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Detection of Aflatoxigenic *Aspergillus Flavus* in Maize Grains and Soils in Sulaimani Province using Molecular Approaches

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Abstract

Aflatoxins are potent carcinogens produced *by A. flavus* as secondary metabolites. They have the ability to contaminate a large number of food, which ultimately affect the human population. Investigating the incidences of aflatoxigenic species of *A. flavus* in the local area samples were the aim of current study. Samples were collected randomly from soil and maize grains in the Sulaimani governorate. Morphological features and specific PCR-based protocol were used for identification of *A. flavus* isolates. This PCR protocol was based on the multi-copy internal transcribed region of the rDNA unit (ITS1-5.8S-ITS2 rDNA), which allowed discrimination from other closely related species. Molecular approach has been used for detection of aflatoxigenicity of the *A. flavus* isolates. In this study out of eighteen isolates only seven isolates were molecularly and morphologically identified as *A. flavus*. The aflatoxigenicity of these seven isolates were detected by molecularly method which successfully amplify two structural genes (*aflD* and *aflO*) out of three structural genes, and one regulatory gene (*aflR*). Furthermore, no specific amplicon for (*aflP*) was obtained in this study.

1. Introduction

Food and feed contamination by mycotoxins is a significant food safety issue in the world, especially in developing countries. Mycotoxigenic fungi have the ability to contaminate a wide range of food and animal feed with one or more mycotoxins [1,2]. Mycotoxins produced by a varieties of fungi [3].There are now around 300 to 400 compounds recognized to be mycotoxins [4,5]. Some of these mycotoxins are important and focused on, including: aflatoxins, ochratoxins, deoxynivalenol, zearalenone, fumonisin, T- 2 toxin, and T-2 like toxins. One of the most common foodborne fungi is the aflatoxin producer. Contamination of agricultural commodities by aflatoxins are a serious problem due to the substantial health effect it has on humans and animals [3].

Aflatoxins are potent carcinogenic, mutagenic and teratogenic secondary metabolites and aflatoxins are produced predominantly by *Aspergillus flavus* and *Aspergillus parasiticus* [6,7,8,9]. In humans, the risks associated with aflatoxins consumption have been well recognized, and the International Agency for Research on Cancer (IARC) has designated aflatoxins as a human liver carcinogen [4]. The aflatoxinogenic fungi can contaminate several food commodities including cereals, maize (corn), cotton, groundnuts (peanuts), pistachio nuts, and walnuts [9,10,11]. Maize used as a source of human and livestock food. It is also one of the crops subject to the most critical mycotoxin problems throughout the world. Furthermore, it is

one of the main cereals in Kurdistan region, which is annually produced in large amounts to be used in local cereal products [12].

There are many cultural and highly specific and sensitive methods for determining aflatoxins in culture, such as culture-based methods that determine if a sample is contaminated with aflatoxigenic fungi [10,13,14]. In many of these methods, more specialty and experts require for detection the presence of aflatoxins. Recently, DNA based detection systems have been introduced as powerful tools for detecting and identifying the aflatoxin producing fungi. One of which, includes the use of polymerase chain reaction (PCR) as it generates multiple copies of the region to be amplified easily and more accurately than many other methods being used [15,16].

Here, we used molecular approach for accurate identification and detection of aflatoxigenicity of isolates of *A. flavus*, which allowed us to investigate the incidences of aflatoxigenic species of *A. flavus* in the local area samples.

2. Materials and Methods:

2.1 *Survey Sites*:

Composite soil samples were collected from the surface layer (0.0 -0.3 m) of the dominant cropped soils at different sites within Sulaimani Governorate. The sites encompassed (Kanimara,Dukan, Halabja, Peramagron, Rania, Khwrmal and Twella), that included agricultural soils cultivated with maize and different vegetables. Maize grains were collected from corn fields in (Halabja, Hawraman and Rania) regions. All samples were collected in between (2^{nd} of July 2014 -10^{th} of Oct 2014).

