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Aflatoxin production and genes assay in *Aspergillusflavus* isolated from spices

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ABSTRACT

Aflatoxin fungi; *Aspergillusflavus* were investigated in some common spices, ten isolates belong to *A. flavus* were detected by using microscopic identification. The toxin production was assay by using total aflatoxin rapid test strips (TARS) and the results showed that the all isolates able to produced toxin, the using of TARS offer a very easy and rapid method to detect coexist aflatoxin products in spices. The PCR technique was used to detect four aflatoxin genes *apa-1, omt-1, ver-1* and *nor-1,* these genes are regulating and building the toxin pathway in *A. flavus,* the PCR result showed the presence of two genes*apa-1* and *omt-1* in all isolates; while there is no evidence to presence the other two rest genes *nor-1* and *ver-1* in any isolates. This may due to the variation in the nucleotides sequences within the isolates which prevent the primers to annealing with DNA template or may due to the PCR reaction condition which including very variable parameter for each single PCR reaction.

Key words: *Aspergillusflavus* fungi, Aflatoxin, Aflatoxin genes, Spice, PCR.

INTRODUCTION

Aflatoxins (AFs) are secondary metabolites produced by filamentous fungi *Aspergillus,* particularly *flavus* and *parasiticus* (1). There are four naturally occurring AFs, B1, B2, G1 and G2 with AFB1 the most common and toxic. Many disease have been documented on several animals: carcinogenic, mutagenic and immuno-suppressive effects of aflatoxins (2).

Human liver cancer and levels of aflatoxins in the daily diet are strictly correlated as epidemiological studies show (3). Molds are widely distributed as environmental contaminants (4), in fact, under favorable conditions of temperature and humidity, molds grow on many commodities including cereals, oil seeds, nuts, herb-teas and spices (5).

Aflatoxin belongs to a group of fungal toxins known as mycotoxins. It was discovered some 30 years ago in England following a poisoning outbreak causing 100,000 turkey deaths. Mycotoxins have received considerable attention due to their significance in agricultural loss and human health. Amongst the mycotoxins that are known to cause human diseases, aflatoxins have been studied most. Aflatoxin poses a potential threat to food safety.

Despite of many studies on the mycotoxins in agricultural products, only a few are concerned with spices and herbal medicines that are more and more common in our daily diet and play an important role in the economy. Furthermore, previous works show that aflatoxins levels are not reduced by domestic cooking with either microwave or conventional gas oven heating (6) and that AFs do not decompose at the temperature of boiling water during the preparation of the drink (7).

It is therefore important to have a simple and quantitative method of analysis to control levels of aflatoxins in food. As aflatoxin is epidemiologically implicated as carcinogen in humans and an environmental contaminant which is widespread in nature, its possible chronic toxicity is therefore of greater concern than acute toxicity. Human and animals can be exposed directly from the ingestion of foods contaminated by Aflatoxin. Several methods have been used to detect Aflatoxinic fungi, such as the traditional dilution plating method, chemical analysis and immunological assays in food and feed. (8)

Unlike bacterial toxins that are primarily peptides and therefore encoded by a single gene, fungal toxins such as the aflatoxins are multi-ring structures and therefore require a sequence of structural for their biological synthesis. Studies in genomic function have been shown that at least more than 25 genes have been determined to involve in aflatoxin

biosynthesis, these genes clustered within a 70 kb DNA region in chromosome aware involved in biosynthesis pathway. Among these genes the *ver-1* gene codes for versicolorin A dehydrogenase which converted the versicolin A to strigmatocystin, the omt A gene is involved in the conversion of sterigmatocystin to O-methylsterigmatosystin, the *nor-1* codes for reductase that converts norsolorinic acid to averanti and *aflR* gene is a regulatory gene that activates the pathway genes. (Pham and Dam, 2010) by controlling the expression of the *nor-1* and *ver-1* genes (9).

MATERIALS AND METHODS

Sampling:

All spices samples were collected from local market, which includes pasta spices, chicken dry soupspices and black pepper.

***Aspergillusflavus* isolation:**

All spices samples were grinding and 1 g from each sample was added to 10 ml of distilled water in 20 ml vial, mixed and diluted by using series dilution method to get 10-3 concentration and 0.1 ml of suspension was cultured in potato sucrose agar and incubated for 3-4 days at 28 ˚ C.

