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Molecular Characterization Of MDR_3 And MDR_4 Genes Of *Aspergillus Fumigates* Isolated From Lung Disease Patients

Prof. Dr. Amal J. Kadhim
Assist. Prof. Dr. Yass K. Abbass
Assist Prof. Khwam R. Hussein

Abstract:

Background:Triazoles are the mainstay of treatment for aspergillosis. 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.

Aim: The aim of this study is to investigate the triazole-resistant of *A. fumigatus* and underlying MDR pump genes in viable clinical isolates which obtained from patients suffering pulmonary infections in Thi-Qar province.

Methods: The conventional Polymerase Chain Reaction (PCR) was used to confirm antifungal resistance by detecting the presence of MDR pump genes (*MDR3* and *MDR4*).

Results: The results of using this technique showed that *A. fumigatus*isolates were positive to MDR pump genes with 90% and 96% for *MDR3* and *MDR4* genes, respectively.

Conclusions: Our study revealed that the MDR pump genes are predominant in azole resistance isolates. Furthermore, PCR was proven to be highly effective method for identifying these genes.

Key words: ***Aspergillusfumigatus,*** **MDR genes, azole resistance**

Introduction: Azole resistance is an emerging problem in *Aspergillus*infections caused by *Aspergillusfumigatus*, with increasing reports of azole treatment failure. Although azole resistance can develop during azole therapy, exposure to azole compounds used in the environment appears to contribute to a greater extent (Van Der Linden *et al.*, 2013;White *etal.*, 2017). Surveillance studies increasingly report geographical spread of azole resistance in environmental and clinical *A. fumigatus* isolates, including in Europe, Asia, Middle East, Africa and most recently North and South America (Vermeulen *etal.*,

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2013;Chowdhary *et al.*, 2014;Wiederhold *et al.*, 2016). *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 (Snelders *et al.*, 2008;Van Der Linden *et al.*, 2015). More recently, and perhaps more disturbing, cases of azole-resistant invasive aspergillosis started to be reported in patients without prior azole exposure (Chowdhary *et al.*, 2014). Generally, two routes of resistance development are distinguished: through long-term azole patient therapy and via the application of azole compounds in the environment (Snelders *et al.*, 2008; Camps *et al.*, 2012). Resistance mutations are also believed to develop in the environment when the fungus is exposed to azole compounds that exhibit anti-*Aspergillus* activity (8).

Fungi have to beat intracellular toxin accumulation in order to successfully colonize human hosts (8) . This is achieved by efflux pumps, of which there are two main categories: ATP-binding cassette (ABC) proteins, primary transporters that take advantage of ATP hydrolysis, and major facilitator superfamily (MFS) pumps, secondary transporters that use the proton-motive force across the plasma membrane(10). In *A. fumigatus*, at least 49 ABC family transporters and 278 MFS genes have been described, which is more than four-times then number identified in yeasts like *Saccharomyces cerevisiae*(11). Multidrug resistance (MDR) pumps, which are involved in the active extrusion of antimicrobial molecules. The aim of this study is to investigate thetriazole-resistant of *A. fumigatus* and underlying MDR pump genes in viable clinical isolates which obtained from patients suffering pulmonary infections in Thi-Qar province.

Materials and Methods:

Antifungal susceptibility:*A. fumigatus* isolates which used in the study were collected from immunocompromisedpatients suffering from pulmonary problems in Al-Hussain Teaching Hospital in Thi-Qar province,south of Iraqand during the period from January to June 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). Antifungal susceptibility tests wereperformedusing disk diffusion method (Adeniyi*et al*, 1996).

PCR amplification:PCR techniquewasused for the amplification of target gene (MDR_3 and MDR_4), the same procedure for each template and set of primers was used. Each reaction mixturewas contained 20 µl PCR buffer (10 mMTris-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 dTTPBoehringer 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

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min at 94°C, 30 sec at 58°C, and 2 min at 72°C, and then for 30 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).The program SPSS 11.5 was used for data elaboration and analysis. A chi-squared test for samples was used for statistical analysis. Data were compared at a significance level of 0.05.

