Molecular analysis of *afIR* gene in *Aspergillus flavus* isolated from Iran

¹Infectious and Tropical Diseases Research Center, Health Research Institute,
Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

²Department of Medical Mycology, Medical School, Ahvaz Jundishapur University
of Medical Sciences, Ahvaz, Iran

mjzarrin@yahoo.co.uk

Aspergillus flavus produces the most potent carcinogens, aflatoxins, when it contaminates agricultural crops. *afIR* gene regulates aflatoxin-related genes and it has been identified in four species of *A. flavus*, *A. parasiticus*, *A. sojae* and *A. oryzae*. Contamination of agricultural commodities with aflatoxin is a grave risk to humans and animals' health. Aflatoxin related genes are clustered in a 75 kb region of genome in *A. flavus*. Investigations obviously demonstrated that aflatoxin biosynthesis needs the *afIR* gene product and an entirely functional aflatoxin biosynthetic cluster.

The purpose of the current study was to investigate the presence of the *afIR* gene in *A. flavus*.

Material and methods. Forty-two *A. flavus* isolates including 10 references, 25 clinical and 7 environmental isolates were analyzed in this study. The isolates were identified by morphology. To characterize morphologically, the conidial arrangement, phialides, vesicles and conidiophores were observed microscopically. Using PCR, the *afIR* gene was amplified with primers *afIR1* and *afIR2*. PCR were carried out to amplify an 800 bp DNA fragment of *afIR* gene. Some amplicons were sequenced. The sequences were searched in NCBI database and analyzed with MEGA5 software.

Results and discussion. Out of 42 *A. flavus* isolates, an 800 bp band was amplified for 35 isolates. No band was observed for seven isolates including 4 clinical and 3 environmental isolates. Data analysis demonstrated that 100% of reference strains and 84% of clinical strains produced the expected fragment while it was only 57.14% for environmental isolates. The sequences had 100% identity with *A. flavus afIR* gene which was deposited in the NCBI database.

Conclusion. In conclusion, molecular analysis of the *afIR* gene showed that this gene was not amplified from some strains of *A. flavus*; therefore, perhaps it lacks the gene or it is greatly abnormal. Additional re-

searches are needed to verify whether the strains with lack of *afIR* gene have a loss of function in production of aflatoxin or other mechanisms of regulation exist.

Keywords: *Aspergillus flavus*, aflatoxin, *afIR* gene.

Introduction. Aflatoxins (AFs) are a highly toxic and carcinogenic metabolites produced by *Aspergillus* species especially *Aspergillus flavus* and *Aspergillus parasiticus* on food and agricultural commodities. *A. flavus* is a pathogenic fungus that infects humans, animals and plants. *A. flavus* creates a variety of diseases based on the condition of immune system in hosts [1]. This fungus produces the most potent carcinogens, aflatoxins, when it contaminates agricultural crops.

Among the various types of aflatoxins, the most significant are aflatoxin B1, B2, G1, and G2.

A. flavus generates only AFB1 and AFB2, whereas the other aflatoxigenic species also produce AFG1 and AFG2 [2-4]. Contamination of different agricultural crops such as maize, peanuts, cottonseed and chillies by aflatoxins can happen as a result of infection by this fungus.

The aflatoxin B1, B2, G1 and G2 in group 1 were classified as human carcinogens by International Agency for Research on Cancer (IARC) [5,6]. Their research showed that the aflatoxin related genes were clustered in a 75 kb region of genome. *afIR* gene regulates aflatoxin-related genes and it was identified in four species of *A. flavus*, *A. parasiticus*, *A. sojae* and *A. oryzae* [7].

The purpose of this research was to clarify the presence of the *afIR* gene in *A. flavus* based on sources of the strains in Iran. Investigations clearly demonstrated that aflatoxin biosynthesis needs the *afIR* gene product and a completely functional aflatoxin biosynthetic cluster. The current study investigated the presence of the *afIR* gene in *A. flavus*.

