International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

A Study of the Expression of *Aflatoxin B1* Regulatory Gene in Clinical and Environmental *Aspergillus flavus* using Real-time PCR

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Abstract

This recent study investigated the differences in the expression of Aflatoxin B1 regulatory gene among clinical and environmental (*Aspergillus flavus*) isolates. Samples were subjected to RNA isolation, cDNA synthesis and PCR amplification. Electrophoresis demonstrated clean bands 798 bp upon amplification with aflR1 primers. The ability of *A. flavus* confirmed the presence of the aflR2 gene in product size 400 bp in all the studied isolates with a different gene expression (Sentence not clear. Rephrase). There was a direct correlation between the *aflr* gene expression and the isolates source. The source of isolates significantly influenced the *aflr* gene expression. It appeared that the *aflr* gene expression increased at the *A. flavus* isolated from patients with aspergillosis with a gradual gene expression in *A. flavus* isolated from grains of *Zea mays (maize) and* with no clear expression in the *A. flavus* isolated from rice grain. These results indicate a suitable molecular method for detecting and controlling the spread of *A. flavus* in the grainer and in hospitals to avoid infection.

Keywords: *Aspergillus flavus*; Aflatoxin B1; gene expression; *aflR*

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1. Introduction

Mycotoxigenic filamentous fungi have the ability to contaminate a wide range of food and animal feed with one or more aflatoxin [1]. In temperate climates, food and feeds, are vulnerable to being attacked by aflatoxigenic *Aspergillus flavus*, which acts as an opportunistic pathogen under certain conditions, being responsible for most cases of aspergillosis [2]. The production of aflatoxins (AFAs) in food and feed can occur at pre-harvest, during drying, processing or storage. Due to the toxicity and carcinogenicity of mycotoxins, infected products which will be consumed by humans or animals are a serious health risk and therefore, are controlled and observed very closely [3].

Real-time PCR is highly responsive and has the ability to quantify rare transcripts and minute changes in gene expression. Using realtime PCR to assess and monitor the ability of the fungus to activate mycotoxin biosynthesis genes under different environmental conditions is useful as an early indication of contamination in a specific food chain. Moreover, it has been previously reported that mycotoxin genes are induced and not expressed constitutively [4,5].

Aflatoxin B1 (AFB1) is mutagenic and teratogenic and can depress cell-mediated immunity. It also plays a role in the invasion of the lung and formation of an aspergillosis ball [2]. AFB1 acts synergistically with hepatitis B virus infection to boost its carcinogenic potency thirty-fold in hepatitis B surface antigen positive populations. The International Agency for Research on Cancer (IARC) has evaluated AFB1 as a Group 1 carcinogen producing liver cancer in humans [6,7].

The purpose of this research was to understand and ascertain how the source of *A. flavus* isolate influences the regulation of Aflatoxin B1 production, and to investigate the correlation of the fungus source (clinical and environmental) with expression of Aflatoxin B1 gene and phenotypic production.

2. Materials and methods

2.1 A. flavus isolates

A total of 12 *A. flavus* isolates were collected from various sources (Table 1)

Table (1): *Aspergillus flavus* isolates

No.	*A.flavus* Isolate	Source
1	**AF1**	**Rice grain**
2	**AF2**	Grainer/ Baghdad-Iraq
3	**AF3**	**Aspergillosis patients**
4	**AF4**	Chest and respiratory diseases specialized centre,

		Ministry of health, Baghdad, Iraq
5	**AF5**	***Zea maize* grain** Grainer / Baghdad, Iraq
6	**AF6**	
7	**AF7**	
8	**AF8**	
9	**AF9**	
10	**AF10**	
11	**AF11**	
12	**AF12**	

Isolates were identified on the basis of morphological and microscopic features (Figure 1). Isolates subcultured on Sabouraud Dextros Agar medium at $4C^0$ were used for DNA extraction.