Morphological and microscopic characterization of *A. flavus*:

Morphological and microscopic features consider an important methods to diagnose *A. falvus*, growth period, colony shape and color, colony texture and growth speed have been observed (10), slide culture technique used for microscopic diagnosis to get a pure picture of mycelium and conidia.

Aflatoxin Assay:

Aflatoxin prepared by using Shotwell`s method (11), 50 g of rice added to petri dishes and soaked in water for one hour, the soaked water decant and the petri dishes sterilized by autoclave then inoculated with A. flavus spores and incubated for 7 days at 28 ˚C with keeping wetting through incubation period.

Aflatoxin detection:

The total Aflatoxin detected by using total aflatoxin rapid test strips, it's an immune chromatographic test using colloidal gold immunoassay method. It relies on the competition between Aflatoxin residues in the sample and the Aflatoxin on test line.

Samples preparation:

For Aflatoxin test; 3g of *A. flavus* mycelium with rice was taken into 50 ml centrifuge tube, 10 ml of Acetonitrile added and vortex for 2 minutes and centrifuged at 4000r/min. at room temperatures for 10 min., 6 ml of supernatant moved into another centrifuge tube, then blow to dry by air at 56 ˚C , 0.3 ml of double distilled water with 1 ml of n- hexane added and mixed for 30 seconds the centrifuged at 4000r/min. at room temperature for 5 min., the up solution layer removed and the down solution layer toked for test by putting 60 µl in the sample collect region in the strip and the read the results within 3-5 min.

Fungal Genomic DNA Isolation:

Potato sucrose liquid medium was used for grow the *A. flavus*, 50 ml medium in 200 ml flasks inoculated with fungal spores and incubated for 7 days at 28 ºC(12), method used to isolate the fungal DNA and the method includes:

1. Transferred the mycelium from flask to mortar, crushed in liquid nitrogen by pestle, and obtained fine powder.
2. 6 ml of extraction buffer (1.4 NaCl, 100 mMTrisHCl, 200 mM Na2EDTA, 2% CTAB) added to fungal powder in test tube.
3. 6 ml of chloroform : isoamyl (24:1) added to tube and mixed.
4. The mixture centrifuged at 4000r/min.
5. The upper aqueous layer moved to another test tube and 6 ml of phenol : chloroform (1:24) added and centrifuged.
6. The upper aqueous layer removed and 6 ml of cold isopropanol added and mixed to obtain white filamentous which dissolved in TE buffer (10 mMTris, 1 mM EDTA) and stored in -20 ˚C.

Preparation of DNA samples by diethylether extraction:

To improve DNA yielding diethylether used:

1. In polypropylene tube, diethylether mixed with an equal volume of water and vortex for 10 seconds to allowed phases to separated.
2. Added an equal volume of ether (top phase) to DNA and vortex then centrifuged for five second, discarding top (ether) layer and the operation was repeated.
3. The ether removed by placing opened samples in fume hood for 15 min to evaporate all ether.

DNA concentration and purity:

The DNA concentration and purity measured by Biodrope spectrophotometer.

PCR Amplification of Aflatoxin Genes fragments:

All 10 *A. falvus* isolates were tested for amplification of Aflatoxin genes. The oligonucleotide primers in the PCR were:

Fw Ver-1: 5'-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3' Rv Ver-1 :5'-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3' Fw*Omt-1*: 5'-GGC CCG GTT CCT TGG CTC CTA AGC-3 Rv*Omt-1*: 5'-CGC CCC AGT GAG ACC CTT CCT CG-3 Fw*Nor-1*: 5'-ACC GCT ACG CCG GCA CTC TCG GCA C-3' Rv Nor-1: 5'-GTT GGC CGC CAG CTT CGA CAC TCCG -3' Fw*Apa-2*: 5'-TAT CTC CCC CCG GGC ATC TCC CGG-3' Rv*Apa-2*: 5'-CCG TCA GAC AGC CAC TGG ACA CGG-3', (13)

The primers were purchased from Biolabs (England). PCR analysis was carried out with a DNA thermal cycler (Sensoquest, Germany). Amplification was performed in 25 µl reaction mixture containing 12.5 µl Master mix Approximately 0.2 ng of each genomic DNA was used in each PCR reaction with 1.5 of 10 pmol of each primer, PCR mixture were heated at 94°C for 5 min and then subjected to 30 cycles consisting of denaturation at 94°C for 30s, annealing at 55-65°C for 1 and extension at 72°C for 1 min. A final 10 min. extension step at 72 °C was also included. 25 µl of each PCR product was electrophoresed on 1% agarose in 1X TE buffer and stained with 0.5µg/ml ethidium bromide. The PCR products were visualized with UV under UV transilluminator after 45 min running the gel and compared with a standard DNA size marker (Biolab, England).

