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Molecular detection of *CYP51* genes in *Aspergillus fumigatus*

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Abstract

Triazoles are the mainstay for aspergillosis treatment. However, azole resistance is an emerging problem reported worldwide in *Aspergillus* infection mainly caused by *A.fumigatus*. Increase azole resistance in *A.fumigatus* has reported with treatment failure and become a significant challenge in effective management of aspergillosis. In the current study , *A.fumigatus* isolates were resistant to itraconazole and ketonazole with percentage of 36% and 44% respectively. The conventional Polymerase Chain Reaction (PCR) was used to confirm antifungal resistance by detecting the presence of *CYP51A* and *CYP51B* genes. The results of using this technique showed that *A. fumigatus* isolates were positive to these genes with 100% percentage. Our study revealed that the CYP51 genes are predominant in azole resistance isolates. Furthermore, PCR was proven to be highly effective method for identifying these genes.

Keywords: Aspergillus fumigatus, CYP51A, CYP51B, azole resistance.

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الكشف الجزيئي لجينات*CYP51* للفطر *Asprgillus fumigatus*

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الخلاصة:

تشكل مركبات الترايزول العلاج الاساسي لعلاج داء الرشاشيات.رغم ذلك تبرز هنالك مشكلة مقاومة الفطر للعلاج بمركبات الازول المرتبطة اساسا مع اصابة الانسان بالفطر *A.fumigatus* و المسجلة في اماكن عديدة حول العالم. ازدياد مقاومة الفطر للعلاج بهذه المركبات ارتبط بصورة وثيقة مع حالات فشل العلاج و اصبح يشكل تحدي كبير تجاه التدابير الفعالة ضد مرض الرشاشيات. .في الدراسة الحالية كانت عزلات الفطر *A.fumigatus* مقاومة لكل من المضادات الفطرية itraconazole و ketonazole بنسبة ٣٦% و ٤٤% على التوالي. استخدم تفاعل البلمرة التسلسلي لتاكيد وجود المقاومة الفطرية من خلال الكشف عن وجود جينات CYP51 بنوعيها *CYP51A* و *CYP51B* . كانت نتائج الدراسة الجزيئية هي وجود كلا الجينين المدروسين بنسبة 100% . تثبت الدراسة الحالية بان هذه الجينات مهمة في عزلات الفطر المقاومة لمركبات الازول كما ان تقنية PCR هي طريقة فعالة في الكشف عن هذه الجينات.

Introduction:

Azoles are the mainstay of oral therapy for aspergillosis . Azole resistance in *Aspergillus* has been reported infrequently, resistance to Voriconazole (VOR) was described in laboratory mutants (1) and clinical isolates (2). *A. fumigatus* becomes increasingly resistant to azole, cross resistance to multiple azoles is frequently observed, with the majority of resistant isolates being resistant to more than one azole (8 ; 4). Although increase in the number of different azole compounds available for drug therapy (itraconazole was first licenced in 1997, voriconazole in 2002, posaconazole in 2006 and isavuconazole in 2015) seems not to have halted the increase in resistance (5). Cross resistance to multiple azoles is frequently observed, with the majority of resistant isolates being resistant to more than one azole (6). Mortality rates in case series of patients with culture-positive azole-resistant invasive aspergillosis varied between 50% and 100% (7 ; 8). Azole resistant *Aspergillus* has been isolated in azole naïve patients, in azole exposed patients and in the environment (9). Resistance is mainly due to a point mutation in the 14 asterol demethylase

(CYP51A) gene, the target of azoles (10).In *A. fumigatus*, target site alterations are the most commonly reported resistance mechanism, with over 30 CYP51A mutations identified (9). The aim of the present study was to detect *CYP51* genes in *A. fumigatus* isolates which were collected from clinical sources.

Materials and Methods:

Antifungal susceptibility: *A. fumigatus* isolates which used in the study were collected from immunocompromised patients suffering from pulmonary problems in Al-Hussain Teaching Hospital in Thi-Qar province, South of Iraq, during the period from January to October 2016. The clinical specimens (n=25) of *A. fumigatus* isolates were tested for antifungal resistance. These fungal isolates were grown at 37°C on SDA (Sabouraud dextrose agar).All *A.fumigatus* isolates were tested for susceptibility to Itraconazole and Ketonazole by Minimum Inibitory Concentration (MIC) assays, agar well diffusion method were used to evaluate the antifungal resistance (11) .

PCR amplification: PCR technique was used for the amplification of target genes (*CYP51A* and *CYP51B*), the same procedure for each template and set of primers was used. Each reaction mixture was contained 20 µl PCR buffer (10 mM Tris-HCl [pH 9.0], 1.5 mM MgCl2 , 30 mM KCl2, 1.0% Triton X-100), 1 U of Taq DNA polymerase (Promega, USA), 250µM of deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP Boehringer Mannheim GmbH, Mannheim, Germany), 5 pmol of each primer, and 2 µl of sample DNA. Ultrapure sterile molecular water was added to a final volume of 20 µl. Oligonucleotides primers were used for amplifications in PCR are indicated in Table(1). Amplification was performed in a thermal cycler (Bio-Rad, USA) for one cycle of 5 min at 94 °C, 30 sec at 60 °C, and 2 min at 72 °C, and then for 40 cycles of 30 sec at 94 °C, 45 sec at 58 °C, and 2 min at 72 °C, followed by one final cycle similar to the previous one but with 1 min at 72 °C for all genes in the study. The PCR products were analyzed by electrophoresis on 1.5% agarose gels at 80 V. for 1 h in

1X TBE, depending on their sizes and were visualized by transillumination after staining with ethidium bromide (12).