2.2 *Fungal isolation:*

2.2.1 *Isolation fungi from the Soil samples:*

Soil samples were subjected for fungal isolation within two days of collecting. Fungi were isolated by using soil dilution plate method on (PDA) and (AFPA). Both Potato-Dextrose Agar (PDA) and Aspergillus Differentiation Agar media (AFPA) were prepared according to the instructions as indicated by the manufacture. After adding (100 mg\1L) chloramphenicol antibiotic for prevention of bacterial growth, they were sterilized by autoclaving at 121ºC for 15 min under 1.5 bar pressure. The Petridishes were then incubated at 28º C. The plates have been observed after (5-7) days [17,18,19,20].

2.2.2 *Isolation fungi from the Maize samples:*

Maize grains were collected from three different locations of Sulaimani Governorate. Direct plate method used for isolation of fungi from maize samples. This method previously described by [21]. This step was followed by surface sterilization of the grains in NaOCL for 120 seconds, then the maize grains were washed off using dH_2O. Later on, drying was performed by using sterile Whatman filter paper, and plated on sterile (PDA) at the rate of 10 grains per plate. Thirty seeds were plated per plant and incubated at a room temperature of 28 ^{o} C.

2.3 *Identification of Aspergillus species:*

2.3.1 *Morphological identification:*

Morphological characteristics such as growth rate, colony diameter, aerial mycelium and pigmentation of fungi or colony reverse color, conidiophore, vesicle, metulae, phialides and conidia are generally basic and essential tool for identification of *Aspergillus* species. Macroscopic colony characters were examined under the dissecting microscope after *Aspergillus* isolates have been grown on selective and differential media (AFPA), and (PDA) at 28^{o} C and 25^{o} C respectively for seven days.

After *Aspergillus* isolates have grown on both media under the above mentioned incubation conditions for macroscopic analysis, new subcultures of *Aspergillus* isolates were prepared on (AFPA) and (PDA) for studying microscopical characters. Most microscopic characters such as (conidiophore, vesicle, metulae, phialides and conidia) were examined by (Slide Cultures Technique) as described by (Shekhany, 2008). Slides were prepared from these cultures by using lactophenol cotton blue as a mounting medium then examined under light microscope. Macroscopic and microscopical characters have been used for identifications of isolated fungi as reported by [10,22,23,24].

2.3.2 *Molecular identification:*

PCR-based methods that target DNA are considered a good alternative for rapid diagnosis due to their high specificity and sensitivity, and have been used for the detection of aflatoxigenic strains of *A. flavus* [6,10,25]. The genomic DNA from the all seven isolates were extracted and prepared by using DNA extraction kit (Bio Basic) as described according to manufacturer's instruction.

DNA used as template for PCR amplification. We used a PCR protocol based on multi-copy sequences (ITS) (ITS-1, 5.8 S and ITS-2) of rDNA specific to *A. flavus*, which allows distinction from other aflatoxigenic molds, in particular from *A. parasiticus*, and organisms commonly found on grains [6,10,25,26]. Specific PCR assays were carried out using the purchased primers FLA1 (5'-GTAGGGTTCCTAGCGAGCC-3') and FLA2 (5'-GGAAAAAGA TTGATTTGCGTTC-3') for *A. flavus*.

PCR reactions were performed in the Eppendorf Mastercycler Gradient. The PCR amplification protocol for *A. flavus* was as follows: 1 cycle of 5 min at 95 °C, 26 cycles of 30s at 95 °C (denaturation), 30 s at 58 °C (annealing), 45 s at 72 °C (extension) and, finally, 1 cycle of 5 min at 72 °C [6,26].

Amplification reactions were carried out in volumes of 25 µl containing 4 µl (10 pg–100 ng) of template DNA, 1 µl of each primer (20 mM), 2X PCR reaction buffer, 4 mM of $MgCl_2$, enzyme stabilizer, loading dye, and 0. 5 mM of dNTPs (100mM) and Taq DNA polymerase (1unit \ 10µl) supplied Prime Taq Premix GeNetBio (Korea). PCR products were detected on 2% agarose ethidium bromide gels in TAE 1X buffer (Tris–acetate 40mM and EDTA 1.0 mM). The 100-bp DNA ladder GeNetbio (Korea) was used as the molecular size marker.