RESULTS AND DISCUSSION:

Aflatoxin detection:

The petri dishes in 7 days old of rice medium culture were taken for aflatoxin detection, The fungus colonies were granular, velvety or wooly and yellow or yellow – brown in color; the reverse color was golden to red - brown(14), figure (1).

Figure (1): *A. flavus* grown in rice culture medium for aflatoxin production

Ten *A. Flavus* isolates were obtained from all samples according to morphological specifications, to determine the aflatoxins production; the total aflatoxins rapid test strip was used , the results showed that all isolates were aflatoxins producers by giving positive results " red T line was invisible " which mean the content of aflatoxins in sample is higher than 3ng/g (3ppb) , figure (2).

Figure (2): Total aflatoxins rapid test strip, showing the positive result for all ten isolates represented with red line.

The Total Aflatoxins rapid test strip is an immuno-chromatographic test, using colloidal gold immunoassay method. It relies on the competition between Total Aflatoxins residues in the sample and the Total Aflatoxins immobilized on T line on the membrane for the Total Aflatoxins antibody-dye conjugate. Since the drug has been presenting in the sample, it will compete with the drug immobilized on T line, to the limited amount of dye-antibody. As a sufficient amount of drug in the sample is presenting, the drug will saturate the antibody. Consequently, it will show an extremely light or even invisible T line, indicating a positive result. On the other hand, if there is a negative sample (or the amount of the drug is lower than the minimum detectable concentration) it will generate two obvious lines in both the T and C line section.

The use of Aflatoxins rapid strip test gives an easy, cheap , sensitive and fast method to testing samples for Aflatoxins residue existing in corn, rice, peanut and feed sample. The total test time only need 5 minutes comparing with other complex, difficult, expensive and long methods. The isolation of Aflatoxigenic fungi from common foods show the ability of fungi to grow on various nutrient sources and gives a high risk indicator to health which must be sterilized and save for nutrition,(15).

DNA isolation:

The CTAB DNA isolation method gave a pure genomic DNA with high quality and quantity as shown in figure (3).

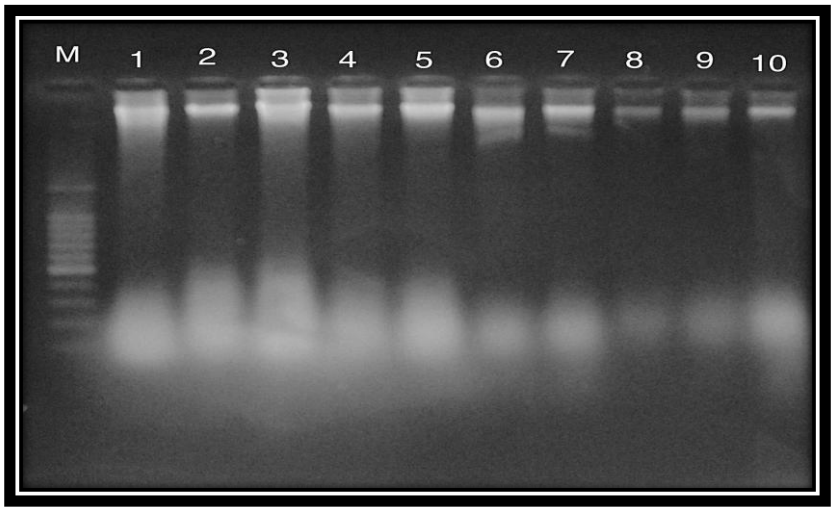

Figure (3): Fungal Genomic DNA for ten Isolates (1-10) in agarose gel with high molecular weight, M: Molecular marker

