Original Article

Determination of antifungal susceptibility patterns among the environmental isolates of *Aspergillus fumigatus* in Iran

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Abstract

Background: In recent years, triazole-resistant environmental isolates of *Aspergillus fumigatus* have emerged in Europe and Asia. Azole resistance has been reported in patients who are treated with long-term azole therapy or exposure of the fungus spores to the azole fungicides used in agriculture. To date, a wide range of mutations in *A. fumigatus* have been described conferring azole-resistance, which commonly involves modifications in the *cyp51A* gene. We investigated antifungal susceptibility pattern of environmental isolates of *A. fumigatus.*

Materials and Methods: In this study, 170 environmental samples collected from indoors surfaces of three hospitals in Iran. It was used β-tubulin gene to confirm the all of *A. fumigatus* isolates, which was identified by conventional methods. Furthermore, the antifungal susceptibility of itraconazole, voriconazole, and posaconazole was investigated using broth microdilution test, according to European Committee on Antimicrobial Susceptibility testing reference method.

Results: From a total of 158 environmental molds fungi obtained from the hospitals, 58 isolates were identified as *A. fumigatus* by amplification of expected size of β-tubulin gene (~500 bp). In this study, *in vitro* antifungal susceptibility testing has shown that there were not high minimum inhibitory concentration values of triazole antifungals in all of the 58 environmental isolates of *A. fumigatus*.

Conclusion: Our findings demonstrated that there was not azole-resistant among environmental isolates of *A. fumigatus.* Medical triazoles compounds have structural similarity with triazole fungicide compounds in agriculture, therefore, resistance development through exposure to triazole fungicide compounds in the environment is important but it sounds there is not a serious health problem in drug resistance in environmental isolates in Iran.

Key Words: Azole resistance, *cyp51A* gene, triazole

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INTRODUCTION

Aspergillus fumigatus spores are present in soil and air which is one of the most common invasive aspergillosis (IA) which is associated with significant morbidity and mortality in the immunocompromised host such as solid organ and hematopoietic stem cell transplant recipients and patients receiving

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chemotherapy.[1,2] Present treatment options of IA include three classes of antifungal agents: Polyenes, echinocandins, and triazoles.[3,4] Azole resistance in clinical *A. fumigatus* isolates is important, and a number of *A. fumigatus* isolates with *in vitro* itraconazole (ITC) and voriconazole (VRC) resistance have been reported over the recent years.[5,6] The azoles inhibit the ergosterol biosynthesis pathway through the inhibition demethylation of sterol 14-a-demethylase (CYP51).[7] Azole resistance is commonly due to mutations in *cyp51A* gene target for azole antifungals.[8,9] This resistance mechanism was recovered both in clinical and environmental samples (soil, compost, seeds, air, and water).[10] Two patterns of azole resistance have been reported; exposure to azoles compounds in the patient and exposure of the fungal spores to azole fungicides used in agriculture.[11,12] Triazole fungicide compounds such as bromuconazole, tebuconazole, epoxiconazole, and difenoconazole as herbicides and plant growth have structural similarity with medical triazoles compounds.[13] If azole-resistant *A. fumigatus* spores distribute in the environment, inhalation of resistant spores with immunocompromised patients will develop azole-resistant aspergillosis.[14] The standard antifungal susceptibility tests have been described for detection of azole resistance *Aspergillus* spp. with the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing (antifungal susceptibility testing [AFST]-EUCAST).[15,16] Unfortunately, *in vitro* triazole testing is not routinely performed in Iran and we lack comprehensive studies on the antifungal susceptibility patterns of environmental *A. fumigatus* isolates. In this study, we determined *in vitro* AFST of *A. fumigatus* environmental isolates against medical triazole, including ITC, VRC, and Posoconazole (POS).

MATERIALS AND METHODS

Air and surface samples from hospitals environment

This study was performed in three central hospitals of Tehran and Isfahan from June 2014 to September 2014. The bone marrow transplant, hematology-oncology wards, Intensive Care Unit (ICU), and Neonatal Intensive Care Unit were selected as sampling sites since they include high-risk patients. A total of 90 air samples and 80 surface samples (doorknobs, bedside tables, and windows) were analyzed.

Morphological identification of the collected molds isolates

Air samples were collected from multiple locations of hospitals by petri-dish trapping technique at a height of 1 m. Samples from indoor surfaces with cotton swab moistened on sabouraud dextrose agar (SDA) and were incubated for a week at 37°C. Afterward, all environmental molds isolates were identified by conventional macroscopic and microscopic morphology.[17]

DNA extraction

DNA was extracted as described by Camps *et al*.[18] In brief, the *A. fumigatus* isolates were cultured on SDA. A loop full of a fresh colony was harvested and added to 200 μl of breaking buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM ethylenediamine tetraacetic acid) with glass beads. After shaken, 200 μl of phenol-chloroform-isoamyl alcohol (25:24:1) saturated with pH 8.0 aqueous buffer was added, and the samples were incubated for 5 min while they were shaken. After centrifugation for 5 min, the upper phase containing the DNA was transferred to a new tube and was kept at –20°C until use.