2.4 *Determination of aflatoxigenic potential of A.flavus*:

2.4.1 *Detection of aflatoxin producers based on cultural characteristics in Aspergillus differentiation media*:

In this method, aflatoxigenic *A. flavus* species have been distinguished from other *Aspergillus* species based on the revers orange color on the plates after seven days of incubation at 28 °C. Orange color in the reverse of the colonies is due to the reaction of ferric ions from ferric citrate present in the medium with the aspergillic acid molecules synthesized by *Aspergillus* species [10,15,27].

2.4.2 *Molecular detection of aflatoxigenic A.flavus isolates:*

Recently, molecular methods have been developed to distinguish and identify aflatoxinogenic strains of *A.flavus* from other food-borne fungi [27]. Nowadays polymerase chain reaction (PCR) is extensively used for aflatoxin detection as it is easy to employ, fast, and accurate [16]. Several research groups [28, 29,30] have tested the possibility of applying PCR-based detection techniques for the presence of genomic DNA by conventional PCR. Some others, however, used Reverse Transcription PCR (RT-PCR) or Real-time PCR to detect the expressivity of the aflatoxin biosynthetic genes.

Cluster genes in aflatoxin biosynthesis pathway contain structural and regular genes, *nor-1, omtB,* and *omt-1* are structural genes that coding for key enzymes and *aflR* is a regulatory gene that plays a key role in the production of aflatoxin and is affecting on the structural genes and activate transcription. Four primer pairs have been used for specific implication of (*aflD, aflO, aflP*, and *aflR* genes table (1) [31]. The housekeeping gene tub1 coding β-tubulin may be chosen as a system control for DNA amplification.

Amplification reactions were performed by using the Prime Taq Premix GeNetBio (Korea) kit with 25 µl reaction mix containing 4 µl (10 pg–100 ng) of template DNA, 1 µl of each primer (20 mM), 2X PCR reaction buffer, 4 mM of $MgCl_2$, enzyme stabilizer, loading dye, and 0. 5 mM of dNTPs (100mM) and Taq DNA polymerase (1unit \ 10µl). PCR products were detected on 2% agarose ethidium bromide gels in TAE 1X buffer (Tris–acetate 40mM and EDTA 1.0 mM). The 100-bp DNA ladder (100-1500) GeNetbio (Korea) was used as the molecular size marker. Cycling parameters were: 5 min at 94 °C, for 35 cycles, 30 s at 94 °C, 60 s at 50 °C, 90 s at 72 °C, with a final extension at 72 °C for 7 min in a DNA thermal cycler [32].

Table- 1: Primers used in this study, target gene, sequence & expected PCR size.

Primer code	Gene	Primer sequence (5' to 3')	Expected size (bp)	Optimal annealing Temp. (°C)
Nor1- (F)	*aflD*	ACGGATCACTTAGCCAGCAC	990	50
Nor1- (R)		CTACCAGGGGAGTTGAGATCC		
omtB(F)	*aflO*	GCCTTGACATGGAAACCATC	1333	50
omtB(R)		CCAAGATGGCCTGCTCTTTA		
Omt1-(F)	*aflP*	GCCTTGCAAACACACTTTCA	1490	50
Omt1-(R)		AGTTGTTGAACGCCCCAGT		
AflR-(F)	*aflR*	CGAGTTGTGCCAGTTCAAAA	999	50
AflR-(R)		AATCCTCGCCCACCATACTA		
Tub1-(F)	*tub1*	GCTTTCTGGCAAACCATCTC	1498	50
Tub1-(R)		GGTCGTTCATGTTGCTCTCA		

3. Results:

3.1 *Morphological detection of A. flavus*:

Examination of cultural and morphological features can be used as a key to identify species in the *A. flavus* group and to differentiate between this group and other fungal groups with similar morphologies [10 ,22,24,33].