Using manual method for extraction DNA is cheaper from using the extraction Kits, but sometimes this methods inhibiting PCR reaction due to the phenol trace with extracted DNA Phenol is a highly reactive organic compound and can be present in samples that have been extracted using PCIA (Phenol-Chloroform Isoamyl Alcohol). The results

obtained when increasing concentrations of phenol were added to the PCR reaction mixture included a reduction in amplification efficiency and a 2 cycle shift in C_T (cycle threshold: The number of cycles required during amplification to reach a significant change in fluorescence), indicating Taq polymerase inhibition and inhibitor binding to DNA respectively. Changes in the melt curve by approximately 3^{o}C also showed evidence of inhibitor binding to DNA, the mechanism of inhibition is likely to include effects of the inhibitor on the polymerase as well as binding to the DNA. Although phenol is a protein denaturant, it may still promote unzipping of DNA through hydrogen bonding to single stranded DNA . Phenol binding to DNA results in a decrease in Tm, which indicates a weakening of the hydrogen bonds between bases and an enhancement in the denaturation process. Traces of phenol can completely inactivate the Taqpolymerase .

The using of diethylether improved DNA yielding by removing phenol contamination which can be inhibition the PCR amplification reaction. Phenol is a highly reactive organic compound and can be present in samples that have been extracted using PCIA (Phenol-Chloroform Isoamyl Alcohol) . The results obtained when increasing concentrations of phenol were added to the PCR reaction mixture included a reduction in amplification efficiency and a 2 cycle shift in C_T (cycle threshold: The number of cycles required during amplification to reach a significant change in fluorescence), indicating Taq polymerase inhibition and inhibitor binding to DNA respectively. Changes in the melt curve by approximately 3^{o}C also showed evidence of inhibitor binding to DNA, the mechanism of inhibition is likely to include effects of the inhibitor on the polymerase as well as binding to the DNA. Although phenol is a protein denaturant, it may still promote unzipping of DNA through hydrogen bonding to single stranded DNA (16). Phenol binding to DNA results in a decrease in Tm, which indicates a weakening of the hydrogen bonds between bases and an enhancement in the denaturation process. Traces of phenol can completely inactivate the Taq polymerase (17).

The extraction mechanisms depending on forming two phases: the organic phase and the aqueous phase. The aqueous phase is water-based and can be an acidic, basic, neutral, or a saturated salt solution. The organic phase is an organic solvent, usually diethyl ether or dichloromethane, which has minimal solubility in water. Organic extraction solvents do not mix with water, they form distinct layers, much like oil and water. The denser liquid is the bottom layer. Compounds can be separated based on which liquid they are more soluble in, (18).

Aflatoxins genes detection by PCR:

Four primers belong to four gene " *Apa-1, Nor-1, Omt-1 and Ver-1*" were investigated among ten *A. flavus*isolates from different spices. All attempts to obtain a clear bands for all aflatoxin genes wrer failed to amplification by using gradient thermocycler in annealing temperature range 55-65 ^{o}C depending on the reference (13), after trying different annealing temperature, the temperature 68 ^{o}C gave a pure bands for two aflatoxins genes "*Nor*-1 and *Omt-1"*.The *Nor*-1gene gave a bands for all *A. flavus* isolates in size 400 bpas shown in figure (4)While the *Omt-1* gave a bands in size 1000 bpas shown in figure (5),The selection of the annealing temperature is possibly the most critical component for optimizing the specificity of a PCR reaction. In most cases, this temperature must be empirically tested. The PCR is normally started at 5°C below the calculated temperature of the primer melting point (Tm).In special cases the primer annealing temperature may need to be raised as high as the extension temperature. In fact, high-temperature annealing should result in enhanced specificity, because the hybridization of the primer to the template DNA occurs under more stringent conditions, combining primer annealing and primer extension steps results in a two-step PCR protocol. Primer extension, in most applications, occurs effectively at a temperature of 72 °C and seldom needs optimization. In contrast, in two-temperature PCR experiments, the annealing-extension temperature may be in the range of 60 to 70 °C. Again, the optimal temperature can easily be determined with the gradient function of the thermocycler machine, (19)