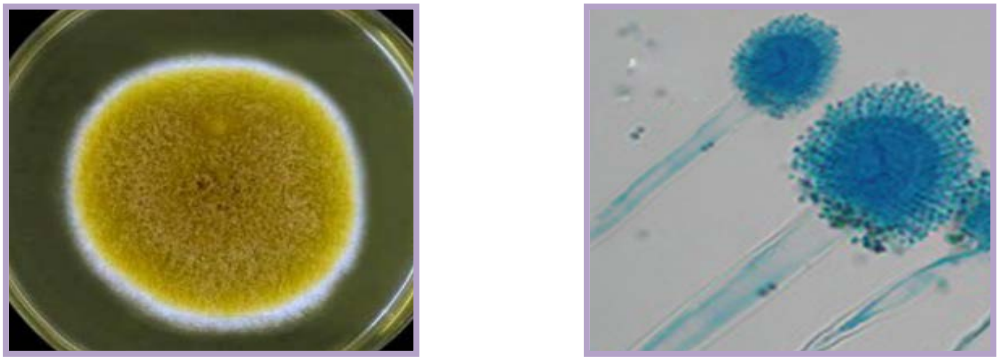

Figure (1): Growth of *A. flavus* on culture media at 28°C for 7 days Growth of *A. flavus* on PDA medium & Microscopic feature of *A. flavus* (staining with lactophenol cotton blue) showing conidial head (40 X).

2.2 Isolation of RNA and cDNA synthesis

After 7 days of *A. flavus* incubation on SDA medium, the entire mycelial colony was removed. The mycelia were quickly frozen in liquid nitrogen and stored at -80 °C until extracted. Total RNA were isolated using a Gene aid total RNA purification mini kit (Taiwan) according to the manufacturer's instructions. Isolated RNA was treated with RNase-free DNase I (Biobasic, Canada) for 20 minutes at 37°C. DNase I was inactivated at 65°C for 10 minutes. The integrity of the RNA was verified after separation by electrophoresis on a 1.5% agarose gel containing 0.5% (v/v) ethidium bromide. First-strand cDNA was synthesized from 500ng of total RNA using the reverse transcription system (Bioneer, Korea). The reaction solution was used as a template for the reverse transcriptase polymerase chain reaction (RT-PCR).

2.3 Polymerase Chain Reaction (PCR):

Conventional PCR (program and reactions): primers were selected according to a previously published study [8]. The primers (Table:2) amplified the Aflatoxin B1 Regulatory Region (RR) for *A. flavus* isolates and B-actin (reference gene). cDNA was amplified using primers listed in Table 2. All primers were supplied by the Alpha DNA Company, Canada.

Table (2): Primers used for amplification of *aflR1* and *aflR2* actin cDNA sequences.

Gene	Primer	Sequence5'-3'	**Step in AFA biosynthesis pathway**	Genbank accession number	Reference
aflR1	Forward	AACCGCATCCACAATCTCAT	Positive regulator of AFB biosynthesis	264763	[8]
	Reverse	AGTGCAGTTCGCTCAGAACA			
aflR2	Forward	GCACCCTGTCTTCCCTAACA		264764	[8]
	Reverse	ACGACCATGCTCAGCAAGTA			

Apolymeras chain reaction was initiated with a hot start method with the cDNA template using a Lab net Thermo cycler (USA). PCR was performed in 25 µL volumes. Each reaction mixture was heated to 95ºC for 10 minutes. A total of 30 cycles, each cycle consisting of denaturation at 94ºC for 0.3 minutes, annealing at 55ºC for 0.45 minutes, and extension at 72ºC for 1.15 minutes, and a final extension at 72ºC for 10 minutes. 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 with 0.5 mg/mL ethidium bromide.

