Detection of aflatoxins by HPLC and the expression of biosynthetic *nor-1* gene of aflatoxin and *ocrA* gene of ochratoxin.

Mohamed N. Hassan[1], Ashraf S.A. El-Sayed[2], Hend M.S. Nada[1]

[1]Bacteriology, Mycology and Immunity Department, Faculty of Veterinary Medicine, Zagazig University, Egypt. [2] Microbiology Department, Faculty of Science, Zagazig University, Egypt

ABSTRACT

Due to the high toxicity of aflatoxins and its effects on public health and animal wealth, food and feed. The determination of mycotoxins in commodities is the only way of control. The identification of aflatoxigenic Aspergillus isolates through detection of *nor-1* gene was tried. Ochratoxin has been widely detected in cereal-derived products. Its production by *A. ochraceous* and its biosynthetic gene *ocrA* was evaluated. For this purpose, 500 different cereal samples were collected from different markets in Sharkia Province. Five strains of *Aspergillus* spp. have been screened for their ability to produce aflatoxins after cultivation in Czapek,s Dox media. Aflatoxin B1was detected in average amount 4.9-200 ng/µl through using HPLC technique. A Real time PCR (RT-PCR) technique was applied to determine the gene expression of structural *nor-1*, which catalyzed the first step in aflatoxins biosynthetic pathway.

Keywords: aflatoxins, biosynthetic nor-1 gene, ocrA gene.

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1. INTRODUCTION

Mycotoxins are a group of secondary metabolites produced by fungi such as *A.flavus, A. parasiticus, A.ochraceous, Fusarium tritinctum, Fusarium poae* and *penicillium* species. There are many types of mycotoxins, but the most problematic and associated with disastrous outbreaks in field are aflatoxins, ochratoxins, patulin and T-2. Aflatoxins attracted special attention by their ability to contaminate hundred kinds of agro –products such as cereals, plant oil, nuts, soya sauce and even tea leaves. The ability of aflatoxin production had been reported in various species of the genus Aspergillus mainly in section Flavi group (Rodrigues et al., 2009). Aflatoxins are secondary metabolites produced by many species of Aspergillus, particularly *Aspergillus flavus* and *Aspergillus parasiticus* (Blesa et al., 2003). Not all *A. flavus* and *A. parasiticus* are aflatoxins producer, about 60% of isolates were producer (Razzaghi-Abyaneh et al., 2006). Four major aflatoxins AFB1, AFB2, AFG1, AFG2 are based on their fluorescence and U.V light, and their two metabolites AFM1 and AFM2, but the most toxic mycotoxin currently known is the Aflatoxin B1. Naturally *A. flavus* produce aflatoxins AFB1, AFB2, while *A. parasiticus* produce the four mycotoxins (Dorner 2004). Aflatoxins are highly toxic, mutagenic and carcinogenic compound, chemically. They are a group of difuraroco-marine derivative. The reason why gene expression of producer and non-producer strains is seeming to be an essential to trace the incriminated strains and its pathway in the environment. Among the variety of modern

well established methods for assessment of aflatoxins quantities in food and feed, is the HPLC technique relies on physical and chemical structure of toxins provides an acceptable, accurate and alternative methods to evaluate the status of aflatoxins in contaminated food and feed. Numerous publication describes cultural, biological, analytical and genetical methods. Ochratoxin has been widely detected in cereals-derived products, coffee beans, dried fruits, spices, beer and wine. Four ochratoxins (A, B, C and D) are present. The major field problematic mycotoxin among this group is ochratoxin A (OTA). Ochratoxin A (OTA) is a mycotoxin produced by *A. ochraceous, Penicillium verrucosum* and *P. nordicum* (Larsen et al. 2001 and Castella et al., 2002) and other number of Aspergillus species (Frisvad and Samson 2000 and Abarca et al., 2001). It is a common food and feed contaminant enter body through consumption of food products. This myco-toxin is a nephrotoxic, carcinogenic and by virtue of ability to cross the placenta, it considered embryotoxic and teratogenic (WHO, 2002). The most important toxic effect of this mycotxin is its nephrotoxicity leading to conditions such as "Balkan Endemic Nephropath". Numerous publication describes cultural, biological, analytical and genetical methods. The development of a rapid, sensitive method for detection and differentiation of potential aflatoxigenic species in foods and feed is ultimate goal to avoid any potential health risk, hence determination is the only way to mycotoxin control (Valasek and Repa, 2005). Animal toxicity including the possible carry-over mycotoxins or their metabolites, into the human food chain (Bryden, 2012). (Fente et al. 2001) rely on multiplex Polymerase chain reaction (PCR) and real time Polymerase chain reaction (RT-PCR) has proved to be avery precise and rapid biomolecular technique for detecting genes or their transcripts involved in Aflatoxin biosynthesis by *A.flavus* and *A.parasiticus* (Somashekar et al., 2004 and Scherm et al., 2005).

