ORIGINAL REPORT

In Vitro Susceptibility of Aflatoxigenic and Non-aflatoxigenic *Aspergillus flavus* Strains to Conventional Antifungal Agents

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Abstract- Presently appearance of resistance to antifungal agents among *Aspergillus* species is dramatically increasing. The objective of this study was to look at the *in vitro* activities of antifungal drugs against Iranian clinical (from nail, bronchoalveolar lavage, paranasal sinus) *isolated A. flavus* strains. The susceptibility of 45 aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* strains were evaluated to six antifungal agents (caspofungin, itraconazole, amphotericin B, ketoconazole, fluconazole, nystatin) using CLSI M38-A2 broth microdilution method. The results indicated that 57.1%, 28.6% of aflatoxigenic and 25.8%, 6.5% of non-aflatoxigenic isolates were susceptible to caspofungin, amphotericin B respectively. All isolates but one aflatoxigenic strain were sensitive to ketoconazole. All 45 strains showed to be resistant to nystatin. Also 64.28%, 92.9% of aflatoxigenic and 64.51%, 100% of non-aflatoxigenic isolates were resistant to fluconazole and itraconazole in ranking order. There was no statistically significant difference between the susceptibilities of aflatoxigenic and non-aflatoxigenic strains of *A. flavus* to tested antifungal agents.

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Keywords: Antifungal agents; *Aspergillus flavus*; Broth microdilution; Susceptibility

Introduction

Like other *Aspergillus* species, the occurrence of *Aspergillus flavus* is worldwide especially in tropical and subtropical regions (1,2). The vital importance of *A. flavus* has been grown in the last years. Although *A. fumigatus* is responsible for the majority (85 to 90%) of the various clinical manifestations (3-5) *A. flavus* is second leading cause of invasive and non-invasive aspergillosis among other *Aspergillus* species (6,7). Also *A. flavus* has an economic importance of aflatoxins, very potent carcinogens (8). The incidence of invasive fungal infections particularly those caused by *Candida* spp, *Cryptococcus neoformans* and *Aspergillus* spp. has increased over the past few decades (9,10).

In spite of recent progress in treatment, death rate remains extremely high. Only amphotericin B and itraconazole were available for treatment of aspergillosis, but alternative new antifungal agents such as anidulafungin, caspofungin, voriconazole have recently approved for the treatment of these infection. Based on the results from different studies the *in vivo* resistance to amphotericin B has been reported among *Aspergillus* species (11,12). So the aim of this study was to investigate the *in vitro* activity of 6 common antifungal agents against Iranian clinical *A. flavus* strains.

Materials and Methods

Here we performed CLSI M_{38} – A2 and CLSI M_{27}-A3 microdilution method in order to assessment susceptibilities of 6 common antifungal agents against *A. flavus* strains and *C. krusei* ATCC 6258 respectively. The test organisms were comprised of 45 *A. flavus* archived strains isolated from various sites and specimens (nail, bronchoalveolar lavage, paranasal sinus) referred to Medical Mycology Laboratory, School of Public Health, Tehran University of Medical Sciences from January 2004 to December 2006. All 45 isolates had previously been identified by standard mycological methods and molecular characteristics (13). The qualitative and quantitative

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determination of the aflatoxins produced by the *A. flavus* strains was performed using HPLC. Out of these 45 isolates, 14 showed to be aflatoxigenic and 31 were non-aflatoxigenic (14). Each isolate was maintained as spore suspension in sterile distilled water and stored at room temperature until they were used.

In the present study, we used standard powders of amphotericin B, fluconazole, nystatin, itraconazole, ketoconazole (all from Sigma, USA), caspofungin (Merck and Whitehouse, USA).

Quality control was insured by testing *C. krusei* ATCC 6258 and reference *A. flavus* ATCC 204304 on each run. MIC ranges for quality control and reference isolates were within established values.

Antifungal stock preparation

Amphotericin B, itraconazole, nystatin were dissolved in dimethyl sulphoxide (Merck, Germany) ketoconazole was dissolved in methanol, fluconazole and caspofungin were dissolved in de-ionized water.