The color of the colonies was used for first identification of *A. flavus*. After seven days of incubation at 28°C on AFPA, and at 25°C on PDA, plates were observed for macroscopic characteristics such as colony color, colony diameter, colony reverse color figures (1) and (2). Table (2) illustrates the macro and micro morphological characteristics of *A. flavus*.

Table (2) Morphological features used to identify *A. flavus*.

Morphological Characteristics	Name of medium	
	AFPA	PDA
Colony color\ Texture	White \ Granular	Yellow to Green\ Granular
Colony diameter (mm)	15-18	35-40
Colony reverse color	Yellowish orange	Hyaline
Seriation	Biseriate\Uniseriate	Uniseriate\ Biseriate
Vesicle Shape	Globose	Globose
Conidia Head Shape	Radiate	Radiate

The size of the colonies are observed after seven days of incubation on both media.

Figure (1) Colony characteristics of *A. flavus* : Name of Medium: AFPA, Colony color: White , Colony Diameter: (18) mm, Colony Reverse Color: Yellowish orange After seven days of incubation at 28°C.

Figure (2) Colony characteristics of *A. flavus* : Name of Medium: PDA, Colony color: Yellow to Green, Colony Diameter: (35) mm, Colony Reverse Color: Hyaline, After seven days of incubation at 28°C.

A. fluvus is morphologically characterized by yellow green woolly or granular colonies on PDA. The colonies are flat, with radial grooves. This species is microscopically characterized by conidiophores that are hyaline and globose vesicles bearing chains of conidia. Conidia appeared globose to subglobose, pale green in color [24,34]. Conidial heads are typically radiate figure (3) and biseriate figure (4), but having some heads with phialides borne directly on the vesicle figure (5).

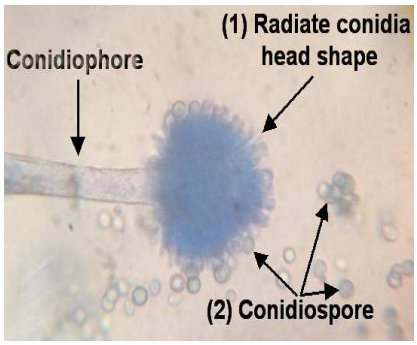

Figure (3) Conidial heads shape and Conidiospore: 1) Radiate conidia head shape. 2) Chain of Conidiospores. Microscopic observation of the fungal isolate under 400x magnification (lactophenol cotton blue).

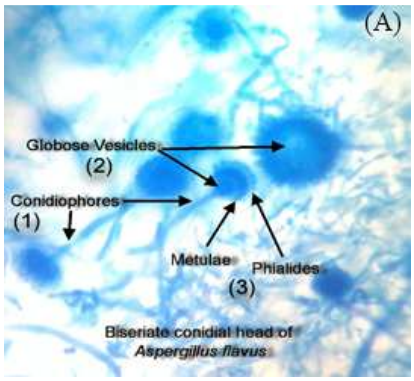

Figure (4) Biseriate conidial head of *A. flavus*: 1) Aspergillum like spore bearing conidiophore. 2) Globose vesicles. 3) Vesicles bearing (Metulae and Phialides) biseriate conidial head. Microscopic observation of the fungal isolate under 400x magnification (lactophenol cotton blue).

Biseriate conidial head has been used as a primary separation key of *A. flavus* from *A.parasiticus*. However not all *A. flavus* isolates consistently produce metulae [10].

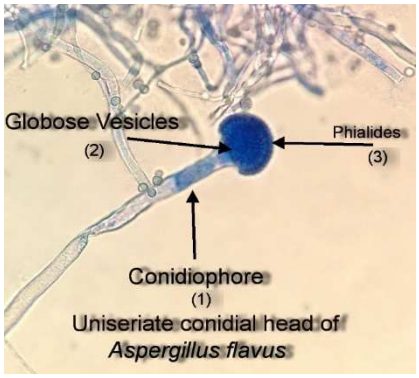

Figure (5) Uniseriate conidial head of *A.flavus*: 1) Aspergillum like spore bearing conidiophore. 2) Globose vesicles. 3) Phialides borne directly on the vesicles.
Microscopic observation of the fungal isolate under 400x magnification (lactophenol cotton blue).