The aim of the present study was to apply HPLC technique, to quantify Aflatoxin from different Aspergllius cereal isolates and to detect structural *nor-1* gene expression by RT-PCR. *Nor-1* gene is a key structural gene in biosynthetic pathway of aflatoxins it encodes an enzyme that catalyze the conversion of the first stable aflatoxin biosynthesis intermediate norsolorininc acid to averantin. Concerning ochratoxin production by *Aspergillus ochraceous* the detection of *ocrA* gene by PCR was used.

2. Material and methods

2.1. Aflatoxin standard and references Aspergillus strains:

Aflatoxins B1, B2, G1 and G2 standards were offered from animal health institute. Standard solutions were prepared in methanol and stored in a dark container at –20°C until the time of use. Strains of *Aspergillus* spp. were graciously offered from Botany department, Faculty of Science, Zagazig University (*A. parasiticus* and *A. oryzea* strains)

2.2. Fungal strains isolated from cereals:

Local *Aspergillus* species were isolated from different cereal grains. *Aspergillus* strains were isolated and purified on Czapek,s Dox Agar and Sabouraud Dextrose broth.

2.3. Preparation of spore suspension

Strains were grown on Czapeks slant for 7 days at 30 °C. Spores were harvested in sterilized saline. Then re-cultivated on Sabouraud dextrose broth media.

2.4. Culture conditions for aflatoxin production

Czapeks dox media was used as a basal medium for aflatoxin production in stationary cultures. The cultures were incubated at 27 ± 1°C for 7 days. At the end of the incubation, the isolates re-cultivated on Sabouraud dextrose broth media. Then

the cultural matt was collected and extracted by methanol. The extract was used for aflatoxin determination.

2.5. Quantitiation of aflatoxins by HPLC

Extraction, cleanup and determination of aflatoxins for HPLC technique: The aflatoxins determination by HPLC method was done according to (AOAC, 1995). The detector was set at ex= 365 nm, Em= 425 nm. The mobile phase was composed of: toluene: ethyl acetate: formic acid: methanol (90:6:2:2, v/v). The flow rate was: 1.5 ml / min. Calibration curves for each aflatoxin were determined, using a series of standard solutions prepared in methanol. Linear calibration graphs were obtained by plotting the peak area against the aflatoxin amount injected. Quantification of aflatoxins was performed by comparing the peaks areas with the calibration curve.

2.6. Preparation of genomic DNA

Aspergillus strains was performed according to a QIAamp DNeasy Plant Mini kit. For this purpose, the strains were grown for 5days at 30°C in Czapeks dox agar slant. After that the mycelium was harvested by filtration, transferred to a mortar, frozen in liquid nitrogen in -80°C and ground. The fungal mycelium was put in a tungsten carbide bead were added to a 2 ml safe-lock tube, 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) were added. Tubes were placed into the adaptor sets, which were fixed into the clamps of the tissue lyser. Disruption was performed in two 1–2 minutes high-speed (20– 30 Hz) shaking steps. The mixture was incubated for 10 min at 65°C and mixed 2 or 3 times during incubation by inverting tube. Hundred and thirty µl Buffer P3 was added to the to the lysate, mixed, and incubated for 5 min on ice. The lysate was centrifuged for 5 min at 14,000 rpm. The lysate was pipetted into the QIA shredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuged for 2 min at 14,000 rpm. The flow-through fraction from step 16 was transferred into a new tube without disturbing the cell-debris pellet. One and half volumes of Buffer AW1 was added to the cleared lysate, and mixed by pipetting. Six hundred and fifty µl of the mixture from step 8 (including any precipitate that was formed) were pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifugated for 1 min 8000 rpm and the flow-through was discarded. The DNeasy Mini spin column was placed into a new 2 ml collection tube. 500 µl Buffer AW2 was added and centrifuged for 1 min at 8000 rpm and the flow-through was discarded. Five hundred µl Buffer AW2 was added to the DNeasy Mini spin column, and centrifuged for 2 min at 14, im000 rpm to dry the membrane. DNeasy Mini spin column was transferred to a 1.5 ml or 2 ml micro centrifuge tube, and 50 µl Buffer AE were directly pipette onto the DNeasy membrane. It was incubated for 5 min at room temperature (15–25°C), and then centrifuged for 1 min at 8000 rpm to elute.