Afterward 100 µl of two fold dilutions of each antifungal agent was dispensed into the wells of a 96-well flat-bottom microtiter plate. All plates stored at -70^{o}C up to they were required.

Inoculum preparation

To prepare inoculum, all isolates cultured on potato dextrose agar at 35^{o}C for seven days. The colonies were covered with normal saline containing Tween 20 and conidia were collected with aid of the tip of sterile transfer pipette. Conidia were then counted by hemocytometer and the cell density adjusted to 0.4-2.5×10^4 CFU/ml. For *Candida krusei*, an inoculum of 0.5-2.5×10^3 CFU/ml was used according to CLSI M_{27}-A3 (15).

Antifungal susceptibility testing

100 µl of each spore suspension was dispensed into microdilution wells and incubated for 48 h at 35^{o}C. Minimum Inhibitory Concentration (MICs) and Minimum Effective Concentration (MEC) were determined. MICs for Amphotericin B, nystatin and itraconazole were defined as the lowest drug concentration that showed 100% inhibition and 50% growth reduction for ketoconazole، compared to the control.

Since assessment of *in vitro* activity of echinocandins against *Aspergillus* spp. complicated, CLSI recommended the MEC defined as the lowest drug concentration at which short, stubby and highly branched hyphae are existed on microscopic examination (16).

The end point was read visually by aid of mirror. Interpretive breakpoints for antifungal agents against *C. krusei* ATCC 6258 and A. *flavus* isolates were according to CLSI M_{27}-A3 and CLSI M_{38}-A2 respectively (15,16).

Statistical analysis

The results were analyzed with the SPSS 19 software, and t-test and McNemar's test were used for comparison of susceptibilities between aflatoxigenic and non-aflatoxigenic strains. *P*-values less than 0.05 were considered statistically significant**.**

Results

MEC and MIC frequency distribution of *Aspergillus flavus* are shown in Table 1. Of all aflatoxigenic 13 (92.9%), 8 (57.1%), 4 (28.6%), 1 (7.1%) were susceptible to ketoconazole (MIC_{90}=8), caspofungin (MEC_{90}=64), amphotericin B (MIC_{90}=4), and itraconazole (MIC_{90}=16) respectively, whereas none of isolates were sensitive to nystatin (MIC_{90}=16) and 9 (64.28%) strains revealed to be resistant to fluconazole (MIC_{90}=64). Among non-aflatoxigenic strains, 31 (100%), 8 (25.8%), 2 (6.5%) were susceptible to ketoconazole (MIC_{90}=8), caspofungin (MEC_{90}=64) and amphotericin B (MIC_{90}=4) in ranking order. Although 31 (100%) were resistant to nystatin (MIC_{90}=16) and itraconazole (MIC_{90}=16), but 20 (64.51%) showed to be resistant to fluconazole (MIC_{90}=64).

A remarkable observation in this study was that when activities of caspofungin and ketoconazole were compared against toxigenic and non-toxigenic *A. flavus* strains, caspofungin tended to show slightly higher effect (MEC=0.25) against aflatoxigenic isolates while ketoconazole revealed higher activity against non-aflatoxigenic isolates (MIC=0.5) (Table 1).

On the other hand, nystatin, fluconazole and itraconazole were not active against all isolates. Although amphotericin B was slightly more active against aflatoxigenic than non-aflatoxigenic strains, but in general amphotericin B had limited activity against *A. flavus* strains.

Caspofungin showed lower activity than ketoconazole, but it was more active than four other antifungal agents. Finally there was no statistically significant difference between susceptibilities of aflatoxigenic and non-aflatoxigenic isolates tested (Table 2).

Table 1. MEC and MIC frequency distribution of aflatoxigenic and non-aflatoxigenic *A. flavus* strains.

