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Research Article

Using Aflatoxin B1 Regulatory Gene Sequencing to Study Similarity and Difference between *Aspergillus flavus* Strains, Isolated from Patients with Aspergllosis

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Abstract: Aflatoxins are carcinogenic fungal secondary metabolites produced by *Aspergillus flavus* and other closely related species, The work in this study presented the first detailed molecular analyses of AFLAR2 gene in the strains of *Aspergillus flavus* isolated from patient with aspergillosis infections In this study, rapid assessment of 14 isolates of *A. flavus* was accomplished using an indigenously designed primer pair for the Aflatoxin regulatory gene aflR2 in Polymerase Chain Reaction (PCR). Specificity was assayed in pure culture systems using DNA extracted from 14 different *A. flavus* straines as PCR template. Positive amplification was achieved only with DNA from 10 *A. flavus* that produce Aflatoxin B1.

Keywords: AFLAR2, aspergillosis, *Aspergillus flavus*

INTRODUCTION

Aspergillus flavus act as opportunistic pathogen able under certain conditions, responsible for most cases of aspergillosis, the most common systemic filamentous fungal infection worldwide (Fridkin and Jarvis, 1996). The incidence of aspergillosis has increased during the last two decades (Rogers, 1995; Denning, 1998; Wald *et al*., 1997). Infection of *A. flavus* occurs by inhaling conidia which may colonize airways prior to invasion to cause diseases in human lung and liver through production of aflatoxins (Cleveland *et al*., 2009; Hedayati *et al*., 2007; Sigler and Verweij, 2003).

Many biological and health effects of aflatoxins on body organs and body systems were recorded like the effect of aflatoxins on mitochondrial DNA when the reactive aflatoxin-8, 9-epoxide preferentially binds to mitochondrial DNA (WHO, 2008). Effect of aflatoxins on protein synthesis when binds and interferes with enzymes and substrates that are needed in the initiation, transcription and translation processes involved in protein synthesis (Clifford and Rees, 1967).

The aflatoxin regulatory gene (aflR) encodes a positive regulator, Aflr, which activates aflatoxin pathway gene transcription (Chang *et al*., 2002). The aflR gene, a regulatory gene for aflatoxin biosynthesis, encodes predicted Aflr protein contains a GAL4-type zinc-finger motif that transcriptionally activates most of the structural pathway genes, such as ver-1 and nor-1 (Trail *et al*., 1995; Ehrlich *et al*., 1999). Although, The aflR gene which regulates these clustered genes, has been identified in *A. flavus*, *A. parasiticus*, *A. sojae* and *A. oryzae* (Zong Lee *et al*., 2006). Also have effect on immune system and causing suppression of cell-mediated immune responses, reduction of phagocytosis and depression of complement and interferon production (Samson, 2001; Rigo *et al*., 2002).

The aims of the study were to study Similarity and difference between *Aspergillus flavus* strains, isolated from patients with aspergllosis based on aflatoxin B1 regulatory gene sequencing.

METHODOLOGY

***A. flavus* isolates:** A total of 14 *A. flavus* isolates isolated from patients suffering from Aspergillosis, (as clinically identified by a physician), from Chest and respiratory diseases specialized center, Ministry of health Baghdad governorate (this specialized center generally accepted patients from different Iraqi governorate). All Isolates identifications were made on the basis of morphological and microscopical features and were sub cultured on Sabouraud Dextros Agar medium at 4°C, for using it in DNA extraction *A. fumigatus* isolates isolated from patients comes from different governorates Baghdad, Salahaldeen, Basrah, Kerbala, Najaf, Dyala, Wasit, Sulaimania and the paients suffering.

Genomic DNA isolation: Total genomic DNA of all the studied isolates was extracted manually using CTAB method according to the method described by Sambrook *et al*. (1989) to produce a rapid extraction and high quality extracted DNA. Purity and concentration of DNA was measured by spectrophotometer (Maniatis *et al*., 1982). Genomic DNA integrity was detected by running on 1% agarose gel electrophoresis followed by staining with ethidium

bromide and visualized under UV light (Farber *et al*., 1997).