Real-time PCR (program and reactions): The Real-Time PCR reaction was performed for *aflR* expression analysis using *Accu Power* Green Star qPCR PreMix Kit (Bioneer-Korea). This pre-mix contains SYBR Green dye for monitoring the amplification process. For each reaction, 0.5 µL of each *aflR* primer (10 pmol/µL) and 1.5 µL of DNA (100 ng/µL) were added. The final volume was adjusted to 20 µL with DEPC-distilled water. The Real-Time PCR programme conditions for *aflR* detection consisted of initial denaturation at 95°C for 5 minutes and 40 cycles at 95°C for 1 minute, 60°C for 45 seconds and 72°C for 1 minute, followed by a melting curve analysis at 60-95°C. To confirm amplification specificity, the amplified fragments were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide. Quantitation of relative expression was determined by the $2^{-(\Delta\Delta CT)}$ method [14]. Each sample was run in triplicate. For an estimation of the standard curve, online software was used to convert DNA concentration to log copy number (http://www.uri.edu/research/gsc/resources/cndna.html).

3. Results and discussion

The cDNA from all examined *A. flavus* isolates produced clean bands upon amplification with *aflR1* primers with a 798 bp product size. The ability of *A. flavus* confirmed the presence of *aflR2* gene in product size 400 bp. (Sentence not clear. Rephrase)

Most of the work cited in the literature involved monomeric or multiplex PCR, which detects aflatoxiginic strains of *A. flavus*, *A. parasiticus* and *A. nomius*. Our current results agreed with [9] who confirm the ability of *A. flavus* (environmental isolates) to produce aflatoxin B1. It is recognized that food constituents can interfere with *Taq* polymerase giving false negative results [6].

The primers were specific for *aflR* gene fragment, the size of the amplicons corresponded to the expected size and no additional or non–specific bands were observed. Nested PCR was mainly used to confirm the authenticity of the primary PCR. For *A. flavus*, the 798 bp amplicon was reconfirmed by using it as a template in the nested PCR. The nested PCR primers generated the expected amplicon size of 400 bp [7].

Conventional and SYBR green real time PCR were used for the amplification of genes *aflR* and β-actin. Melting curve analysis was used to indicate the presence of target genes and the lack of primer dimers or amplification of other DNA targets (Figure 2 a, and Figure 2 b). Absolute and relative gene expression of the *aflR* gene was estimated using the standard curve and the reference gene (B-actin) (Figure 4).

In order to study gene expression, the efficiency of QPCR was determined by using several serial decimal dilutions of eluted PCR bands. The logarithm of gene copy numbers was estimated according to cDNA concentrations using online software. The DNA copy numbers are shown in Figure 5.

The present study showed that there was a direct correlation between *aflr* gene expression and the source of the examined isolates. The influence of the isolate source on *aflr* gene expression was significant. *Aflr* gene expression increased in *A. flavus* isolated from patients with aspergillosis, was modest in *A. flavus* isolated from grain of *zeal mays*, and was not detected from rice grain isolates (Table 3).

The source of the isolate may affect gene expression due to differences in growth condition [10].This may lead to differentiations in the mechanism of adaptation through production of enzymes and other secondary metabolites that facilitate tissue invasion and colonization.

The increased expression of *aflR* in *A. flavus* isolated from patients with aspergillosis might result from an elevated basal level of AFLR due to increased *aflR* copy number in the transformants. Consequently, this allows it to overcome the suppressive effect of nitrate on aflatoxin pathway gene transcription. Enhanced levels of AFLR would be available to bind to its own promoter sites and thereby activate the transcription of *aflR*. Activation of transcription could be modulated by the highly acidic domain [11].

O' Callaghan [12] established that a high number of spores may lead to a rise in the expression of *aflR* a regulatory gene which interacts and activates *aflR*, the principle regulatory gene, which then activates the aflatoxin structural genes [13]. At certain parameter combinations of 20, 25 and 30°C the expression of the *aflr* gene was enhanced when compared to the other genes in the cluster. This was often accompanied by increased biosynthesis of AFB1 compared to the neighbouring conditions.

