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Study of Azole - Resistant and *Cyp51a* Gene in *Aspergillus Fumigatus*

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Abstract

AIM: The main goal of the present study was to find azole-resistant and molecular analysis of *cyp51A* gene in *Aspergillus fumigatus*.

MATERIALS AND METHODS: Fifty-eight *A. fumigatus* strains including environmental, clinical and reference isolates were assessed in this investigation. Azole susceptibility testing for itraconazole and voriconazole was carried out for *A. fumigatus* isolates. PCR was performed based on *cyp51A* gene sequence for all isolates.

RESULTS: Susceptibility testing verified the minimum inhibitory concentrations (MICs) for itraconazole (0.125 to 2 µg/ml) and voriconazole (0.125 to 4 µg/ml). Nine (15.5%) *A. fumigatus* isolates were resistant to voriconazole with MIC 4 µg/ml. A 1500 bp DNA fragment was amplified using *cyp51A* gene for all tested Aspergillus isolates. The sequences of the fragments showed 99% identity with *A. fumigatus cyp51A* gene in the GenBank. No point mutation was found at *cyp51A* gene codons.

CONCLUSION: In the current study, we detected the voriconazile resistant in *A. fumigatus* isolates. Susceptibility tests should be considered in patients who infected by *A. fumigatus*.

Introduction

Aspergillus fumigatus is one of the most common airborne fungal pathogen which causes invasive aspergillosis. The spores are capable of spreading to air and inhaled and eventually cause infection in a susceptible host. *A. fumigatus* infections occur in excessive morbidity and mortality in immunocompromised hosts [1] [2]. The azoles are antifungal drugs that inhibit the ergosterol biosynthesis pathway by the inhibition of 14α-demethylase. Azoles, for example, itraconazole, voriconazole, and posaconazole are among the advised first-line agents in the management and prophylaxis of aspergillosis [3].

The appearance of azoles resistance in yeast species has encouraged researchers of the mechanisms associated with this resistance. Several genes encoding 14α-demethylase (*ERG11*/*cyp51*) have been identified for fluconazole-resistant of the clinical isolates of *Candida albicans* [4] [5].

The mechanisms for azole drug resistance in *A. fumigatus* appear to be extremely dissimilar from that in *Candida* spp. There are two various but related *Cyp51* proteins which are encoded by *cyp51A* and *cyp51B* genes [6] [7].

Two models of resistance to azoles have discovered in *A. fumigatus*. First, the *A. fumigatus* could turn into resistant during exposure to azoles in the patient. This model was detected in chronic pulmonary aspergillosis and aspergilloma cases in the United Kingdom [8]. In this pattern, eighteen dissimilar

amino acid substitutions were distinguished in the *cyp51A* gene [8]. Second, the *A. fumigatus* can turn into resistance in the environment during the exposure to azole fungicides which are applied in agriculture and material conservation.

This model was suggested in the Netherlands [9]. In this pattern, a replacement at codon 98 of the *cyp51A* gene combined with a tandem repeat of 34 bp in the promoter (TR/L98H), was detected in 94% of the resistant strains [10].

Treatment of invasive aspergillosis is mainly limited to therapy by the polyene agent amphotericin B, and triazoles such as itraconazole, voriconazole and echinocandin caspofungin. Amphotericin B is very toxic and can consequence in nephrotoxicity [11], while triazoles are fungistatic and subject to development of resistance [12].

Here, we explain the analysis of *cyp51A* gene *A. fumigatus* which is responsible for the phenotype of *A. fumigatus* azole-resistance.

Material and Methods

A total of 58 *A. fumigatus* strains were used in the study including 45 environmental, 9 clinical and 4 reference isolates. The following strains were used as a reference: PTCC 5009, IBRC-M 30033, IBRC-M 30040, IBRC-M 30048. Environmental strains were recovered from soil or air.

One hundred ml of yeast extract peptone dextrose (YEPD) medium in Erlenmeyer flasks was inoculated with 1 ml of thick spore suspension and incubated at 37°C for 72 h 200 rpm under agitation to obtain mycelium growth. The mycelia were harvested, washed with 0.5 M EDTA and sterile dH2O and using liquid nitrogen and a pestle and mortar ground into a fine powder. The DNA was extracted with vivantis is GF-1 plant DNA extraction kit, Malaysia.

The primer sets, P450-A1 (5'-ATGGTGCCGATGCTATGG-3') and P450-A2 (5'-CTGTC-TCACTTGGATGTG-3') was used to amplify an ~ 1500 bp DNA fragment of the full coding sequences of *cyp51A* gene. PCR reactions were carried out with a volume of 30 µl, comprised of 3 µl 10X reaction buffer, 200 µM each dNTP, 2.2 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase (CinnaGen, Tehran, Iran), 30 ng template DNA and 50 pmol of each primer.