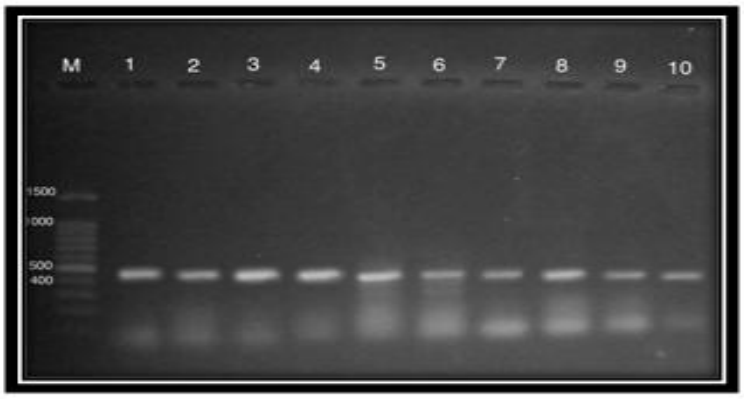

Figure (4): The pure band of *Nor*-1for all ten isolates (1-10) in size 400 bp, M: Molecular weight

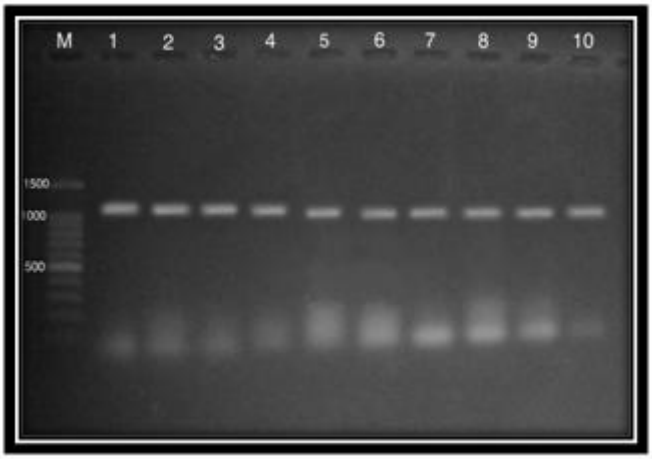

Figure (5): The pure band of *Omt-1* for all ten isolates (1-10) in size 1000 bp, M: Molecular weight

Where is the investigation for the other genes " *Ver-1*and *Apa-1"* were failed, no bands were observed in agarose gel, this may be due to the environment which the strain isolated from, and this what referred by Cotty*et al.* (20)who mentions that there are a differences in aflatoxin production among *Aspergillus* lineages which both *A. parasiticus* and *A. flavus* S_B belong to clades in which aflatoxin-producing ability is highly conserved whereas *A. nomius*and *A. flavus*L isolates belong to clades in which aflatoxin production is highly variable(21). Also considerable variability in numerous traits, including aflatoxin production, probably developed under radiation, the *structure* of *aflR* the pathway specific regulatory gene for aflatoxin biosynthesis, has evolved during divergence of aflatoxin-producing taxa within *Aspergillus* section *Flavi*, as inferred from 28 representative isolates. Sequences difference in the *aflR*coding and promoter regions provide a basis for predicting the roles of environmental and developmental cues in differential regulation of aflatoxin production among aflatoxin-production fungi, (22).

Also the results which described by Geisen (23)' no strong positive PCR signals were found with DNA from aflatoxinogenic*A. flavus*strains. After 30 cycles a weak signal for the *mot-1* gene appeared, and after 40 cycle, a weak band for the *ver-1* gene became visible with DNA from *A. flavus*as the template, but a reaction with the *aflR*-specific primer pair failed. The sequences for the *aflR*gene of *A. flavus*and *A. Parasiticus*are highly homologous, but show distinct differences and failed to detect*aflR gene* in *A. flavus*by PCR,(24).

The using a multiplex PCR approach by targeting three aflatoxinbiosynthetic genes *nor1, ver1* and *omt* have a problems with the specificity, because, while it does appear to distinguish between aflatoxigenic *Aspergillus*species and other non-aflatoxigenic food related species such as *Penicillium, Fusarium, Byssochlamys*and *Geotrichum*spp., it cannot distinguish between aflatoxigenic and non-aflatoxigenic*A. flavus*strains, (23).

Liu and Chu (25), revealed that aflatoxin B_1accumulation was directly related to AFLR expression and was regulated by various environmental and nutritional conditions including temperature, air supply , carbon source, nitrogen source and zinc availability. There is no specific PCR for any one of the four biological produced aflatoxins. Unfortunately the structural genes presently in use for PCR detection of aflatoxin producing fungi are also involved in the synthesis of other fungal toxins such as sterigmatocystin by *Aspergillusversi color* and *Aspergillusnidulans* and therefore lack absolute specificity for aflatoxin producing fungi.