Table1: Oligonucleotide primers used in this work

Primer	Sequence (5'-3')	Product sizebp	Reference
AfuMDR$_3$	CTATATCGGGTCAGTCCTGG GACCCAGAACAAGGAATCCGAC	131	(12)
AfuMDR$_4$	TTCTACGATCCCGATTCAGG GACGACACTAAGCCATATGC	158	(12)

Results and Discussion:

Sensitive test for all isolates of *A.fumigatus* was done against tow antifungal (Itraconazole and Ketonazole) by cell diffusion methods. Azole resistance was presented in 9/25 (36 %) culture-positive patients for itraconazole, andketonazole 11/25 (44%)with a MIC range between 0.01 and 1 mg/ml.This results 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 (Gomez-Lopez *et al.*, 2014;van Paassen *et al.*, 2016). 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 (Dunne *et al.*, 2017;Alvarez-Moreno *et al.*, 2017). These fungicides exhibit chemical similarity to the medical triazoles and have been suggested as possible candidates to induce resistance in *Aspergillus*(20).

Some of the molecular mechanisms of *A. fumigatus* azole resistance such as *AfuMDR3*and *AfuMDR4*geneswhich were recorded a percentage of90%, 96%, respectively(Fig. 1).This agreement with(Slaven *et al.*, 2002;Nascimento *et al.*,

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2003). 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*(Verweij *et al.*, 2009;Chowdhary *et al.*, 2013).

Some of the azole fungicides are of the triazole class and have a similar molecule structure to the medical triazoles(23).It was hypothesized that *A. fumigatus* develops resistance due to use of azole fungicides to combat phytopathogens for crop protection becauseof the molecule similarity of fungicides with medical triazoles,the latter also lose activity. In addition to abundant asexual reproduction,parasexual and sexual reproduction probably also occursin the environment, thereby increasing the fungus's ability to undergogenetic recombination and thus overcome cellular stresscaused by fungicide exposure.Azole fungicides are used globally, thus creating an environment where azole-resistant *A. fumigatus*can thrive. Reduced uptake of the drug into the fungal cell has also been mooted as a mechanism of resistance in *A. fumigatus*(24).*AfuMDR*$_3$ and *AfuMDR*$_4$were identified to be connected with triazole resistance in a study where resistant *A. fumigatus* mutants showed either constitutive high-level expression of both transporters or induction of expression when exposed toitraconazole(ITC). Two out of 23 mutants seemed to be ITC resistant due to overexpression of these genes, although evidence of a direct relationship between them and an ITC resistant phenotype is lacking. *AfuMDR*$_3$ and *AfuMDR*$_4$is a member of the ATP-binding cassette(ABC) proteins family (12). Additionally, *AfuMDR4* has been shown to be induced with VRC in complex *A. fumigatus* biofilm populations and that this contributes to azole resistance (25).Efflux pump overexpression related to azole resistance in *A. fumigatus*, although these have been generated in the laboratory (26).

In addition, fungal pathogens can successfully infect and colonize the host by overcoming the intercellular toxin accumulation by the activation of efflux pumps, in particular adenosine triphosphate–binding cassette transporters and transporters of the major facilitator superfamily. Overexpression of adenosine triphosphate–binding cassette and major facilitator superfamily transporters have been described in azole-susceptible and azole-resistant *A. fumigatus* isolates, with or without azole treatmentamphotericin B exposure (27).