Initial denaturation for 5 min at 94°C was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58˚C for 1 min and extension at 68˚C for 2 min. The PCR product (5 µl) was electrophoresed on 1% agarose gel in TAE buffer and stained with ethidium bromide.

Spore suspension for each isolate was set up in sterile normal saline and adjusted at a concentration of 10^6 spores/ml, consequent to 68 to 82% transmittance at 530 nm [13]. Broth microdilution susceptibility test was carried out as explained in clinical and laboratory standards institute (CLSI) method with modifications. The antifungal drugs used were itraconazole (Sigma-Aldrich, Germany) and voriconazole (Sigma-Aldrich, Germany).

Stock solutions were prepared in 100% dimethyl sulfoxide (CinnaGene, Karaj, Iran), then diluted in RPMI 1640 medium and dispensed into 96-well microdilution trays. The final concentration of voriconazole and itraconazole in the wells ranged from 0.015 to 8.0 µg/ml. The stock spore suspension (10^6 spores/ml) was diluted to a final concentration of 5×10^4 CFU/ml and dispensed into the microdilution wells. The inoculated microdilution trays were kept at 35°C and read after 48 h. The minimum inhibitory concentration (MIC) for voriconazole and itraconazole was described as the lowest concentration that created prominent growth inhibition.

Some *cyp51A* gene amplicons were submitted for direct sequencing (Bioneer Corporation, Daejeon, South Korea). The obtained sequences were searched for in the National Center for *Biotechnology Information* (NCBI) database (http://www.ncbi.nih.gov/).

The sequences showed 99% similarity with *A. fumigatus cyp51A* gene sequences deposited in NCBI database. The computer software package MEGA5 (http://www.megasoftware.net) was employed for sequences alignment.

Results

A total of 58 samples were tested including 45 environmental, 9 clinical and 4 reference isolates, according to the European committee for antibiotic susceptibility testing (Eucast) methodology suggestion breakpoints for *A. fumigatus,* itraconazole and voriconazole [14]. For itraconazole and voriconazole, <2 µg/ml (susceptible), 2 µg/ml (intermediate) and >2 µg/ml (resistant). Susceptibility testing verified the MICs for itraconazole (0.125 to 2 µg/ml) and voriconazole (0.125 to 4 µg/ml) (Table 1).

Nine (15.5%) *A. fumigatus* isolates were resistant to voriconazole with MIC 4 µg/ml. Twelve (20.7%) isolates exhibited intermediate susceptibility to itraconazole with MIC 2 µg/ml. The PCR amplification of *cyp51A* gene with set primers P450-A1 and P450-A2 produced a 1500 bp fragment for all tested *Aspergillus* isolates (Fig. 1).

Table 1: Results of susceptibility testing voriconazole and itraconazole for *A. fumigatus* isolates

Isolate number	Source	MIC (µg/ml) for voriconazole	MIC (µg/ml) for itraconazole
3/M	Environmental	2	1
4/M	Environmental	2	0.5
5/M	Environmental	2	1
6/M	Environmental	0.5	0.5
8/M	Environmental	2	1
11/M	Environmental	1	1
12/M	Environmental	1	2
13/M	Environmental	4	0.5
14/M	Environmental	0.5	1
15/M	Environmental	0.5	2
16/M	Environmental	2	2
17/M	Environmental	4	0.5
19/M	Environmental	2	0.5
20/M	Environmental	1	1
21/M	Environmental	2	1
22/M	Environmental	2	1
23/M	Environmental	0.125	0.5
24/M	Environmental	4	0.5
26/M	Environmental	2	1
27/M	Environmental	0.5	2
28/M	Environmental	2	1
29/M	Environmental	1	1
30/M	Environmental	1	2
31/M	Environmental	2	1
32/M	Environmental	1	1
34/M	Environmental	2	2
36/M	Environmental	2	1
37/M	Environmental	4	0.5
38/M	Environmental	2	1
44/M	Environmental	2	0.5
45/M	Environmental	4	0.5
46/M	Environmental	2	1
6/1	Environmental	2	1
6/2	Environmental	2	2
6/3	Environmental	4	2
7/1	Environmental	2	1
7/2	Environmental	2	0.5
7/3	Environmental	2	0.5
7/4	Environmental	2	0.5
7/5	Environmental	4	0.5
7/6	Environmental	2	1
9/1	Environmental	2	0.5
10/1	Environmental	2	2
11/1	Environmental	2	1
18/1	Environmental	2	0.5
1/B	Clinical	2	1
2/B	Clinical	2	0.5
3/B	Clinical	2	1
4/B	Clinical	2	0.5
70/B	Clinical	1	2
72/B	Clinical	1	1
98j/B	Clinical	1	1
1010/B	Clinical	0.25	2
MEH	Clinical	0.5	2
PTCC 5009	Reference	1	0.125
IBRC-M 30033	Reference	2	0.5
IBRC-M 30040	Reference	4	0.5
IBRC-M 30048	Reference	4	0.5