Material and methods

Organism. Forty-two *A. flavus* isolates including 10 references, 25 clinical and 7 environmental isolates were analyzed in this study. Reference strains were obtained from Pasture Institute of Iran. The clinical isolates were kindly provided by Dr. Hossein Zarrinfar, Dr. Sadegh Khodavaisy and Dr. Parvin Dehghan. The environmental isolates were recovered from soil and air. For obtaining a pure culture, the isolates were subcultured 3 times on Sabouraud dextrose agar (Merck, Germany). The isolates were identified by morphology. To characterize morphologically, the conidial arrangement, phialades, vesicles and conidiophores were observed microscopically.

DNA extraction. DNA was extracted according to Lee et al [8] method with modification. One ml of spore suspension from each isolate was transferred into an erlenmeyer flask containing 50 ml YEPD medium. The flasks were maintained at 200 rpm under agitation on a rotary shaker at 37°C for 48 hours to obtain mycelia growth. The mycelia were harvested with filtering, washed with 0.5 M ADTA and sterile dH₂O and freeze-dried at -70°C for extraction of DNA. The frozen mycelia were then ground into a fine powder using a pestle and mortar.

Approximately 100 mg of mycelium powder was transferred into a 1.5 mL sterile tube and added 400 µl of lysis buffer (100 mM Tris-HCl, pH 8.0, 30 mM EDTA, pH 8.0, SDS 5% w/v).

The microtubes were incubated at 100°C for 20 minutes, and 150 µl of 3 M acetate potassium was added to each tube.

The tubes were kept in 100°C for 20 minutes, and were treated with 150 µl of 3 M acetate potassium. The suspension was incubated at -20° C for 10 minutes, and was centrifuged in 12000 rpm at 4 ° C for 10 minutes. The supernatant was transferred to a new Eppendorf tube and 250 µl Phenol-Chloroform- Isoamyl Alcohol (25:24:1, v/v) was added and it briefly was vortexed and centrifuged in 4° at 12000 rpm for 10 minutes. After transferring of the supernatant to a new microtube, an equal volume of iced-cold 2- propanol was added and kept in -20° C for 10 minutes and centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded and the pellets were washed with 300 µl of 70% ethanol. The ethanol was removed and tube was dried. The DNA was dissolved in 50 µl distilled water.

PCR amplification. The PCR amplification was performed based on Somashekar et al (9) method with modification. The *afIR* gene was amplified with primers *afIR1* (5'-AAC CGC ATC CAC AAT CTC AT-3') and *afIR2* (5'-GGT GCA GTT CGC TCA GAA CA-3'). Amplification was performed in 50 µL reaction containing 2.2 µL MgCl₂, 200 µM each dNTP (dATP, dCTP, dGTP and dTTP), 2.5 U of *Taq* DNA

polymerase (CinnaGen, Iran), 30 ng template DNA, 50 pmol of each primer. The PCR condition was as follow: an initial denaturation step at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 50°C for 90 s, 72°C for 100 s. PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide in TAE buffer.

Sequencing. Several *afIR* gene products were sequenced (Bioneer Corporation, Daejeon, South Korea). The obtained sequences were searched in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>). The sequences had 100% similarity with *A. flavus afIR* gene deposited in the NCBI database. The MEGA5 software (<http://www.megasoftware.net>) was employed for alignment of sequences.

Results. Out of 42 *A. flavus* isolates, an 800 bp band was amplified for 35 isolates using primers *afIR1* and *afIR2* (Figure 1). No band was observed for seven isolates including 4 clinical and 3 environmental isolates. Data analysis showed that 100% of reference strains and 84% of clinical strains produced the target band while it was only 57.14% for environmental isolates. The PCR products of 3 strains were sequenced and analyzed with MEGA5 software. The sequences had 100% identity with *A. flavus afIR* gene which was deposited in the NCBI database.