Polymerase chain reaction amplification

The identity of *A. fumigatus* isolates were confirmed by amplification of the tubulin gene using the primer set Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Btb (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') as described previously.[19,20] Polymerase chain reaction (PCR) amplification was carried out in a final volume of 50 μl. Each reaction contained 0.5 μl of template DNA, 2 pmol of primers (Bt2a and Btb), 10 mM of dNTPs, 10 μl of 5 × HF Buffer, and 2.5U of *Taq* DNA polymerase. An initial denaturation step at 98°C for 30 s followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 61°C for 30 s, and extension at 72°C for 1 min, with a final extension step of 72°C for 10 min. Agarose elctrophoresis is performed to visualize PCR products and viewed under ultraviolet light.[19]

Purified PCR products were directly sequenced in the department of human genetics at Radboudumc in the Netherlands.

In vitro susceptibility testing

A. fumigatus isolates cultured on SDA. *In vitro* susceptibility patterns of ITC, VRC and POS using a broth microdilution test, according to the EUCAST reference method. The minimal inhibitory concentration (MIC) endpoints was defined as the lowest antifungal concentration after 48 h. Stock solutions of the drugs were dissolved in dimethyl sulfoxide in each well and stored at – 70°C until used. Final concentrations of ITC, VRC, and POS range assayed from 0.016 to 16 mg/L and inoculated each well with 100 μl of the 2–5 × 10^5 conidial suspensions. The microdilution trays were sealed and incubated at 35°C. Susceptibility tests were performed at least three times with each strain on different days. In all experiments, the controls were *Paecilomyces*

variotii (ATCC 22319), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258).

Ethical considerations

All samples were collected in accordance with the applicable rules concerning the review of Research Ethics Committees at Tehran University of Medical Sciences and written consents before participating in the study.

Statistical analysis

The Pearson χ^2 test was used to compare proportions and analyze differences in species distribution.

RESULTS

A total of 158 environmental isolates of molds, 58 (36.7%) *A. fumigatus* isolates [Table 1], 93 (58.8%) linked to other molds isolates and 7 (4.4%) unidentified fungal isolates.

Environmental isolates of *A. fumigatus* were identified by amplification of expected size of β-tubulin gene (~500 bp) [Figure 1]. Antifungal susceptibility test results for all 58 environmental isolates of *A. fumigatus* displayed that there were not azole-resistant among these isolates [Figure 2].

The MIC values for resistant isolates of *A. fumigatus* to ITC and VRC are >2 mg/L and for sensitive isolates are ≤1 mg/L and MIC values resistant isolates of *A. fumigatus* to POS is >0.25 mg/L and for sensitive isolates is ≤0.12 mg/L according to EUCAST protocole.[21-23]

DISCUSSION

The emergence of triazole resistance in *A. fumigatus* has been widely reported in recent years among patients with aspergilosis that received long-term azole therapy or in patients through exposure to azole fungicides in agriculture. Azole resistant *A. fumigatus* isolates with mutations in the *cyp51A* gene in Europe (Netherlands, Denmark, Spain, UK, Belgium, Germany, and France) has been attributed to the use of azole fungicides in agriculture.[11,12,24] In this study, environmental *A. fumigatus* isolates with amplification of β-tubulin gene were investigated to find the sensitivity of the isolates to triazole drugs. The findings demonstrated that there was not azole-resistant *A. fumigatus* among environmental

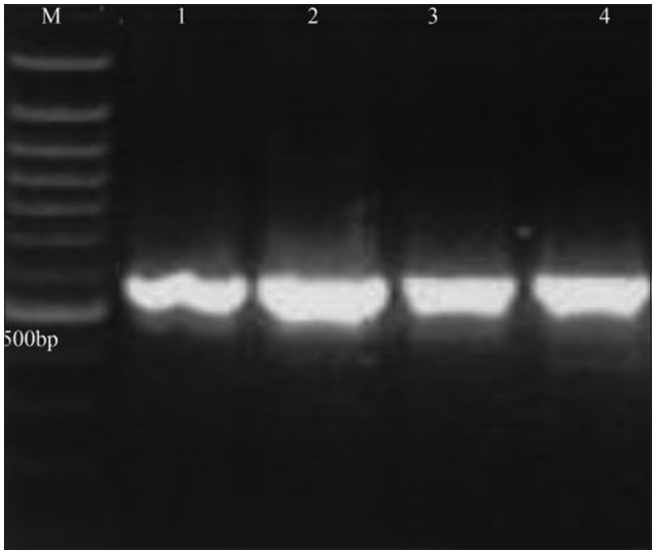

Figure 1: Polymerase chain reaction products of beta-tubulin gene in environmental isolates with ladder 100 bp; 1–4: *Aspergillus fumigatus* (500 bp)