2.7. Real time PCR

The real-time PCR reactions were performed in a Step One Real-Time PCR System- Applied Biosystems, Singapore software v2.2.2. The primers and the internal probe used in the reaction were those proposed by Mayer et al. (2003). The used primer/probe set had the following nucleotide sequence: nortaq-1,5′-GTCCAAGCAACAGGCCAAGT-3′; nortaq-2,5′ TCGTGCATGTTGGTGATGGT-3′; norprobe, 5′ TGTCTTGATCGGCGCCCG-3′ enclosing an amplicon of 66 bp from nucleotide 514 to 580 according to the published sequence of nor-1 for the isolate AF36 of *A. flavus* (AY510455) (Geisen, 1996). For PCR reaction 6 µL of the DNA sample solution was mixed with 12.5 µL of 2x QuantiTect Probe RT-PCR Master Mix solution containing; PCR buffer (Tris-CL, KCL, (NH4)2SO4,8 µM MgCl2, dNTP mix. Hot Start Taq DNA Polymerase and Fluorescent dye, 0.5 µL of each primer (50 pmol) ,125 µL probe (30 pmol.), and 5.25µL sterile Rnase, Dnase free water. The amplification thermal profile was: 4

min at 94°C following of 40 amplification cycles at 95 °C for 30 s, 53 °C for 30 s and 72 °C for 20 s.

2.8. Ochratoxin A gene expression by PCR (according to Patino et al., 2005)

Ochratoxin A gene was amplified using the primers at a concentration of 1µl of each primer (20 pmol), 6µl template DNA, 12.5µl of Emerald Amp GTPCR mastermix (2x premix). PCR conditions were as follows: denaturation at 95°C for 5min; 35 cycles of 94°C for 30sec, primer-specific annealing temperature at 35°C for 90 secs, extension at 50°C for 30 secs and a final extension at 72°C for 1min. The reaction was carried out in the Biometra Thermal Cycler.

2.9. Gel electrophoresis

The PCR products were resolved by electrophoresis in a 1.5% agarose gel in 100ml TBE buffer. The amplified products were visualized under UV, Biometra Co.) and compared with a standard DNA size marker.

3. RESULTS

3.1. Aflatoxin production and determination by fluorescent HPLC

The quantification of aflatoxins was done after detection by fluorescence detector comparing the retention time and response of the peak area of standard solution (Fig. 1) with that of the injected sample. In (Table: 1) shown that the culture of the isolates*; A. flavus* 1, *A. flavus2* and *A. parasiticus* were found to be aflatoxins producer Table (1), Fig. (2-3), while the culture of the isolate *A. niger* and *A. oryzea* were failed to give any detectable amounts of Aflatoxin consequently considered non producer as shown in (Table 1), Fig.4.

3.2. Expression of the structure genes (nor-1) of aflatoxin by Real time PCR.

The results showed that the DNA extract of the isolates *A. flavus, A. flavus* 2 and *A. parasiticus* have the nor-1 gene. Represented electrophoresis band pattern of Real time PCR products shown in (Fig 5). The RT-PCR results obtained with genomic DNAas a template indicated that all tested aflatoxigenic isolates *A. flavus1, A.flavus2* and *A. parasiticus* expressed *nor-1* gene with significant expression levels in cereal isolate, while *A. niger* and *A. oryzea* were showed undetectable *nor-1* expression levels Fig. (5), (Table 2). Significant positive correlation was noted between mean expression level of *nor-1* and the quantum of Aflatoxin production by isolates (Table 3).