Figure1: PCR product of *afIR* gene with primers *afIR1* and *afIR2*

Notes: Line M, 100 bp ladder; Line 1, S12; Line 2, PFCC 106-139; Line 3, PFCC 124; Line 4, PFCC 170; Line 5, PFCC 173; Line 6, Z1; Line 7, Z2; Line 8, Z6; Line 9, Z9; Line 10, Z10; Line 11, M24; Line 12, M25; Line 13, Negative control without DNA.

Discussion. Aflatoxins are a group of secondary metabolites generated commonly by *A. flavus* and *A. parasiticus*. Significant importance is related with the presence of aflatoxins in food and feed due to their carcinogenic, mutagenic and teratogenic effects. Aflatoxins were distinguished in many agricultural products and processed products. In human, acute aflatoxicosis lead to death and chronic aflatoxicosis lead to suppression of immune system and cancer [10].

The *afIR* gene, regulates aflatoxin clustered genes [7, 11]. Absence or atypical *afIR* gene would be an acceptable indicator that aflatoxin can not be produced by a strain [7]. Previous investigations have revealed that the AFLR protein be able to bind to the

promoter of each aflatoxin synthesis gene and turn on the gene expression [12, 13]. Furthermore, the *aflR* gene has a function of autoregulation [14].

In this study, the *aflR* gene was generated successfully for 35 out of the 42 tested strains of *A. flavus*. Therefore, no *aflR* PCR products were detected in 7 isolates. These results suggested that the *aflR* gene is absent or considerably dissimilar in some *A. flavus* isolates. Nucleotide analysis demonstrated 100% similarity for the *aflR* gene sequence of 3 *Aspergillus* strains. The *aflR* gene in 23 out of 34 *A. flavus* strains was amplified in Taiwan [7]. van den Broek et al in Switzerland showed that 93% of *A. flavus* and 27% of *A. oryzae* isolates lack either or both *aflR* and *omt-1* genes [15].

Conclusion. In conclusion, molecular analysis of the *aflR* gene showed that this gene was not amplified from some strains of *A. flavus* including

4 clinical and 3 environmental isolates; therefore, perhaps they lack the gene or they are greatly abnormal. Additional researches are needed to verify whether the strains with lack of *aflR* gene have a loss of function in production of aflatoxin or other mechanisms of regulation exist. The current study demonstrated the PCR amplification of *aflR* gene has specificity for detection and it can be used for recognition of aflatoxigenic fungi in food samples.

Prospects for further researches are detection of *aflR* gene from foods.

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МОЛЕКУЛЯРНИЙ АНАЛІЗ ГЕНА *afIR*, ВИДІЛЕНИЙ З ІЗОЛЯТІВ *ASPERGILLUS FLAVUS***В Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran****Ерфанінеджад Мар'ям, Заррін Маджид**

Резюме. При зараженні сільськогосподарських культур *Aspergillus flavus* продукуються сильні канцерогени - афлатоксини. Ураження сільськогосподарської продукції афлатоксинами становить серйозну небезпеку для здоров'я людини і тварин. Ген *afIR*, ідентифікований у чотирьох видів *A. flavus*, *A. parasiticus*, *A. sojae* та *A. Oryzae*, є регулятором інших генів, пов'язаних з афлатоксинами. Кластер генів, пов'язаних з афлатоксином, представлений в регіоні в 75 т.п.н. генома *A. flavus*. Дослідження показали, що для біосинтезу афлатоксина необхідний продукт гена *afIR*, і повністю функціональний кластер біосинтезу афлатоксина. Метою цього дослідження було вивчення наявності гена *afIR* у *A. flavus*. В даному дослідженні були проаналізовані 42 ізоляти *A. flavus*, включаючи 10 еталонних, 25 клінічних і 7 екологічних ізолятів. Ізоляти ідентифікували на підставі морфологічного аналізу. Морфологічну оцінку конідіального розташування, філіад, бульбашок і конідієносців виконували шляхом мікроскопії. Проводили ампліфікацію гена *afIR* за допомогою ПЛР з використанням праймерів *afIR1* та *afIR2*. Шляхом ПЛР ампліфікували фрагмент ДНК гена *afIR* в 800 п.н. Окремі амплікони секвенували. Послідовності були знайдені в базі даних NCBI, і проаналізовані за допомогою програмного забезпечення MEGA5. З 42 ізолятів *A. flavus* послідовність в 800 п.н. була ампліфікована для 35 ізолятів. Смуги не спостерігалися для семи ізолятів, включаючи 4 клінічних і 3 екологічних ізолята. Аналіз даних показав, що 100% еталонних штамів і 84% клінічних штамів продукували очікуваний фрагмент, тоді як для екологічних ізолятів цей показник становив лише 57,14%. Послідовності мали 100% ідентичність з геном *afIR A. flavus*, депонованих в базі даних NCBI. Молекулярний аналіз гена *afIR* показав, що цей ген не виявлено у деяких штамів *A. flavus*, так як, ймовірно, він або відсутній, або істотно аномальний. Додаткові дослідження необхідні для перевірки можливості втрати штамми з відсутністю гена *afIR* функції синтезу афлатоксинів або існування інших регуляторних механізмів.