3.2 *Molecular Identification of A.flavus*:

Seven out of eighteen *A. flavus* isolates included in this study were identified by specific amplification of a DNA fragment of expected size (500 bp) in PCR by using specific primers FLA1 and FLA2 figure (6) [6,25]. PCR based methods that target DNA are considered a good alternative for rapid diagnosis due to their high specificity and sensitivity, and have been used for the detection of aflatoxigenic strains of *A. flavus* [6].

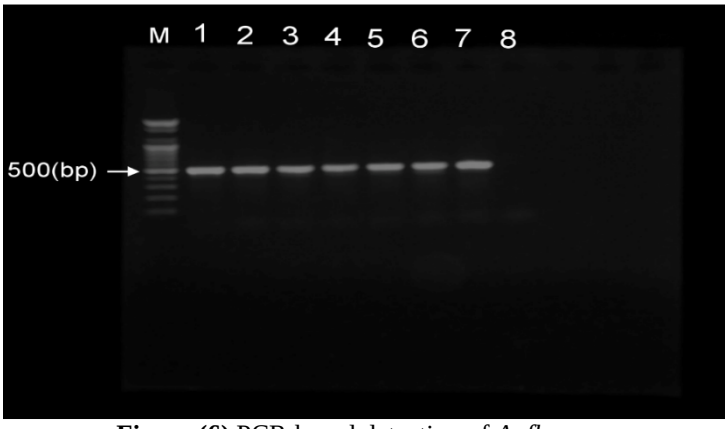

Figure (6) PCR-based detection of *A. flavus*.
Lanes (1–7) PCR amplification product (500 bp) by using primers FLA1/FLA2 and DNA from *A. flavus* isolates. *Lane* (8) Non DNA template. M: (100) bp DNA molecular size marker. Reactions were carried out in volumes of 25 µl containing 4 µl (10 pg–100 ng) of template DNA, 1 µl of each primer and PCR Master mix.
PCR products were detected on 2% agarose ethidium bromide gels in TAE 1X buffer.

3.3 *Cultural method for aflatoxin detection:*

There has been much interest in developing and using cultural method for detecting aflatoxins in fungal cultures. Various analytical methods have been reported for the detection of aflatoxins. Screening was done by using orange color characteristics in Aspergillus differentiation media [13,15].

3.3.1 *Detection of aflatoxin producers based on reverse color change on AFPA*:

Aspergillus differentiation media (AFPA) is a selective and differential medium which is used for detection of aflatoxin producing *Aspergillus* species. As it can be seen from figure (7), after an incubation time of 7 days at 28 ºC, an orange color is developed in the reverse side of the plate. This is because of ferric ions from ferric citrate present in the medium reacts with the aspergillic acid molecules synthesized by *Aspergillus* species.

Figure (7) *A. flavus* on AFPA, after 7 days incubation at 28ºC, with characteristic orange color on reverse side of plate.

3.3.2 *Detection of aflatoxin producing strains by conventional PCR method:*

For molecular diagnosis of aflatoxin producing strains of *A. flavus,* polymerase chain reaction (PCR) was carried out for three structural genes (*aflD, aflO,* and *aflP*) and (*aflR*) is a regulatory gene. The housekeeping gene tub1 coding β-tubulin has been chosen as a system control for DNA amplification. PCR results revealed that all toxigenic isolates of *A. flavus* included in this study contained two structural (*aflD* and *aflO*) genes out of three structural genes, and regulatory gene (*aflR*) has been detected in all toxigenic isolates, figure (8). Non DNA templates running for each primers have been used to detect there were no any contamination in our PCR results.