3.3. Detection of the gene of ochratoxin A by conventional PCR.

The result showed that a band of *ocrA* gene expressed on agarose gel electrophoresis at 406 bp as shown in Fig. (6)

Table (1): HPLC quantification of aflatoxins produced by selected *Aspergillu*s spp. Isolates

Fungal Species Tested	Retention time (min)	Amount of injected methanol extract	Amount of toxin detected ng/µl	Type of aflatoxin (B1, B2, G1 and G2)
A.flavus 1	7.808	20 µl	4.9	B1
A.flavus 2	7.80	20 µl	200	B1
A.niger	7.84	20 µl	0.00	--
A.parasiticus (control strain)	7.808	20 µl	50	B1
A.oryzea (control strain)	7.84	20 µl	0.00	--

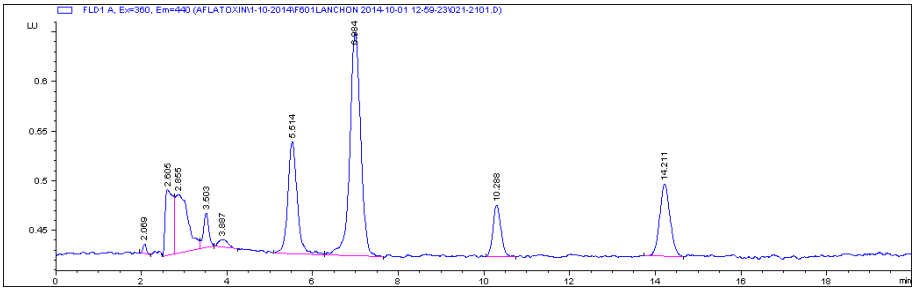

Fig (1) Stander curve of four aflatoxins

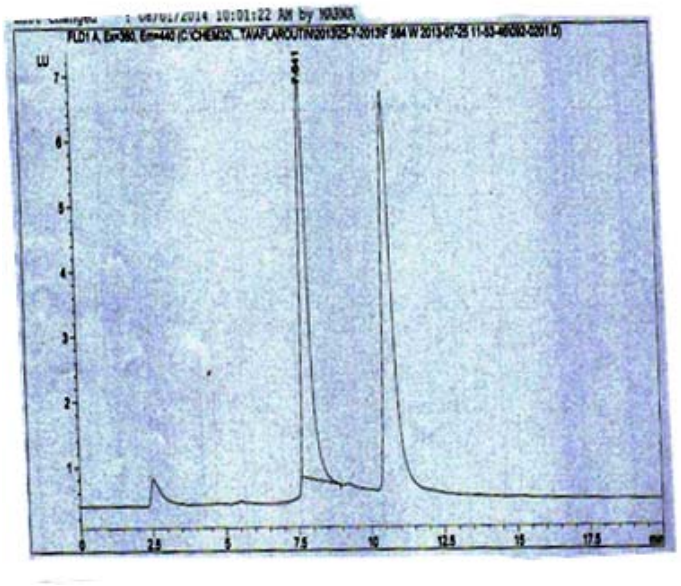

Fig (2) Quantification of aflatoxin with high performance liquid chromatography produced by *A. flavus 2*