1 2 3 4 5 6 M 1 2 3 4 M

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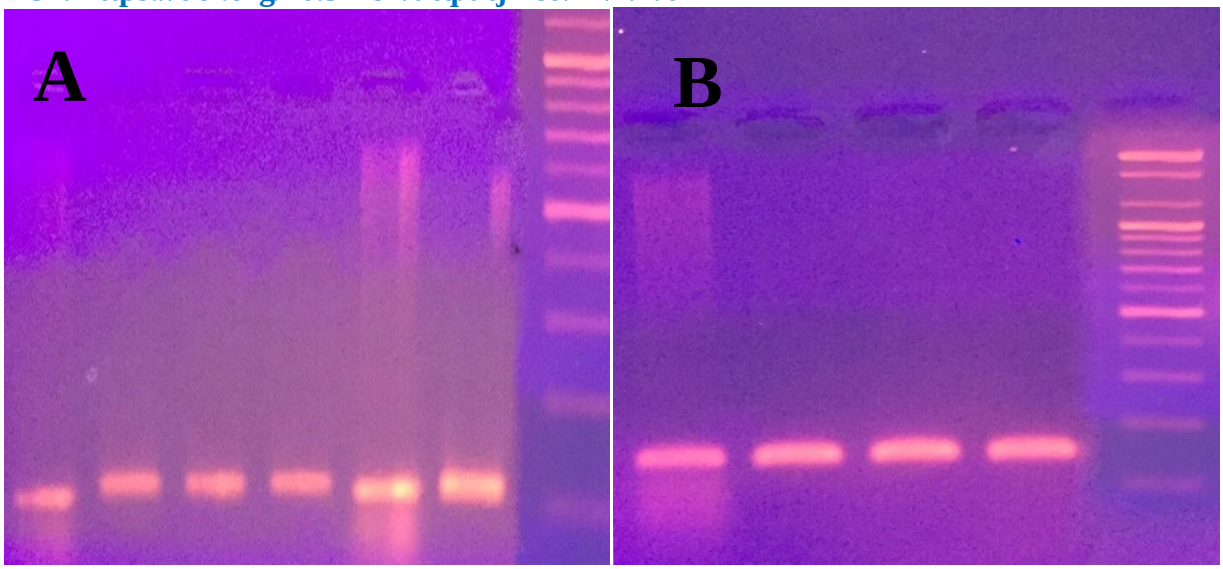

Figure 1: Detection of *A. fumigatus*pump genes asshown in gel-red stained agarose gel of PCR products (A: *AfuMDR3*:131bp; B: *AfuMDR4*:158bp).

Conclusions: Clinical and environmental triazole resistance in *A. fumigatus*is a growing public health concern that has become a worldwide problem. This work recommend forfurther studies to be investigated the azole resistance in other areasto understand the prevalence of resistance especially to understand the relationship between the overexpression of pump efflux and azole resistance in *A. fumigatus,*and to adjust therapeutic options where resistant isolates are present. In addition, the development of molecular methods to detect azole resistance in culture-negative infections should be done.

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التوصيف الجزيئي لجينات *MDR3* و *MDR4* للفطر *A.fumigatus* المرافق للامراض التنفسية

امال جميل كاظم ا.د ياس خضير عباس[1] ا.م.د خوام ريسان حسين[2]

[1] قسم علوم الحياة. كلية التربية للعلوم الصرفة .جامعة ذي قار

[2] المعهد التقني في الناصرية.الجامعة التتقنية الجنوبية

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 itraconazoleو ketonazole بنسبة ٣٦% و ٤٤% على التوالي. استخدم تفاعل البلمرة التسلسلي لتاكيد وجود المقاومة الفطرية من خلال الكشف عن وجود جيناتالضخ MDR بنوعيها *MDR*3 و *MDR*4. كانت نتائج الدراسة الجزيئية هي وجود كلا الجينين المدروسين بنسب ٩٠% و ٩٦% على التوالي. تثبت الدراسة الحالية بان جينات الضخ هي جينات مهمة في عزلات الفطر المقاومة لمركبات الازول كما ان تقنية PCR هي طريقة فعالة في الكشف عن هذه الجينات.