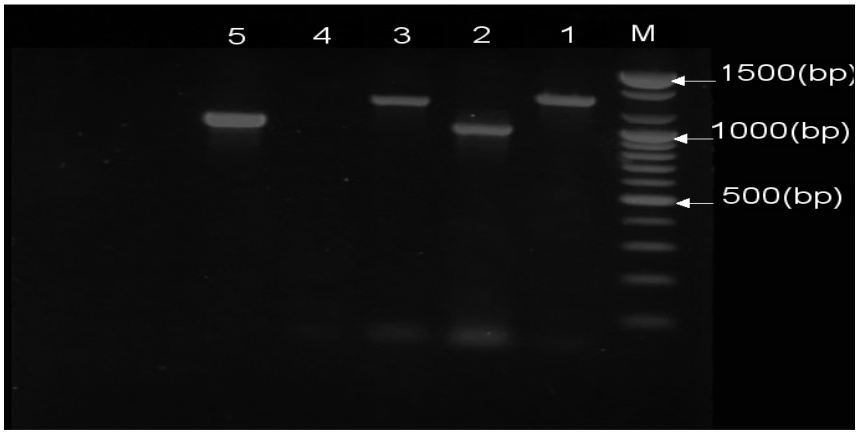

Figure (8) PCR-based detection of Aflatoxigenic *A. flavus.*

Reactions were carried out in volumes of 25 µl containing 4 µl (10 pg–100 ng) of template DNA, 1 µl of each primer, 5.5 µl of RNA DNA free and PCR Master mix. PCR products were detected on 2% agarose ethidium bromide gels in TAE 1X buffer. Gel electrophoresis analysis of PCR products using primers and DNA extracted from isolate one of *A. flavus*. M: (100) bp DNA molecular size marker. *Lanes* (1) DNA of *A. flavus* isolate using housekeeping gene *tub1*primer with 1498bp. *Lanes* (2) DNA of *A. flavus* isolate using *aflD* primer with 990bp. *Lanes* (3) DNA of *A. flavus* isolate using *aflO* primer with 1333bp. *Lanes* (4) DNA of *A. flavus* isolate using *aflP* primer no any product. *Lanes* (5) DNA of *A. flavus* isolate using *aflR* primer with 999bp.

4. Discussion:

4.1 *Morphological identification of the isolates:*

All *A. flavus* isolates included in this study were identified based on their characteristics and description features reported by [10,22,24,23,34]. *A. fluvus* is morphologically characterized by yellow green woolly or granular colonies on PDA its colonies are flat, with radial grooves [34]. Differences in colony diameter of *A. flavus* have been observed, colony diameter on AFPA is smaller compared to its diameter on PDA. Difference in nutrient ingredients of media may make a difference in colony diameter of fungi.

Rodrigues *et al.* [10], reported that microscopical features like metulae and phialides can be used as primary keys to differentiation between *A. flavus* and *A. parasiticus*. Seriation among *A. flavus* and *A.*

parasiticus tend to be vary in a way that *A. parasiticus* is mostly observed having uniserate conidial head. However, *A. flavus* shows biseriate conidial. Figure (4) and (5) show conidial head seriation in *A. flavus*.

4.2 *Molecular identification of the isolates:*

Seven out of eighteen isolates in this study were identified by specific PCR protocol. Molecular methods have been widely applied in the identification of a large number of *Aspergillus* species. Including studies within *A. flavus*, comparing *A. flavus* with other *Aspergillus* species, and even for differentiating aflatoxin producers from non-producers. Several rDNA complex regions and structural aflatoxin genes have been tested for use as molecular markers, with different levels of success [10]. Al-Wathiqi *et al*., [25] successfully used two different sets of primers for identification of *A. flavus*, universal primer for (ITS-1 and ITS-2) (genus specific primers), and (species specific primers) for variable regions of β-tubulin and calmodulin genes. Some genes like Topoisomerase 2 calmodulin, as well as β-tubulin are extensively used to identify fungi at species level. However, the use of these genes is limited to distantly related fungal species. [35,36]. El Khoury *et al*., [37] designed gene-specific primers for the aflatoxin biosynthesis genes. In order to distinguish between *A. flavus* and *A. parasiticus*.