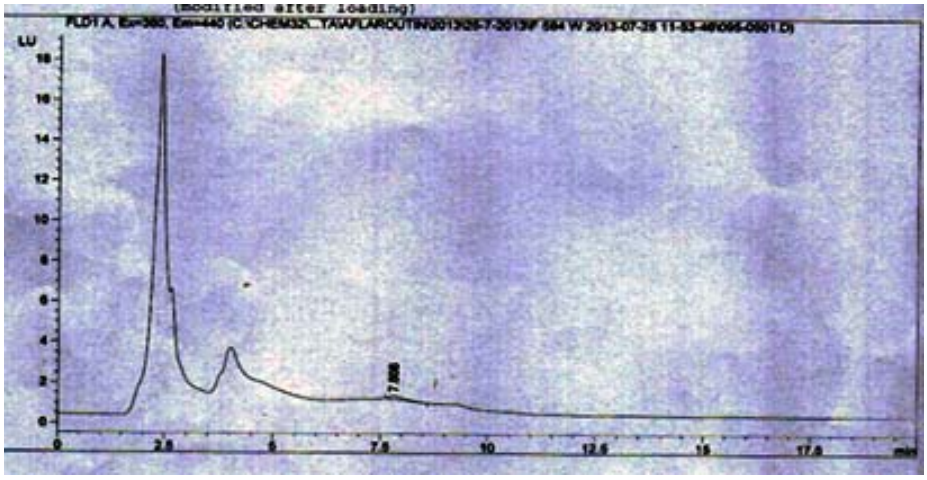

Fig (3) Quantification of aflatoxin in *A.parasiticus* extract by high performance liquid chromatography.

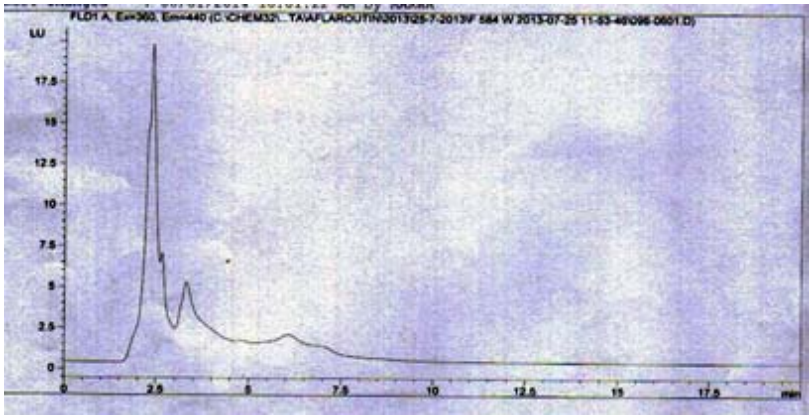

Fig (4) Quantification of aflatoxin in *A.niger* extract by HPLC.

Table (2) Detection of aflatoxin structure gene (nor-1) gene by Real-time PCR

Sample	Aflatoxin result	Threshold cycle
A.parasiticus	Positive	27.33
A.flavus1	Positive	21.98
A.flavus 2	Positive	22.75
A.oryzea	Negative	-
A.niger	Negative	-

Table (3): Correlation between HPLC detected amount of aflatoxin and *nor-1* structural gene expression by *A.flavus*-cereal isolates

Fungal isolates	HPLC Aflatoxin amount	Real time PCR Expression of *nor-1* gene
A.parasiticus (control strain)	50 ng/µl	(27.3)
A. flavus 1	4.9 ng/µl	(21.9)
A.flavus 2	200 ng/µl	(22.7)
A. niger	N.B	N.D
A.oryzea (control strain)	N.B	N.D

4. DISCUSSION

At that early time the development of detection methods represents a challenge to food and feed official authorities, conventional methods and different bioassays were used to distinguish toxigenic from non-toxigenic fungal isolates. Numerous publications are described cultural, biological and analytical chemical methods to distinguish between toxigenic and non-toxigenic fungal isolates. Unfortunately, not all methods receive the same attention, some are failed by time, other criticized by some authors while preferred by others. Because the mycotoxins in general are diverse chemical structures, it is possible to develop one methods to detect relevant mycotoxins. The reason why, further studies are needed to develop rapid, more objectives techniques that solve all aspects of mycotoxin problems. HPLC technique still the accurate method of choice for mycotoxins