Table 1: Oligonucleotide primers used in this work

Primer	Sequence (5'-3')	Product size (bp)	Reference
CYP51A	*F: TGCAGAGAAAAGTATGGCGA R: CGCATTGACATCCTTGAGC	120	(12)
CYP51B	F: AGCAGAAGAAGTTCGTCAAATAC R: TCGAAGACGCCCTTGTG	130	(12)

***F: Forward, R: Reverse**

Results and Discussion: Sensitive test for all isolates of *A. fumigatus* was done against two antifungal (Itraconazole and Ketonazole) by cell diffusion methods. Azole resistance was presented in 9/25 (36 %) positive culture patients for itraconazole, and ketonazole 11/25 (44%) with a MIC range between 0.01 and 1 mg/ml. This results were in agreement with the results obtained by (13) who showed elevated MICs for itraconazole (4 mg/Liter), also the result is close to the results of (14) who showed elevated MICs for itraconazole (>16 mg/L) with tested for susceptibility by broth microdilution also the results of (15 ; 16). Initially, the features that favor the occurrence of drug-resistant strains, such as a short biological cycle, abundant sporulation, and dispersal of spores over long distances (17), are typically observed in *A. fumigatus*. The application of azole fungicides to target phytopathogenic molds in agriculture, including flower production fields, results in azole exposure to ubiquitously present *A. fumigatus* strains in the environment, leading to azole-resistant *A. fumigatus* strains (18 ; 19). These fungicides exhibit chemical similarity to the medical triazoles and have been suggested as possible candidates to induce resistance in *Aspergillus* (20)

Molecular mechanisms of *A. fumigatus* azole resistance such as *CYP51A* and *CYP51B* which were found in those isolates with 100% percentage (Fig:1).

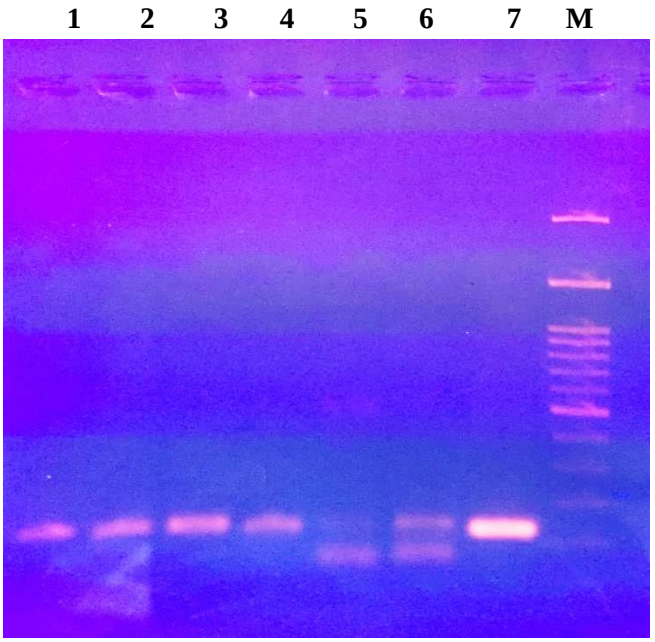

Fig (1): Detection of *A. fumigatus* and genes on agarose gel (2%) stained with ethidium bromide , lanes:1,2,3,4 *CYP51A* (120bp) ; lanes: 5,6,7 *CYP51B* (130bp).

The mechanisms behind drug resistance are more numerous and varied than previously thought. The clinical advances that have been made possible through the use of azole drugs might be threatened by the emergence of azole resistance in *A. fumigatus* (21), (20). There are two CYP51 genes in *A. fumigatus*, cyp51A and CYP51B, both producing similar enzymes. Increased expression of CYP51B as identified by (12) could result in slightly elevated MIC values as seen in these samples. Generally it is thought that CYP51A is responsible for the most important 14-α sterol demethylase activity required for cellular growth (the conversion of lanosterol to ergosterol is a critical step in the genesis and maintenance of the fungal cell membrane). The azole resistance mechanism includes two steps, resistance to down regulation of *CYP51A* and *CYP51B* in the first step and mutation of the azole target as the second step, single drug tolerance is related to the first step, and high

azole tolerance and resistance to multiple azoles would develop in the second step (22). CYP51A is responsible for the most important 14-α sterol demethylase activity required for cellular growth (the conversion of lanosterol to ergosterol is a critical step in the genesis and maintenance of the fungal cell membrane). It has been postulated (23) that CYP51B may be involved in a compensatory function, or become expressed under certain conditions. Overexpression of *CYP51A* gen in *A. fumigatus* has been observed in resistant clinical isolates from a patient who failed azole therapy (24). A set of *CYP51A* alterations leading to azole-resistant phenotype of *A. fumigatus* isolates has been reported worldwide from both clinical and environmental sources. These alterations in the *CYP51A* gene consist of tandem repeats in the promoter region of the gene, combined with mutations in the gene itself. (25), this mechanism is the most frequently identified resistance mechanism in the environmental and clinical *A. fumigatus* strains (20).

Conclusions: Resistance of *A. fumigatus* which isolated from clinical and environmental against triazole is growing as a public health concern that has become a worldwide problem. This work recommend for more further studies bases on molecular azole resistance mechanisms in the resistance isolates, especially the sequencing techniques for *CYP51A* and *CYP51B* genes to appear the mutations that responsible of resistance in *A. fumigatus* ,and to adjust therapeutic options where resistant isolates are present.

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