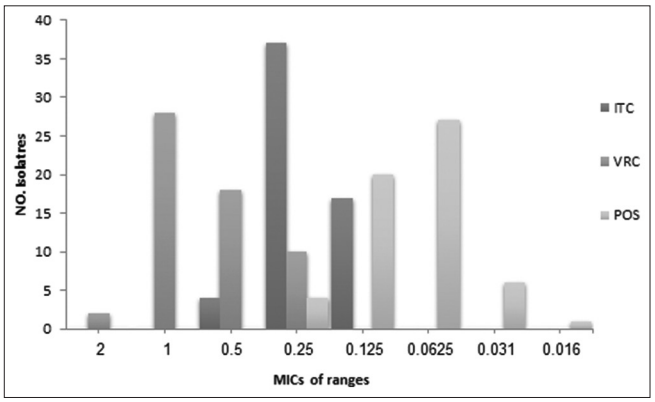

Figure 2: Minimum inhibitory concentration ranges value obtained by testing the susceptibility of *Aspergillus fumigatus* strains to triazole agents

Table 1: Number of *Aspergillus fumigatus* isolates collected from the hospital wards

Molds	Wards				
	Bone marrow transplant	Hematology-oncology	Intensive Care Unit	Neonatal Intensive Care Unit	Total (%)
Aspergillus fumigatus	14	8	23	13	58 (36.7)
Aspergillus niger	2	1	4	1	8 (5.06)
Aspergillus flavus	12	8	6	0	26 (16.45)
Penicillium spp.	5	4	3	3	15 (9.49)
Alternaria spp.	4	6	9	18	37 (23.41)
Cladosporium spp.	0	0	2	5	7 (4.4)
Unknown	2	1	2	2	7 (4.4)
Total	39	28	49	42	158 (100)

isolates, and it should be due to less use of triazole fungicide compounds in agriculture as it shown medical triazoles compounds have structural similarity with triazole fungicide compounds. The authors in a previous study showed 3.5% azole-resistant *A. fumigatus* isolates obtained from patients with long-term use of triazole drugs in Iran.[25] Resistance to the triazole antifungal agents is an emerging public health problem among clinical isolates of *Aspergillus* spp.[26] The main mechanism of azole resistance in *A. fumigatus* is related to the modification of the 14-sterol demethylase target enzyme by several mutations in the *cyp51A* gene.[6,27] Different single nucleotide polymorphisms (SNPs) such as codons G54, L98, and M220 in *cyp51A* gene have been shown in clinical strains that are correlated with triazole resistance but the most frequently reported resistance mechanism is a 34-bp tandem repeat with a substitution at codon 98 (TR34/L98H).[6,28] Badali *et al*. indicated 4% of the *A. fumigatus* isolates from the surrounding environment in Sari (Northern region) and Tehran (Central region) had the mutation in *cyp51A* gene. It seems that hospital in Sari (northern region) is surrounded by an agricultural area for rice and fruits where the usage of fungicides is generally higher than in Tehran.[29] It has been indicated the relationship between environmental azole consumption in agricultural products and development of cross-resistance to medical triazoles, and this may suggest an alternative route of resistance development through exposure to triazole fungicide compounds in the environment.[10] Long-term use of azole drugs exposure in patients and application of azole compounds in the environment might be lead the emergence of azole resistance *A. fumigatus* isolates.[30,31] Similarly, there is reported that out of five triazole fungicides four of them showed significantly higher MICs in the Indian triazole resistant *A. fumigatus* isolates from environmental, and clinical samples compare those of wild-type strains.[32] *A. fumigatus* isolates harboring TR34/L98H mutation were cultured from soil and compost and shown genetic relatedness to clinical resistant isolates.[12] *In vitro* antifungal testing of *A. fumigatus* isolates is not routinely performed in clinical laboratories, therefore true prevalence of resistance in clinical and environmental *A. fumigatus* isolates is not known properly and need to be investigated more. Although it has been reported resistant *A. fumigatus* environmental isolates to antifungal azoles in Europe and India but our findings revealed no resistance among environmental isolates. This result is consistent with the USA environmental reports where none of the *A. fumigatus* isolates obtained from natural soil was found to be azole-resistant.[10]

CONCLUSION

The findings of this study demonstrated that there was not azole-resistant among environmental isolates of *A. fumigatus* obtained from these central hospitals in Iran. However, the widespread use of triazole fungicides compounds in agriculture products and long-term azole therapy might have contributed to environmental azole resistant *A. fumigatus* in the future.

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Conflicts of interest

There are no conflicts of interest.

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