The presence or absent of these genes sometimes doesn't refer to producing aflatoxin as mentioned by (9), which some negative aflatoxin producing strain all genes bans were present, and the presence of all four bands from some non aflatoxinic strains indicates that the absence of aflatoxin production may be due to base pair substitution mutations that result in the formation of non-functional gene products. Also Zachov et al. (26), optimized the PCR for detection of aflatoxin producing strains of *A. parasiticus* and *A. flavus* isolated from feeds, among 50 feed samples; 18 yielded positive growth of aflatoxigenic fungi on agar (AFPA) as evidenced by an orange mat of growth on the reverse colony side, among these 18 cultures 16 were confirmed by PCR as processing the *apa-2* and *ver-1* genes. Beside of that Rahimi et al.(27) concluded that since the *Omt*amplicon resulted from all aflatoxin isolates of *A. flavus* and *A. parasiticus* examined and its absence from non-aflatoxin producing aspergilla, the Omt primers are specific for aflatoxin producing fungi.

No single set of PCR primers can reliably be used to detected aflatoxin producing molds since aflatoxin precursor gene also involved in the synthesis of other fungal toxins such as sterigmatocystin by non aflatoxin producing mold. In addition, the successful amplification of targeted gene sequences cannot be taken as a proof of aflatoxin production since the gene may be cryptic and not expressed due to an undetected mutation external to the amplicon sequence. The use of *ver, nor ,apa* and *omt* genes appears to offer some promise in detecting aflatoxin producing molds particularly

with respect to the ability to distinguish characteristic DNA banding pattern derived from amplicon of appropriate size, (9).

The lack of high level of sensitivity of the PCR when applied to foods without enrichment most probably reflects difficulties in DNA purification and the presence of PCR inhibitors from food present in DNA target preparations. Many primers designed to detect aflatoxins genes, and every gene have more than one primer which gives an idea for the variable within the genes sequences and this what outlined by Shapira *et al*. (28) by using PCR method for detection of aflatoxigenic fungi. The target genes were *omt1* ,*ver 1* and *apa2*, specific PCR products were obtained only with DNA from *A. parasiticus* with all three primers pairs, with the *apa 2* primers failing to give positive signal with other *Aspergillus.* This probably reflect the subtle sequence difference between the *apa 2*gene in *A. parasiticus* (for which primers were designed) and the *apa2* gene in *A. flavu* (23), (29) .

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التحري عن انتاج وجينات سموم الافلاتوكسين في عزلة الفطر*Aspergillusflavus* المعزولةمن التوابل الخلاصة

غيداء صلاح حسين
كلية التربية الاساسية

محمد ابراهيم خليل
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عقيل محمد شريف
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جامعة الموصل

الملخص
أُجري التحري عن الفطريات المنتجة للافلاتوكسين التابعة للجنس *Aspergillusflavus* في عدد من التوابل الشائعة الاستخدام حيث تم تشخيص 10 عزلات من الفطر *A. flavus*باستخدام التشخيص المايكروسكوبي . استخدمت شرائط التحري عن سموم الافلاتوكسين Total AflatoxinRapid Test Strips ووجد ان جميع العزلات لها القدرة على انتاجه،حيث تتميز هذه الطريقة بسهولة وسرعة تشخيص تواجد السموم في المنتجات الغذائية. كما وأُجري التحري عن وجود الجينات المسؤوله عن بناء الافلاتوكسين*nor-1* و *ver-1,* و *omt-1* و*apa- 1*باستخدام تقنية PCR في الفطر *A. flavus* حيث شُخص وجود اثنين من الجينات وهي *omt-1*و*apa- 1* في جميع العزلات المعزولة بينما لم يشخص وجود كل من الجينين *nor-1* و*ver-1,* فيها ، وقد يعزى السبب في هذا الى الاختلاف في تسلسل القواعد النتروجينية بين انواع الجنس الواحد والذي يؤدي الى عدم فعالية البادئات في ارتباطها معDNA القالب او الى ظروف تفاعل الـPCR المستخدمة في البحث والتي تكون متغايرة لكل تفاعل ومكوناته.
الكلمات المفتاحية : فطر*Aspergillusflavus* ، سموم الافلاتوكسين ، جينات الافلاتوكسين ،PCR ، التوابلز