Similarly, earlier observations suggested that aflatoxin B1 production and gene expression were affected by fungal spore counts and humidity from the source [14,15,16,17]

Ehrlich [18,19] showed that expression of the regulatory gene *aflR* was five to ten times lower in weakly sporulated isolates that did not produce aflatoxins or related compounds. curve and reference gene (B-actin) (Figure 4).

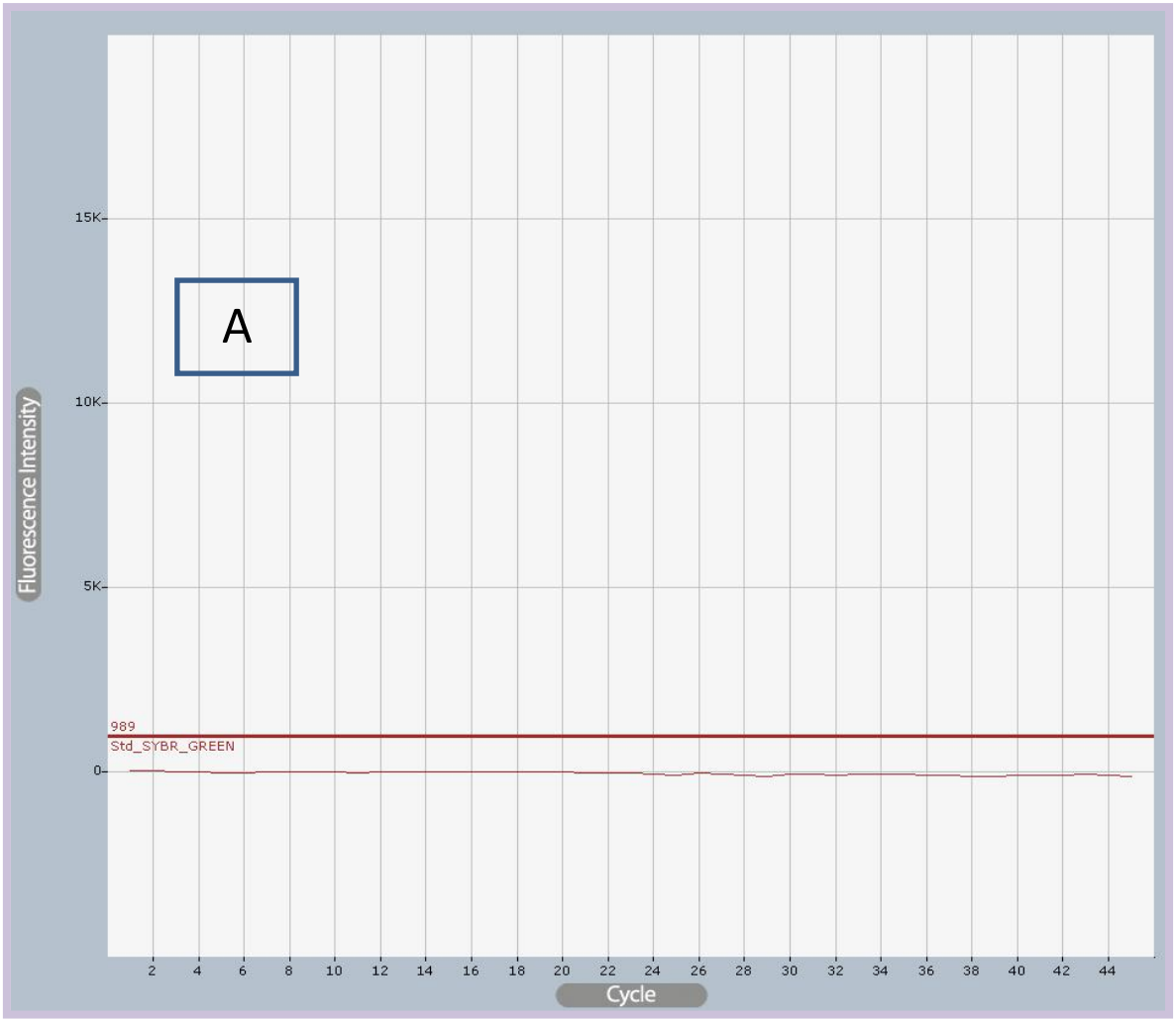

Figure 2 a: Graph shows SYBR Real time PCR amplification curves for *aflR* gene and B-actin gene. A: not expressed