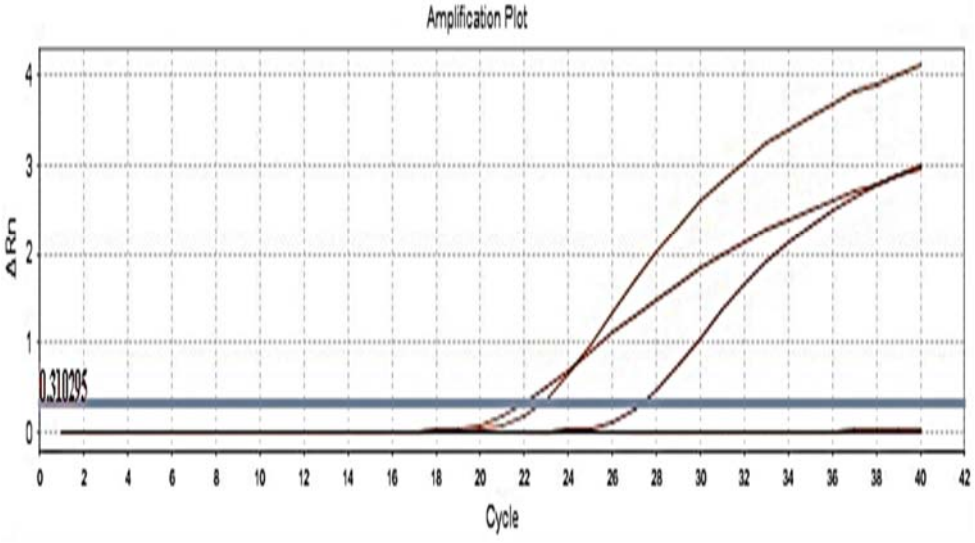

Fig (5) Detection of aflatoxin *nor-1* gene by Real time PCR

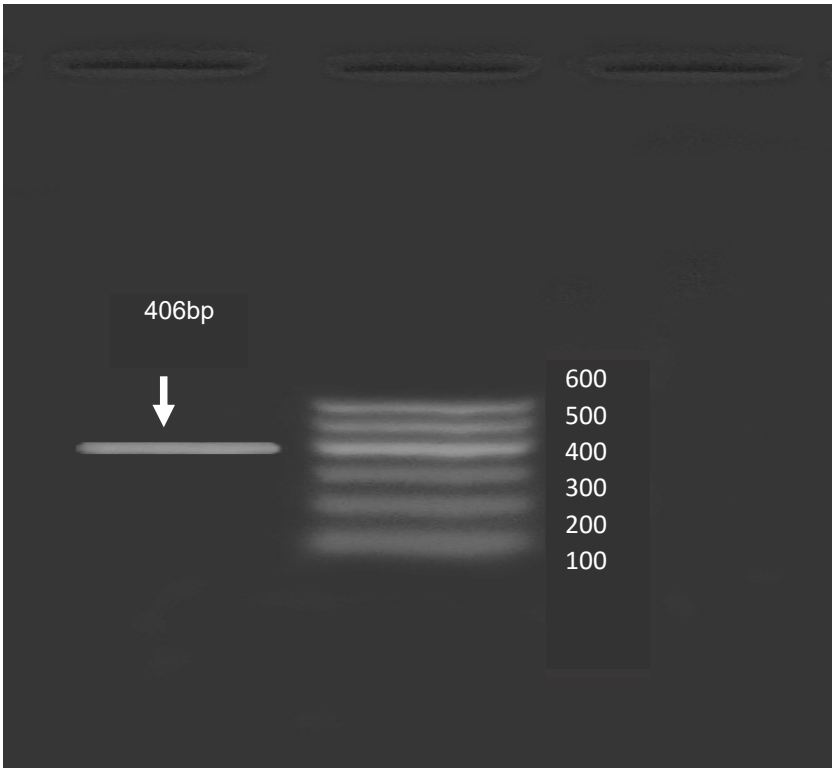

Fig (6) Agrose gel electrophoresis of *ochratoxin A* gene expression, PCR amplification product at 406 bp.