PCR amplification of AFLR2 gene fragments: PCR primers were designed using primer 3 software and were purchased from CinnaGen, Germany, CinnaGen 5X PCR Master Mix, ready to Load (Green) had been used in this study. The polymerase chain reaction was used to amplify the Aflatoxin regulatory gene fragments of aflatoxigenic fungal genomic DNA. The sequence of the forward and reverse primers AFLAR2 of the Aflatoxin regulatory gene was (5¯ GCACCCTGTCTTCCCTAACA 3¯) and (5¯ ACGACCATGCTCAGCAAGTA 3¯), with product size of 400 base pairs (bp) have been patented (Felsenstein, 2004).

PCR conditions: The polymerase chain reaction was performed in 25 mL; each reaction mixture was heated to 95°C for 10 min. A total of 30 PCR cycles, each cycle at 0.3 min at 94°C for denaturation, 0.45 min at 55°C for annealing, 1.15 min at 72°C for extension and a 10 min final extension at 72°C. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in (1x) TBE buffer (50 mM Tris-acetate, 1 mM EDTA, pH 8.0) stained in 0.5 mg/mL ethidium bromide (Noorbacash *et al*., 2009; Nei and Li, 1979).

DNA sequencing and analysis: The PCR products were purified with a High Pure PCR Product Purification kit (Boehringer Mannheim). Sequence reactions of AFLAR1 gene were done by first base company/Malaysia for sequencing of products through used individual sense and antisense primer were used in each sequencing reactions. Homology researches were conducted between the sequence of standard gene BLAST program which is available at the National Center Biotechnology Information (NCBI) online at (http://ncbi.nlm.nih.gov) and using BioEdit program. Sequence ambiguities were identified by comparison with their opposite strand sequences. Sequences of the fragment (AFLAR2) were assembled to yield the entire AFLAR2 gene sequence; the AFLAR2 sequences of each strain were aligned using CLUSTAL X 1.83 (Thompson *et al*., 1997). The alignment of all sequences was visually assessed and optimized when necessary. Phylogenetic evaluation was accomplished by applying neighbour-joining program from the PHYLIP 3.63 package (Chang, 2003). Phylogenetic trees were rooted with *Aspergillus flavus*. Finally, a bootstrap analysis with 1000 replications was performed. Trees were viewed using Tree View (Shapira *et al*., 1996).

RESULTS AND DISCUSSION

Screening of the ability of the strains for aflatoxin production: A total of 14 strains of *A. flavus* isolated from the sputum of aspergillosis infection were assessed for AFB1 production during growth on PDA medium using PCR, elven only of these strains, give positive result for produced AFB1 and three straines give negative results by using AFLAR2 gene (Fig. 1).

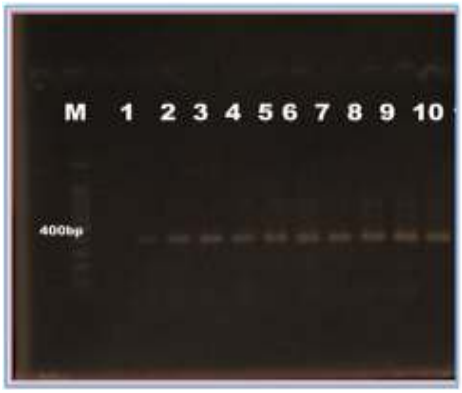

Fig. 1: Amplification of primer (aflR2) detected by PCR, 10 straines+flatoxigenic strains and 3 straines-non-aflatoxigenic strains. Agarose gel (1.5%) visualized under UV after staining with ethidium bromide (product size 400 bp)

PCR showed that all aflatoxin and non-aflatoxin producers strains the gene (aflR2) examined. This discounts the possibility that a lack of aflatoxin production in certain strains is due to loss of the genes from the genome. This is supported by Rodrigues *et al*. (2009) who reported that the loss of production of AFB1 many non-aflatoxigenic *A. flavus* isolates is not caused by large deletions or a complete loss of the aflatoxin gene cluster, but probably results from point mutations.

It is reported that genes involved in the Aflatoxin bio synthetic pathway may form the basis for an accurate, sensitive and specific detection system, using PCR, for aflatoxigenic strains in grains and foods (Thompson *et al*., 1997). In this study, using primer designed to Aflatoxin regulatory pathway gene, aflR, the presence of aflatoxigenic fungi was easily detecting in compared to conventional plating techniques.