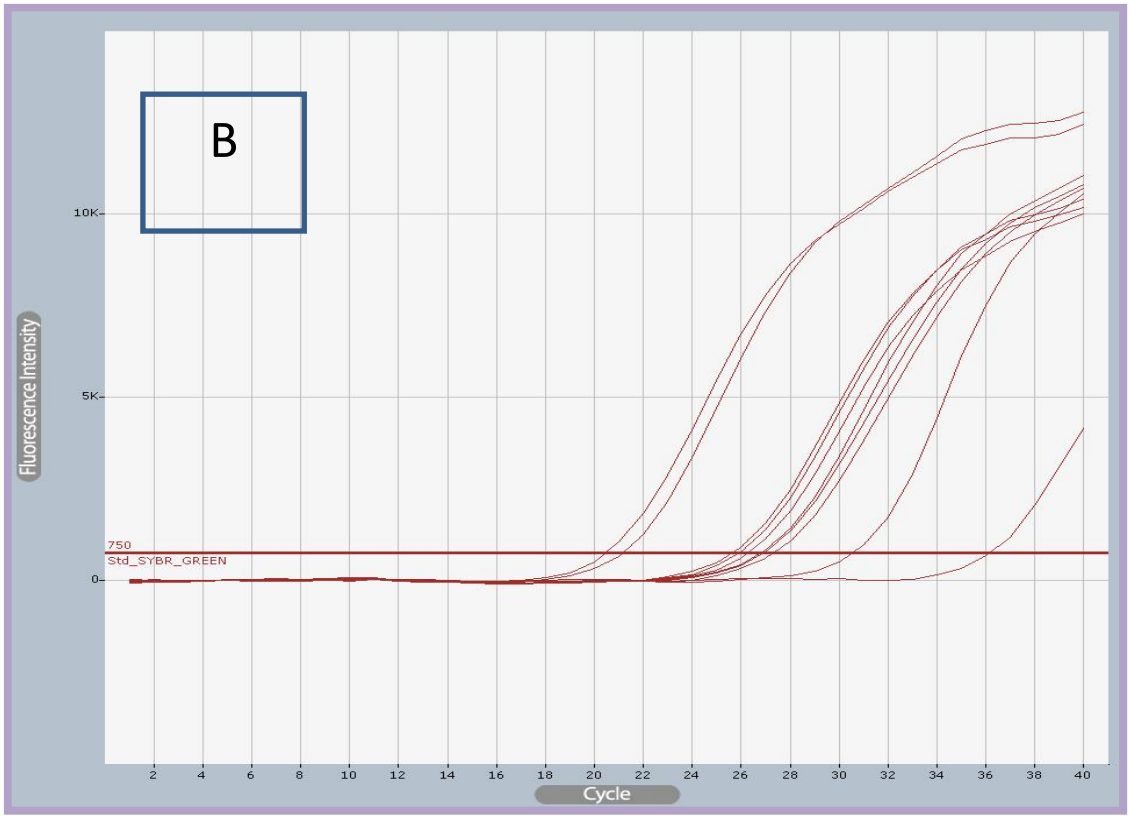

Figure 2 b: Graph shows SYBR Real time PCR amplification curves for *aflR* gene and B-actin gene. B: Expresd