The most widely used DNA target regions to identify *Aspergillus* species are the ones in the rDNA complex, mainly the internal transcribed spacer regions (ITS1-5.8S-ITS2 rDNA) [6,9,10,25,38] . A specific PCR protocol based on multi-copy sequences (ITS1-5.8S-ITS2 rDNA) specific to *A. flavus* was used in this study that allows its differentiation from other aflatoxigenic molds, in particular from *A. parasiticus*, and organisms commonly found on grains [6]. This protocol, however, does not allow discrimination between *A. flavus* and *A. oryzae*, because they have shown to possess high degrees of DNA relatedness and similar genome size [6,10,39] with the exception of few difference in genes involved in aflatoxin biosynthetic pathway [39]. To differentiate between *A. flavus* and *A. oryzae*, we detect two structural genes and one regulatory gene in seven isolates.

4.3 *Detection of aflatoixgenic A. flavus:*

Not all strains of *Aspergillus* are able to produce mycotoxins, therefore, there is a need for screening of their toxin production abilities. There are many advanced and accurate, but expensive and time consuming techniques used to determine aflatoxin concentration, these are: high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and thin layer chromatography (TLC) [15,16].

One cultural based method was used in order rapidly differentiate between aflatoxigenic and non aflatoxigenic *Aspergillus* [10,13,14].

4.3.1 *Detection of aflatoixgenic A. flavus by using the culture based method (Aspergillus differentiation media)*:

(AFPA) is a selective and differential medium for *A. flavus* and *A. parasiticus*. It was used for the rapid detection of aflatoxigenic species. Aflatoxigenic *A. flavus* can produce yellowish orange color on the reverse side of the colonies, when they have been grown on the (AFPA) medium. The coloration was due to reaction of ferric ions, from ferric citrate, with aspergillic acid molecules synthesized by *Aspergillus* species [***10,15].*** *A. flavus* and *A. parasiticus* are capable of producing potent aflatoxins. Several papers reported that toxigenic *A. flavus* isolates generally produce only aflatoxins B1 and B2, whereas *A. parasiticus* isolates produce aflatoxins B1, B2, G1 and G2 [3,8,11]. Our results shows that this medium *(*AFPA) is suitable for primary detection of *A. flavus* and, potentially, of aflatoxins.

4.3.2 *Detection of aflatoixgenic A. flavus by using molecular method (conventional PCR)*:

Microbiological identification, high-performance liquid chromatography (HPLC), thin layer chromatography (TLC) and enzyme-linked immunosorbent assay (ELISA) are examples of the conventional methods used for the detection of aflatoxins. These methods are considered as reliable methods for detection of aflatoxigenic *A. flavus* [13,15,16]. However, these methods are mostly time consuming and requiring labor. Molecular techniques have been widely applied to distinguish the aflatoxin producing and non-

producing strains of *A. flavus* and related species. This has been through the correlation of presence-absence of one or several genes involved in the aflatoxin biosynthetic pathway and the ability-inability to produce aflatoxins. Recently, DNA based detection systems have been introduced as powerful tools for detecting and identifying the aflatoxin producing fungi [16,40,28]. Several PCR based systems have been developed to discriminate between aflatoxin producing and non-producing *A. flavus* strains.

Criseo *et al*., [27], Degola *et al*., [28], Criseo *et al*., [41], Rahimi *et al*., [42], Navya *et al*., [43] and Hussain *et al*., [16], used a multiplex PCR approach based on the amplification of four target DNA fragments (*aflD , aflO, aflP* and *aflR*) for detection of aflatoxigenic *A. flavus*. Latha *et al*., [44] reported that the multiplex PCR could be used as a marker to clearly differentiate between the aflatoxin-producing and non-aflatoxigenic *A. flavus*. Mayer *et al*., [45] have used real-time PCR to monitor the expression of an aflatoxin biosynthetic gene of *A. flavus* in wheat. Houshyarfard *et al*., 2014) reported that the analysis of deletion patterns in aflatoxin gene cluster was a useful marker for the identification of non-aflatoxigenic strains. Furthermore, several research groups have adopted reverse transcription PCR technique (RT-PCR) to detect an mRNA specific for an aflatoxin biosynthetic gene and differentiate aflatoxin-producing from non-producing strains of *A. flavus* [28,32,45].

