with respect to amphotericin B for the clinical (n = 171) and environmental isolates (n = 28), ranging between 0.25—8 and 0.25—4 μg/ml, respectively. Posaconazole and caspofungin had potent activity against all isolates, while the highest MICs were consistently observed for itraconazole, voriconazole, and amphotericin B. Results showed that, in terms of MIC50 and MIC90, all clinical and environmental isolates were highly susceptible to posaconazole and caspofungin, but not voriconazole, itraconazole, and amphotericin B. Eight isolates (4%) had an MIC of ≥ 2 μg/ml for amphotericin B; seven of these were collected from clinical samples. The difference in the MIC90 between the strains did not differ by more than one dilution. Caspofungin, the lone echinocandin tested, exhibited the best activity among the antifungal agents tested, with MECs that were > 4 log2 and > 2 log2 dilutions more active than the MIC50 and MIC90, all clinical and environmental isolates were highly susceptible to posaconazole and caspofungin, but not voriconazole, itraconazole, and amphotericin B. Eight isolates (4%) had an MIC of ≥ 2 μg/ml for amphotericin B; seven of these were collected from clinical samples.

530 nm wavelengths to an OD that ranged from 0.09 to 0.13. The final of the stock inoculum suspensions of each isolate tested ranged from 0.4—3.1 × 104 colony forming units (CFU)/ml, as determined by quantitative colony counts on Sabouraud glucose agar (SGA; Difco). The inoculum suspensions, including mostly non-germinated conidia, were diluted 1:50 in RPMI-1640 medium. Microdilution plates were incubated at 35°C and examined visually for MIC and MEC determinations. The MIC endpoints for amphotericin B, itraconazole, voriconazole, and posaconazole were determined using a reading mirror as the lowest concentration of the drug that prevents any recognizable growth (100% inhibition). MECs for caspofungin were defined microscopically as the lowest concentration of drug that led to the growth of small, rounded, compact hyphal forms, compared to the long, unbranched hyphal clusters observed in the growth control. Epidemiological cutoff values (ECVs) for posaconazole (0.25 μg/ml), caspofungin (0.25 μg/ml), itraconazole and voriconazole (1 μg/ml) and amphotericin B (2 μg/ml) has been described previously by five laboratories as determined by the CLSI M38-A2 microdilution method at 48 h. The strains Paecilomyces variotii (ATCC 22319), Candida parapsilosis (ATCC 22019), and Candida krusei (ATCC 6258) were chosen as quality controls to assess every batch of MIC plates.
significant difference between the susceptibilities of environmental and clinical isolates.

Discussion

*A. flavus* is an airborne fungus frequently isolated from indoor and outdoor environments in Iran [17,4]. *A. flavus*, followed by *A. fumigatus*, are opportunistic pathogens that are frequently associated with sinusitis, keratitis, invasive aspergillosis, and aspergilloma [4,13,17,19,21]. Given the increasing frequency with which *A. fumigatus* and *A. flavus* are recovered from clinical specimens, and the association of these species with resistance to azole agents, *A. flavus* drug resistance patterns have a disproportionate impact on inva-

![Figure 1](image)

**Figure 1** MIC and MEC distribution of the antifungal compounds amphotericin B, itraconazole, voriconazole, posaconazole, and caspofungine against *A. flavus* strains.

*Distribution de la CMI et de la CME des antifongiques : amphoterine B, itraconazole, voriconazole, le posaconazole et caspofungine contre les souches d’*A. flavus*.
sive aspergillosis (IA) treatment strategies and surveillance programs. Therefore, it is essential that the methodology and interpretive criteria of susceptibility testing achieve reproducible results across institutions. New epidemiologic cutoff values (ECVs) were determined for a large set of Aspergillus clinical isolates, which had significant effects on susceptibility rates to antifungal agents [10,13]. Although clinical breakpoints have not been established for most filamentous fungi, ECVs for amphotericin B, itraconazole, voriconazole, posaconazole, and novel azoles have been established for the clinically most important species of Aspergillus. The ECV of amphotericin B for A. flavus was defined as 2 μg/ml, that for itraconazole and voriconazole as 1 μg/ml, that for posaconazole as 0.25 μg/ml, and that for caspofungin as 0.25 μg/ml [10–12].

Triazoles, i.e., itraconazole and voriconazole, are the mainstay of antifungal agents in the management of aspergillosis due to Aspergillus species. The use of either itraconazole or voriconazole as a first-line treatment of invasive aspergillosis due to A. flavus improved the clinical response of patients [28]. These in vitro results related with our in vitro findings; our results revealed that itraconazole and voriconazole MICs for both clinical and environmental isolates were lower than their reported ECVs [10]. Evaluation of the in vitro susceptibility of A. flavus clinical isolates from the USA, South Africa, Latin America, and India revealed that the activity of these antifungals is potent [20,26]. Paul et al. suggested that long-term azole therapy might predispose A. flavus to acquire resistance to voriconazole [24].

Amphotericin B, a polyene agent most frequently used in the treatment of systemic fungal infections, has shown high efficacy in vitro against a broad spectrum of medically important fungi. In the current study, the MIC90 of amphotericin B for all isolates (1 μg/ml) revealed its potent in vitro activity against A. flavus strains. These data are in agreement with the findings of a previous study in which amphotericin B exhibited high activity, but with a lower MIC90 (1 μg/L) and with a range of 0.25 to 2 μg/ml [26]. In contrast, Goncalves et al. reported higher values for amphotericin B (MIC90 ≥ 2 μg/ml; range, 1 to 4 μg/ml) against most species of the Aspergillus section Flavi [14]. In addition, Badiei et al. reported high MIC values for amphotericin B against half of the tested A. flavus isolates [4].

Resistance to echinocandins is very rare, and in our investigation, high MEC values were not defined. Some studies have demonstrated low MECs for caspofungin (< 0.5 μg/ml), which generally exhibited greater in vitro activity than did triazoles and amphotericin B against A. flavus isolates [14,26]. These findings suggest that there are Aspergillus section Flavi strains that are susceptible to caspofungin and anidulafungin. In agreement with the findings of other studies, we observed no significant differences between the in vitro susceptibility data obtained for clinical and environmental isolates [2,15]. However, Araujo et al. reported that clinical isolates had significantly higher MICs than did environmental isolates for amphotericin B, itraconazole, and caspofungin [3].

Until now, little is known about the susceptibility profile of A. flavus in Iran. Therefore, this is the first comprehensive study that provides antifungal susceptibility data for a large collection of environmental and clinically significant A. flavus isolates. Our results suggest that Iranian A. flavus isolates are the most susceptible to posaconazole, followed by caspofungin, voriconazole, itraconazole, and amphotericin B. The in vitro results presented here need to be verified by in vivo studies using appropriate animal models of infections for all Aspergillus section Flavi strains.

Disclosure of interest

The authors declare that they have no competing interest.

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References