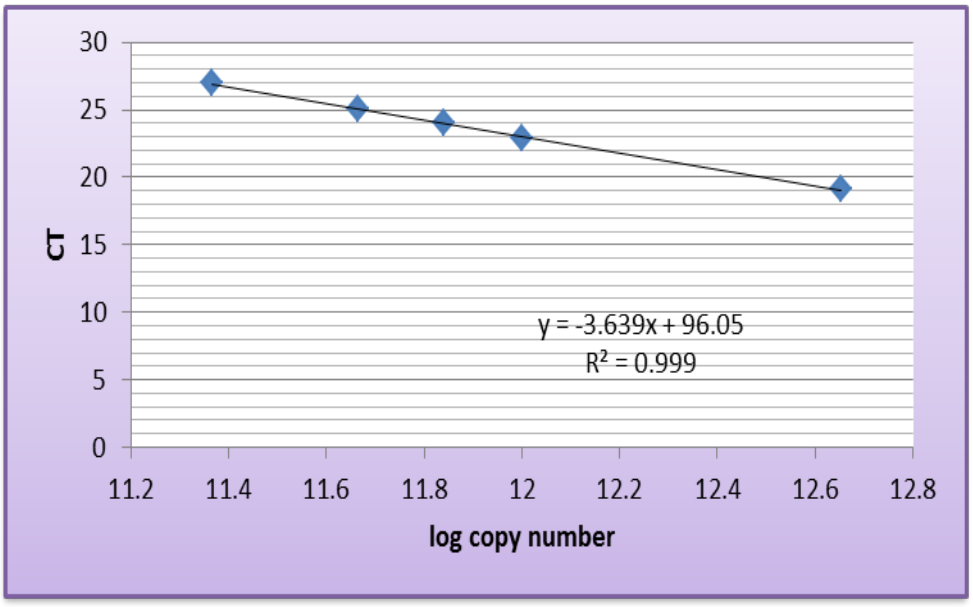

Figure (3): Standard curve shows slope and R^2 values to determine the efficiency of PCR reaction for *aflR* gene amplification. Source [14].

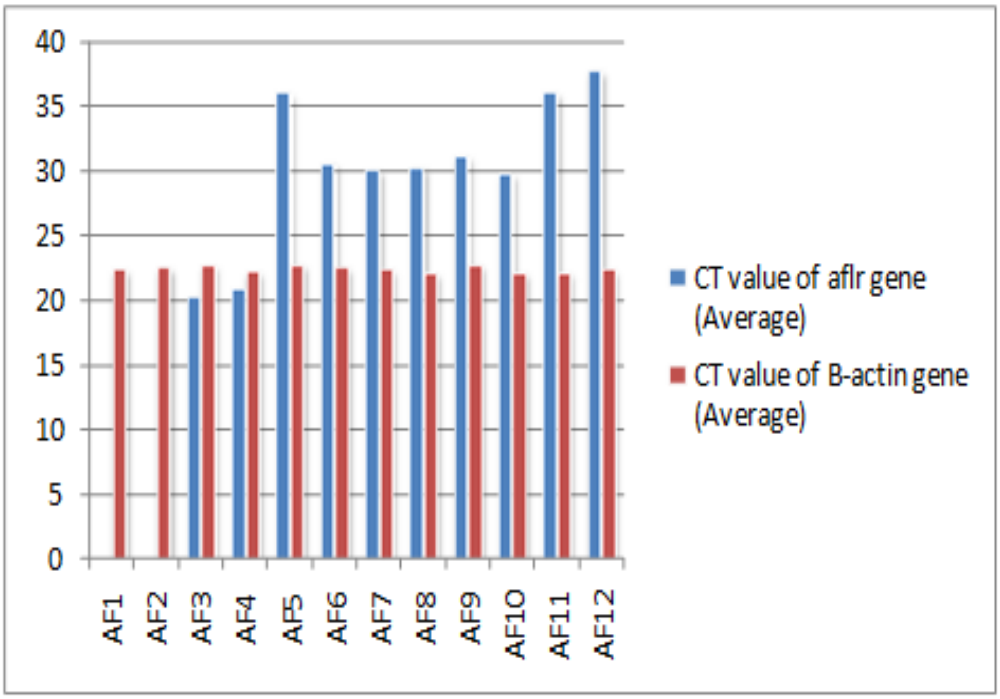

Figure (4): CT values of B-actin and *aflR* genes SYBR green real time PCR amplification.