Species	**MEC or MIC (µg/ml)**												
(No. of isolates) and drug	**0.0313**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**
Aflatoxigenic *Aspergillus flavus* (14)													
Caspofungin			1	2	3	2		1				1	4
Amphotericin B				1	1	2	1	9					
Itraconazole								1		5	8		
Fluconazole											5	2	7
Nystatin								1	1	2	10		
Ketoconazole					1	1	1	4	6				
Non-aflatoxigenic *Aspergillus flavus* (31)													
Caspofungin	1		2	1	4		2	1		2	2		16
Amphotericin B			1		1		1	28					
Itraconazole										12	19		
Fluconazole									2	5	4	2	18
Nystatin								1	1	1	28		
Ketoconazole					1	2	7	12	8				

MEC: Minimum Effective Concentration (Caspofungin only)

MIC: Minimum Inhibitory Concentration

Table 2. *In Vitro* susceptibility pattern of *A. flavus* strains to 6 antifungal agents.

Species (No. of isolates)	**MEC (µg/ml)**				**MIC (µg/ml)**							
	Caspofungin				**Amphotericin B**				**Itraconazole**			
	Range	**50%**	**90%**	**G.M.**	**Range**	**50%**	**90%**	**G.M**	**Range**	**50%**	**90%**	**G.M.**
Aflatoxigenic *A. flavus* (14)	0.125-64	1	64	0.81	0.25-4	4	4	2.21	2-16	16	16	15.77
Non-aflatoxigenic *A. flavus* (31)	0.0313-64	64	64	1.15	0.125-4	4	4	3.2	8-64	16	16	12.23

Table 2. *In Vitro* susceptibility pattern of *A. flavus* strains to 6 antifungal agents (continue).

Species (No. of isolates)	**MIC (µg/ml)**											
	Fluconazole				**Nystatin**				**Ketoconazole**			
	Range	**50%**	**90%**	**G.M.**	**Range**	**50%**	**90%**	**G.M.**	**Range**	**50%**	**90%**	**G.M.**
Aflatoxigenic *A. flavus* (14)	32-64	64	64	95/1	4-16	16	16	22.63	0.5-16	4	8	4
Non-aflatoxigenic *A. flavus* (31)	8-64	64	64	62.59	4-16	16	16	28.62	0.5-8	4	8	3.5

Table 2 presents the MEC**,** MIC ranges and geometric mean for six tested antifungal agents. Since there are no known breakpoints available for antifungal drugs against mold, only herein MEC**,** MIC_{50} and MIC_{90} are presented.

Discussion

The emergence of resistance of invasive infection caused by resistant fungal species emphasizes the importance of an early diagnosis and installation of a timely antifungal therapy. Difficulties in performing antifungal susceptibility testing for filamentous fungi by clinical laboratories have however, limited its routine performance. Susceptibilities to antifungal agents have more extensively been studied for *A. fumigatus* and *A. terreus* than *A. flavus*. In a survey from Taiwan by Hsueh *et al*. (10) reduced susceptibilities to amphotericin B were found in isolates of *A. flavus* and *A. fumigatus*. Same results were reported by Gomez-Lopez *et al.* (17) for *A. flavus* and *A. terreus* from Spain. In other study, *A. flavus, A. nidulans* and *A. terreus* were found to be significantly less susceptible to amphotericin B than *A. fumigatus* and *A. niger* (18). Warris *et al*. (19) documented high MICs for an *A. fumigatus* strain from invasive aspergillosis against azoles. *In vitro* activities of azole and amphotericin B against *A. fumigatus* and *A. niger* have previously been reported by Manavathu *et al.* (20), they observed none of the drugs showed any significant inter-species in their activity, in contrast to Arikan *et al*. (21) report in which amphotericin B exhibited to be slightly less susceptible against *A. fumigatus* than *A. terreus*.

The activities of voriconazole and caspofungin against 32 isolates of *A. terreus* have investigated by Lass-Flörl *et al*. (22), they found voriconazole and caspofungin in highly active against *A. terreus*.

Azole cross-resistance in *A. fumigatus* reported by Mosquera *et al.* (23), they tested susceptibility of 17 clinical isolates of *A. fumigatus* to itraconazole, posaconazole, rovaconazole and voriconazole and have found that posaconazole was the most active against itraconazole-susceptible isolates. *In vitro* resistance to amphotericin B had already been described for *A. terreus* clinical isolates (24,25). To the best of our knowledge, only one local study has been carried out on antifungal susceptibilities of *Aspergillus* species (26). In addition, no study has been done on the newer antifungal drugs such as caspofungin, which may use in therapy of severe cases to manage. Therefore, investigation about the sensitivity of Iranian *A. flavus* isolates seemed to be necessary.