Among the 27 genes involved in aflatoxin biosynthesis only three structural key genes (*aflD, aflO,* and *aflP*) and one regulatory gene (*aflR*) have been used in this study as a target molecular markers to detect the aflatoxigenic *A. flavus*. Scherm *et al*. [32] studied 13 isolates of *A. flavus* and found consistency of (*aflD, aflO* and *aflP*) genes in detecting aflatoxin production ability, further indicating them as potential markers. Most of the genes in the aflatoxin biosynthetic pathway gene cluster are regulated by (*aflR*) [11]. (*aflO,*and *aflP*) are necessary for final formalities of aflatoxins biosynthesis [46,47].

In the present study, molecular method is proved as a rapid and accurate detection system to detect aflatoxigenic *A. flavus*. Our results for all aflatoxigenic isolates shows that bands of the fragments of (*aflD, aflO* and *aflR*) genes visualized at (990, 1333 and 999bp) respectively in figure (8). While no specific amplicon for (*aflP*) was obtained in our condition even if we used the same pair of primers described by the [32]. Criseo *et al*., [27] obtained a four band pattern for all producers and a variable pattern for non-producers of aflatoxin. Results of quadruplex PCR for two aflatoxgenic strains of *A.flavus* (MAM 084 and MAM 096) show very weak signal results for (*aflP*). Also presence of a complete pattern in the non-aflatoxinogenic strains shows that it is not a sufficient marker for differentiation between aflatoxinogenic and some non-aflatoxinogenic strains. Degola *et al*., [28] discarded (*aflP*) because, their results under their experimental conditions demonstrated that the RT-PCR signal was not as significant as that for the other genes. Rodrigues *et al*., [10], Scherm *et al*. [32], Latha *et al*., [44] reported that the expression profile of the three genes (*aflD, aflO,* and *aflP*) were consistently correlated with a strain's ability to produce aflatoxins. Degola *et al*., [28] shows that one strain that apparently transcribed all the relevant genes (*aflD, aflO* and *aflQ*) but did not produce aflatoxin in the medium.

Houshyarfard *et al*., [48] reported that production of aflatoxin is bound to several factors. Firstly, the presence of certain genes. Secondly, the genes should be intact. Meaning that, there should not be major deletions or insertions within the gene regions or regions flanking the gene. Otherwise, deletions of several portions of the aflatoxin biosynthesis gene cluster has been reported to be the main cause for the lack of aflatoxin production.

Degola *et al*., [28] found that screening tests based on PCR detection of aflatoxin biosynthesis genes are not dependable, this is due to the technique that cannot detect mutations outside the primers' targeted region of the gene sequence. No sign of aflatoxigenicity can be observed for eleven isolates when we used the one culture method for detection of their toxigenicity. This is the reason why we consider these isolates as non aflatoxigenic isolates. Non aflatoxigenicity of these isolates may be because of a mutation or gene deletion in one or more genes belonging to the biosynthetic gene cluster [28]. Houshyarfard *et al*., [48] said that the analysis of deletions within the aflatoxin biosynthesis gene cluster for the 15 Iranian non-aflatoxigenic strains of *A. flavus* revealed that *A. flavus* strains had different deletions in the aflatoxin gene cluster.

In this study, it is concluded that seven isolates of *A. flavus* were able to confirm the aflatoxin production by amplifying the three target genes (*aflD, aflO* and *aflR*) as these genes are considered as indicators of aflatoxin production. Not all structural aflatoxin genes which have been detected in current local isolates of *A. flavus*, (*aflP*) was not detected in all toxigenic local isolates.

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