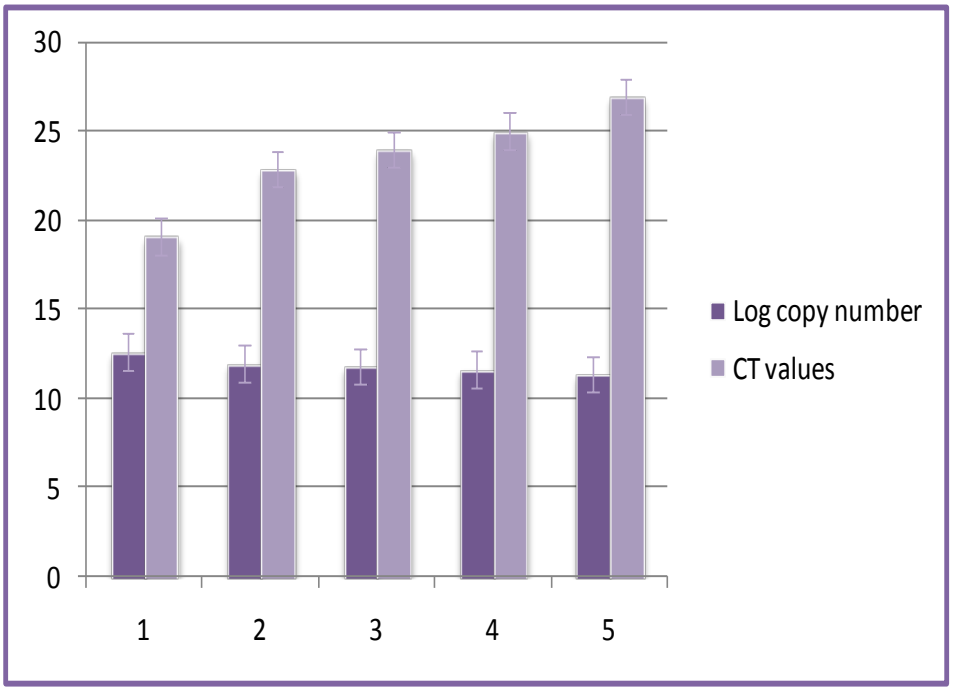

Figure (5): CT values and the number of copies of the DNA template of the gene *aflr*

Table (3): values *aflr* gene expression in Clinical and environmental *A.flavus* isolate.

Sample	CT(*AFLR1*)- CT (*ACTIN*)	Gene expression= $E^{[Ct\ (AFLR1) - Ct\ (ACTIN)}$
AF1	**0**	**0**
AF2	**0**	**0**
AF3	**-2.48**	**1.36**
AF4	**-2.28**	**1.32**
AF5	**13.29**	**0.19**
AF6	**8.09**	**0.36**
AF7	**7.72**	**0.38**
AF8	**8.22**	**0.35**
AF9	**8.49**	**0.34**
AF10	**7.1**	**0.41**
AF11	**13.97**	**0.17**
AF12	**15.5**	**0.14**

4. Conclusion

Contamination of grain stores with fungal toxin-producing is harmful and is a common problem in Iraq, which requires urgent control. We have concluded that during the study of gene expression it is possible for it to be used to control the production of aflatoxin B1 genetically.

Conflict of interest

The authors of this paper declare that they have no conflict of interest.

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Acknowledgment

We thank Dr. Abdul Kareem Jasim Hashim for his efforts in interpreting the results and Mrs. Safa Mujahed Abdullatif (Baghdad University) for providing the primers.

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