Several *cyp51A* gene amplicons including nine voriconazole resistant isolates were sent for direct sequencing. The sequences were searched in the NCBI database.

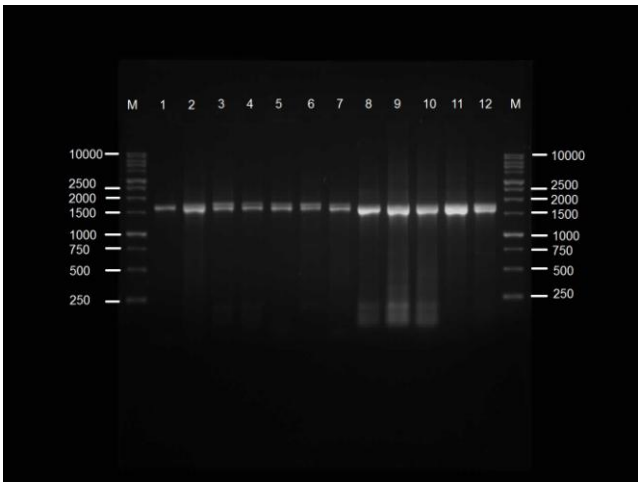

Figure 1: Agarose gel electrophoresis of cyp51A gene products (1500 bp) of Aspergillus fumigatus isolates (lanes 1, 2, reference strains; lanes 3-7, clinical isolates; lanes 8-12, environmental isolates). Lane M, 1 kb ladder; lane 1, 30040; lane 2, 30048; lane 3, 2/B; lane 4, 4/B; lane 5, 72/B; lane 6, 1010/B; lane 7, Meh; lane 8, 7/4, lane 9, 10/1; lane 10, 18/1; lane 11, 19/M; lane 12, 22/M

The sequences showed 99% identity with *A. fumigatus* sequences deposited in the NCBI database. The computer software MEGA5 was applied for sequences alignment. Although nine isolates were found to be resistant to voriconazole, we did not find any isolate with a point mutation in their *cyp51A* gene codons.

Discussion

Rapid recognition of *Aspergillus* infections with a precise assessment of possible drug resistance is vital for successful management of patients with invasive infection. Clinically, triazole resistance rates are different between 2 and 6.6% among samples [15][16]. In 1997 the first case of *A. fumigatus* itraconazole resistant was reported [17]. Research from the Netherlands reported that 3 of 114 *A. fumigatus* isolates were resistant to itraconazole although all had MICs for all of them were low for voriconazole [18].

Some researchers reported that a point mutation that substituted the glycine at codon 54 of CYP51A was created itraconazole resistant in *A. fumigatus* [19][20]. Mechanisms of resistance to azole have been explained in other fungi, particularly *C. albicans*. Triazoles are the foundation of therapy with voriconazole the first-choice treatment for invasive aspergillosis [21]. Nevertheless, azole resistance reports have appeared, not only after long time azole usage [8] but also after a short time using and in azole-naive cases [10].

Resistance to azole in *A. fumigatus* has been connected with mutations in *cyp*51*A* gene that is a target for antifungal azoles. The occurrence of *cyp*51*A* mutations has been related to a failure in treatment.

Management of invasive aspergillosis is complicated because of negative cultures is frequent, and many laboratories do not carry out susceptibility assessments on isolates of *Aspergillus*. Therefore, the frequency of azole-resistant to *Aspergillus* likely underdiagnosed, with a possible risk of unsuitable treatment.

In our study MICs for itraconazole between 0.125 to 2 mg/ litre and voriconazole between 0.125 to 4 mg/ litre were obtained. Nine of the isolates including 7 environmental isolates and 2 standard isolates were resistant to voriconazole with MIC 4mg/ litre. None of the resistance strains showed point mutation in *cyp51A* gene.

We consider that the results of our investigation and the rising reports on azole-resistance propose that susceptibility examinations of *A. fumigatus* isolates must be regularly carried out.

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