Caspofungin was the first approved echinocandins for treatment of invasive fungal infections, such as invasive aspergillosis in patients resistant to other therapies (27). Although observations displayed the resistance development to caspofungin, yet it was more active than other drugs in this study.

As well as other reports our data stated the highest MEC to caspofungin (28-30). Our result is compatible with a case report of aspergillosis due to *A. flavus* and *A. fumigatus* in a 49-year-patient that exhibited *in vitro* resistance to caspofungin by E test method (31).

Despite a complete lack of activity of itraconazole against majority of *A. flavus* strains in the present study, ketoconazole was highly potent *in vitro* against the most of our clinical isolates of *A. flavus*. This finding is in contrast to overviews by Moore *et al.* (32) indicated that ketoconazole is inactive against *Aspergillus.*

The activity of ketoconazole against *Aspergillus flavus* has been investigated by several methods, but there are very few data available regarding the use of microdilution method. Results in present study are compatible with previous study by Messer *et al*. (33) and incompatible with Hsueh *et al*. (10) investigation that showed an increased resistance among *Aspergillus* species. Kumar *et al.* (34) demonstrated that 8% of *A. flavus* strains were sensitive to ketoconazole after 48 h of incubation at 35^0C using macrodilution method. Although our observation considered that ketoconazole is an effective agent with increased MIC, which may leads to resistance in future. The emergence of decreased susceptibility to amphotericin B is so important issue, because this drug is suggested as the first drug of choice for the therapy of aspergillosis by some authors (10) and on the other hand in Iran commonly amphotericin B is used as a drug of choice for patients suspected of systemic fungal infections. Our findings as well as the result of other previous surveys from Iran exhibited high MIC of *A. flavus* strains to amphotericin B (26،35). Also several reports are in agreement with our finding and maintained *in vitro* resistance of *Aspergillus flavus* (21,28,36). Few data are available regarding correlations between MICs and outcome of treatment with amphotericin B for infections caused by *Aspergillus* spp. MIC≤ 2 μg/ml have been reported to be effective in clinical cure for invasive aspergillosis (37).

Lionakis *et al.* (38), Baddley *et al*. (39), Meletiadis *et al.* (40), have also found that *A. flavus* strains were susceptible to amphotericin B. Study by Hsueh *et al*. (10) highlighted the reduced susceptibilities of *A. flavus* to amphotericin B with MIC_{90} of 2 μg/ml.

There are few reports about the *in vivo* activity of nystatin against *A. niger* in man. The *Aspergilli* are not particularly sensitive to nystatin by in vitro testing, but experience with treatment of early cutaneous disease has demonstrated its efficacy in vivo (41,42). Our data as

well as Oakley *et al*. findings state that nystatin has no remarkable *in vitro* activity against *A. flavus*. (43). Elevated resistance to azole among *Aspergillus* species is another important point (17,44). A study by Hsueh *et al*. (10) showed 4.2% of *Aspergillus* isolates were resistant to itraconazole (MIC≥ 8 µg/ml). Similar findings reported about itraconazole by other authors (38,39). In the present survey, in contrast to previous study from Iran all *A. flavus* isolates but one aflatoxigenic were resistant to itraconazole (MIC=16) (26). We also observed high MICs to fluconazole; such a high resistance was found in investigations carried out by Moore *et al*. (32), Messer *et al.* (33) and Sabatelli *et al.* (44).

Our data indicated that ketoconazole and caspofungin tended to be more active than the other agents. There was no significant difference between the susceptibilities of aflatoxigenic and non-aflatoxigenic strains of *A. flavus* have been tested. In conclusion, the emergence of decreased susceptibility to amphotericin B, caspofungin and complete lack of sensitivity to itraconazole among *A. flavus* strains have a great concern. Therefore more epidemiological studies are needed to identify the emerging pathogens، effect of other triazoles such as voriconazole, the rate of resistance development to antifungal agents and molecular mechanisms of resistance.

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