determination. Aflatoxigenic fungi were selected from the *Aspergillus* spp. cereal isolates. For quantification of aflatoxin using the high performance liquid chromatography (HPLC). It was shown that *A. flavus* 1, *A. parasiticus and A. flavus2* were aflatoxins producer with average amount 4.9 ppb, 50 ppb and 200 ppb respectively. These result was agreement with that obtaind by Scherm et al. (2005), Riba et al. (2010), Yazdani et al. (2010), Sourabie et al. (2012), Almoammar et al. (2013), Alwadaia *et al*. (2013), Nair et al. (2014), and Hagagg et al. (2014a) who found that the same species produce Aflatoxin but with different amount. But the result was disagreed with Nosrati and modiri (2014) who thought that *A.niger* and *A.candidus* were aflatoxin producer. the same conclusion reached by Martins et al. (2014) and Moafie et al. (2014) they claimed that *A.niger* was aflatoxin producer, while, Al Hamoud et al. (2012) found atoxigenic strain of *A. flavus*. Moreover, Mostafa and Amer (2013) isolated 17 isolates of *A.flavus* and found that seven strains only Aflatoxin producers.

The observed differences in aflatoxin producing ability (Table 1) could be explained by differences in expression of aflatoxin biosynthetic and regulatory genes between toxigenic isolates from cereals, food due to variation in environmental conditions. Transcriptional expression of the key aflatoxin biosynthetic gene nor-1 gene which encoding an enzyme catalyze the conversion of the first stable aflatoxin biosynthesis intermediate norsolorininc acid to averatin. *nor -1* gene was investigated by Real time PCR and as shown in (Table 2) *A.flavus* 1, *A.flavus 2* and *A.parasiticus* have expressed the *nor-1* gene in different levels. While genomic presence of the structural gene not significantly detected by *A.oryzea* and *A.niger* (Table 2). These result was in agreement with that obtained by Yousefi et al. (2009) Iheanacho (2012) and Passon et al. (2010), and disagreed with Mayer et al. (2003) who found that *A. Oryzea* expressed the *nor1* gene. While, Mahmoud (2015) found that *A.flavus* expressed (*nor-1*) and other strains expressed a regulatory alfR. Moreover, Graz and Buttner (2008) found that *A.flavus* have alfR gene and expressed it with different levels. While, Scherm et al. (2005) stated that there are some other genes, alfR and alfQ present in toxigenic strains of *A.flavus* and *A.parsiticus* controlled in aflatoxin biosynthesis.

From the above mentioned argument, it could be concluded that, the structural genes including *nor-1* presently in use for PCR detection of aflatoxin producing fungi are also involved in the synthesis of other fungal toxins as stregmatocystin by *A.versicolor* and therefore lack of absolute specificity for aflatoxin producing fungi. In addition, the genomic presence of several structural genes involved in aflatoxin biosynthesis not guarantee the production of aflatoxins. The actual differences as we speculated arose from environmental factors or from increase the transcriptional expression of genes involved in aflatoxin biosynthesis by *A. flavus* and *A. parasiticus* isolates. This is exploring the impossibility of genetical discrimination between aflatoxin producer and non. Significant positive correlation was noted between mean aflatoxin production and the mean relation expression level of *nor-1* gene (Table 3). *Ochratoxin A* gene from *Aspergillus ochraceous* by using a specific primers and the *ocrA* gene amplicon has been detected on agarose gel electrophoresis at 406 bp. This result was agreed with that obtained by Patino et al. (2005), and disagreed with Salgg et al. (2005) who found that *ocrA* gene expression un significant. Our results are partly agreed with that of Sartori et al. (2006) found ochratoxin gene amplicon band at 372 bp. While, Fungaro and sartori (2009) and Schmidt et al. (2004) found that *A. ochraceous* gene was expression *OCR- A* gene at 260 bp.

The obvious variance in the above result may be due to technical aspects. As we know unlike bacterial toxins that are

primarily a peptides are therefore encoded by a single gene. Mycotoxin in general such as ochratoxins are multi-ring structures and therefore require a sequence of adequate structural genes for their biological synthesis. Other explanation might be either due to nearly collected DNA or over growth collected leading to change in pattern of purine and pyrimidine bases.

Again it is established that no food or feed substance can escape fungal contamination, consequently, the possibility of mycotoxins elaboration by potentially toxigenic contaminants exists. Therefore, to get free mycotoxins commodities is far reaching. The reason why a philosophy of Guideline or allowed permissible limits find it is way in developing countries. It is aim to minimize the risk of mycotoxins in food, feed and their processing chains to a controlled limit avoiding health threat.

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