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

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Aspergillus flavus produces the most potent carcinogens, aflatoxins, when it contaminates agricultural crops. *aflR* 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 *aflR* gene product and an entirely functional aflatoxin biosynthetic cluster.

The purpose of the current study was to investigate the presence of the *aflR* 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 *aflR* gene was amplified with primers *aflR*1 and *aflR*2. PCR were carried out to amplify an 800 bp DNA fragment of *aflR* 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* *aflR* gene which was deposited in the NCBI database.

**Conclusion.** In conclusion, molecular analysis of the *aflR* 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 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.

**Keywords:** Aspergillus flavus, aflatoxin, *aflR* 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. *aflR* 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 *aflR* gene in *A. flavus* based on sources of the strains in Iran. Investigations clearly demonstrated that aflatoxin biosynthesis needs the *aflR* gene product and a completely functional aflatoxin biosynthetic cluster. The current study investigated the presence of the *aflR* 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 Pasteur Institute of Iran. The clinical isolates were kindly provided by Dr. Hossein Zarrinfar, Dr. Sadegh Khodavaisi 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, philiades, 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 YEIP 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 0.5M 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°C 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 aflR gene was amplified with primers aflR1 (5’-AAC CGC ATC AAC AAT CTC AT-3’) and aflR2 (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: 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 aflR 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 aflR 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 aflR1 and aflR2 (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 aflR gene which was deposited in the NCBI database.

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 aflR gene, regulates aflatoxin clustered genes [7, 11]. Absence or atypical aflR 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

Figure 1: PCR product of aflR gene with primers aflR1 and aflR2

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.
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 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 omr 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 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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Резюме. При заражении сельскохозяйственных культур Aspergillus flavus продуцируются сильные канцерогены - афлатоксины. Поражение сельскохозяйственной продукции афлатоксинами представляет серьезную опасность для здоровья человека и животных. Ген aflR, 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. гена A. flavus. Доследование показало, что для биосинтеза афлатоксина необходим продукт гена aflR, и повсюду функциональный кластер биосинтеза афлатоксина. Метою этого доследования было вивчення наявності гена aflR у A. flavus. В даному доследжені були проаналізовані 42 ізоляти A. flavus, включаючи 10 етанальних, 25 клінічних і 7 екологічних ізолятів. Ізоляти ідентифікували на підставі морфологічного аналізу. Морфологічну оцінку конидиального розташування, філіад, бульбашок і конідієносцев виконували шляхом микроскопії. Проводили амплифікацію гена aflR за допомогою ПЛР з використанням праймерів aflR1 та aflR2. Шляхом ПЛР амплифікували фрагмент ДНК гена aflR в 800 п.п. Окремі амплікони секвенували. Послідовності були знайдені в базі даних NCBI, і проаналізовані за допомогою програмного забезпечення MEGAS. З 42 ізолятів A. flavus послідовність в 800 п.п. була амплифікована для 35 ізолятів. Сумагу не спостерігалася для семи ізолятів, включаючи 4 клінічних і 3 екологічних ізолятів. Молекулярний аналіз гена aflR, депонований в базі даних NCBI, показав, що цей ген не виявлено у деяких штамів A. flavus, так як, имовірно, він або відсутній, або істотно аномальний. Додаткові доследження необхідні для перевірки можливості втрати штамами сільськогосподарської продукції афлатоксинами або існування інших регуляційних механізмів.

Ключові слова: Aspergillus flavus, афлатоксин, ген aflR.