Ключові слова: *Aspergillus flavus*, афлатоксин, ген *afIR*.

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МОЛЕКУЛЯРНЫЙ АНАЛИЗ ГЕНА *afIR*, ВЫДЕЛЕННЫЙ ИЗ ИЗОЛЯТОВ***ASPERGILLUS FLAVUS* В Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran****Эрфанінеджад Марьям, Заррин Маджид**

Резюме. При заражении сельскохозяйственных культур *Aspergillus flavus* продуцируются сильные канцерогены - афлатоксини. Поражение сельскохозяйственной продукции афлатоксинами представляет серьезную опасность для здоровья человека и животных. Ген *afIR*, идентифицированный у четырех видов *A. flavus*, *A. parasiticus*, *A. sojae* и *A. Oryzae*, является регулятором других генов, связанных с афлатоксинами. Кластер генов, связанных с афлатоксином, представлен в регионе в 75 т.п.н. генома *A. flavus*. Исследования показали, что для биосинтеза афлатоксина необходим продукт гена *afIR* и полностью функциональный кластер биосинтеза афлатоксина. Целью настоящего исследования было изучение наличия гена *afIR* у *A. flavus*. В данном исследовании были проанализированы 42 изолята *A. flavus*, включая 10 эталонных, 25 клинических и 7 экологических изолятов. Изоляты идентифицировали на основании морфологического анализа. Морфологическую оценку конидиального расположения, филиад, пузырьков и конидиеносцев выполняли путем микроскопии. Проводили амплификацию гена *afIR* с помощью ПЦР с использованием праймеров *afIR1* и *afIR2*. Путем ПЦР амплифицировали фрагмент ДНК гена *afIR* в 800 п.н. Отдельные ампликоны секвенировали. Последовательности были найдены в базе данных NCBI и проанализированы с помощью программного обеспечения MEGA5. Из 42 изолятов *A. flavus* последовательность в 800 п.н. была амплифицирована для 35 изолятов. Полосы не наблюдались для семи изолятов, включая 4 клинических и 3 экологических изолята. Анализ данных показал, что 100% эталонных штаммов и 84% клинических штаммов продуцировали ожидаемый фрагмент, тогда как для экологических изолятов этот показатель составлял только 57,14%. Последовательности имели 100% идентичность с геном *afIR A. flavus*, депонированным в базе данных NCBI. Молекулярный анализ гена *afIR* показал, что этот ген не был обнаружен у некоторых штаммов *A. flavus*, так как, вероятно, он либо отсутствует, либо существенно аномален. Дополнительные исследования необходимы для проверки возможности потери штаммами с отсутствием гена *afIR* функции синтеза афлатоксина или существования других регуляторных механизмов.

Ключевые слова: *Aspergillus flavus*, афлатоксин, ген *afIR*.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of coauthors of the article.

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