Previous researches demonstrated that the Aflr protein can bind the promoter region of aflatoxin synthesis gene and activate aflR gene expression (Farber *et al*., 1997), so this explain presence of aflR gene and un ability of some isolates to produce aflatoxin that due to failing in expression of aflR gene because Aflr protein was absent or un employed. The aflR gene has an auto regulation function, absence of the aflR gene or the presence of an abnormal aflR gene would be a strong indicator that isolates cannot produce aflatoxin (Noorbacash *et al*., 2009).

Notably, although there is no evidence of aflatoxin production by the nonaflatoxin producing fungi like *A. oryzae* and *A. sojae*, some genes (nor-1, ver 1, omt-A and aflR) needed for aflatoxin biosynthesis are present, but not expressed, in these fungi (Noorbacash *et al*., 2009; Nei and Li, 1979).

Table 1: Sequencing of sense and antisense flanking the AFLAR2gene for 10 *Aspergillus flavus* strains

1CAGTCATGGAACACGGAAACCATGCCGATTTCTTGGCCGAG TCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACC CCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCACCG TTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAAACAC TGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTAC GGCTGACGGACGGTGAGGACAGTTCGTGCAACCTGATGACG ACTGATATGGTCATCTCGGGGAACAAGAGGGCTACCGATGC GGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTA	2CAGTCATGGAACACGGAAACCATGCCGATTTCTTGGCCGA GTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGA CCCCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCA CCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAA ACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCA ACTACGGCTGACGGACGGTGAGGACAGTTCGTGCAACCTG ATGACGACTGATATGGTCATCTCGGGGAACAAGAGGGCTA CCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAG GATGGCTA
3CAGTCATGGAACACGGAAACCATGTCGATTTCTTGGCCGAG TCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACC CCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCACCG TTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAAACAC TGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTAC GGCTGACGGACGGTGAGGACAGTTCGTGCAACCTGATGACG ACTGATATGGTCATCTCGGGGAACAAGAGGGCTACCGATGC GGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATG	4CAGTCATGGAACACGGAAACCATGTCGATTTCTTGGCCGA GTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGA CCCCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCA CCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAA ACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCA ACTACGGCTGACGGACGGTGAGGACAGTTCGTGCAACCTG ATGACGACTGATATGGTCATCTCGGGGAACAAGAGGGCTA CCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAG GATGGCT
5CAGTCATGGAACACGGAAACCATGTCGATTTCTTGGCCGAG TCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACC CCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCACCG TTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAAACAC TGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTAC GGCTGACGGACGGTGAGGACAGTTCGTGCAACCTGATGACG ACTGATATGGTCATCTCGGGGAACAAGAGGGCTACCGATGC GGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTA	6CAGTCATGGAACACGGAAACCATGTCGATTTCTTGGCCGA GTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGA CCCCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCA CCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAA ACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCA ACTACGGCTGACGGACGGTGAGGACAGTTCGTGCAACCTG ATGACGACTGATATGGTCATCTCGGGGAACAAGAGGGCTA CCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAG GATGGCTA
7CAGTCATGGAACACGGAAACCATGTCGATTTCTTGGCCGAG TCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACC CCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCACCG TTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAAACAC TGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTAC GGCTGACGGACGGTGAGGACAGTTCGTGCAACCTGATGACG ACTGATATGGTCATCTCGGGGAACAAGAGGGCTACCGATGC GGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTA	8CAGTCATGGAACACGGAAACCATGTCGATTTCTTGGCCGA GTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGA CCCCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCA CCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAA ACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCA ACTACGGCTGACGGACGGTGAGGACAGTTCGTGCAACCTG ATGACGACTGATATGGTCATCTCGGGGAACAAGAGGGCTA CCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAG GATGGCTA
9CAGTCATGGAACACGGAAACCATGTCGATTTCTTGGCCGAG TCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGACC CCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCACCG TTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAAACAC TGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAACTAC GGCTGACGGACGGTGAGGACAGTTCGTGCAACCTGATGACG ACTGATATGGTCATCTCGGGGAACAAGAGGGCTACCGATGC GGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGATGGCTA CT	10CAGTCATGGACACGGAAACCATGTCGATTTCTTGGCCGA GTCGACGGGGAGTCTTTTCGACGCGTTTTTGGAAGTAGGGA CCCCCATGATCGACCCGTTCCTCGAGTCGGCCCCACTACCA CCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTAGCACTACAA ACACTGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCA ACTACGGCTGACGGACGGTGAGGACAGTTCGTGCAACCTG ATGACGACTGATATGGTCATCTCGGGGAACAAGAGGGCTA CCGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAG GATGGCTAC

Similarity and difference between producers *Aspergillus flavus* strains based on regulatory gene sequencing:

Sequencing of AFLAR2 gene: Sequencing of coding regions aflR2 Transcription factor containing a zinc Enzyme cluster DNA binding motif Positive regulator of AFB biosynthesis Step in AFAs biosynthesis pathway of amplified product for all samples included in our local study into *A. flavus* were done seeking for detection similarity, difference and presence of any mutation within these sequence related to regulatory of aflatoxin B1 were assembled to yield the entire aflR2 gene sequence; the aflR2 sequences of each strain were aligned using CLUSTAL X 1.83 (Rodrigues *et al*., 2009).

Alignment of AFLAR2Gene af all samples with data published for known sequence seeking for enough homology. Ahomology with AFLAR2Gene of *Aspegillus flavus* from Gene Bank was done using the BioEdit software. Compatibility 100% of the forward gene was found with AFLAR2 (flank DNA sense and antisense of the gene) with standard AFLAR1 of Gene Bank results as shown in Table 1.

Moreover, we also found that reversed AFLAR2gene (flank DNA sense and antisense of the gene) match entirely with standard AFLAR1 of Gene Bank results as shown in Table 1.

Also result of sequencing analysis not detects any mutations in exon of regulatory gene AFLAR2 for 10 *A. flavus* isolates.

Previous researches demonstrated that the Aflr protein can bind the promoter region of aflatoxin synthesis gene and activate aflR gene expression (Zong Lee *et al*., 2006).

This may be a result of sequence variability which occurs within the entire aflR gene of *Aspergillus* section *Flavi*. It was found that particular sequence variability differentiates some species in *Aspergillus* section *Flavi*

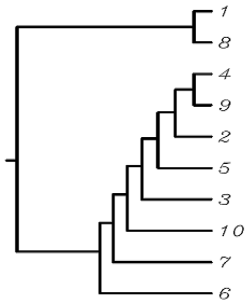

Fig. 2: Neighbour-joining trees of AFLAR2 gene sequences for *Aspergillus flavus* strains based on AFLAR2 gene sequences

and can be used to identify functionality of the AFLR protein (Cleveland *et al*., 2009).

Similarity and difference based on luster analysis: The genetic diversity and the relationships among *A. flavus* isolates were evaluated using sequencing for the aflatoxin b1 regulatory gene varied greatly in their ability to resolve variability among straines. Some straines differentiated into sub-group, while others assembly together (Fig. 2).

To determine the similarity between 10 *A. flavus* isolates this occur through intersection between each two isolates Dendrogram was constructed based on Nei and Li (1979) genetic distance using UPGMA cluster analysis and depicted genetic relationships among 10 isolates of *A. flavus*, Fig. 2 showed two major clusters.

The first main group: Included two straines 1 and 8.

The second main group: Included subgroups, included the strains 2, 3, 4, 5, 6, 7, 9, 10 Differentiation of *Aspergillus flavus* is difficult, due not only to interspecific similarities, but also to intraspecific variability (Sigler and Verweij, 2003).

Individual *A. flavus* isolates was varied between them according to their genetic and morphological characteristic, geographical isolation aflatoxin-producing ability etc., (Shapira *et al*., 1996). All eleven *A. flavus* isolates identical in there morphological characteristics, but genetically varied. The aflR gene is the principal regulator of the aflatoxin production pathway. Felsenstein (2004) showed that relative distribution of aflatoxigenic versus non aflatoxigenic isolates is modulated by many factors including plant species present, cropping history, crop management and environment conditions geographical region. Hedayati *et al*. (2007) was found that 35% of the isolates from shelled pistachio nut in turkey produced aflatoxin on (YES) media was found 28% of isolates from various substrate in china produce aflatoxin. aflatoxin on (YES) media.

DNA sequences which are polymorphic between fungal species, such as Internal Transcribed Spacer (ITS) regions, are good candidates for the detection of a species to the exclusion of all other species. For example, differences in ITS sequences have been used to develop PCR-based assays for the detection of many pathogenic fungal species in host plants without previous isolation of the fungi (Maniatis *et al*., 1982).

Regarding the result of sequencing, its consider excellent way to clear knowledge about the relationship between the strains belong to the same species and produce the same secondary metabolite.

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