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Culture methods and conventional PCR for detection the aflatoxigenicity of *Aspergillus flavus* in local isolates samples

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Abstract

One of the main carcinogenic aflatoxins producers belong to Aspergillus section Flavi is *Aspergillus flavus*. Not all fungi from Aspergillus section Flavi produce aflatoxins. It is important to use reliable and accurate methods to differentiate Aspergillus species in to toxigenic and nontoxigenic strain. Soil and maize grains of Sulaimani governorate were subjected for our study. Primary isolation and identification of isolates performed based on using the morphological features of *Aspergillus flavus* on selective and differential media. PCR-based protocol used for more accurate identification of isolates as *Aspergillus flavus*, which based on the multi-copy internal transcribed region of the rDNA unit (ITS1-5.8S-ITS2 rDNA). Different culture and highly specific sensitive methods used for determining aflatoxigenicity of our isolates. As a culture methods, colony fluorescence, ammonia vapor test and characteristics of *Aspergillus flavus* on aspergillus differentiation media have been used. Accurate detection of aflatoxigenicity of our isolates confirmed by using conventional PCR for detection of the potential gene markers (aflD and aflO),in the aflatoxin biosynthesis. Out of eighteen isolates of *Aspergillus flavus*, aflatoxigenicity seven of them have been detected and confirmed by both culture and molecular methods. In conclusion both culture and molecular methods could be used for rapid detection of aflatoxigenicity of *Aspergillus flavus*.

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Culture based, Aspergillus flavus, Aflatoxin detection, polymerase chain reaction (PCR).

Introduction

Air Food and feed contamination by mycotoxins is a significant food safety issue in the world and developing countries. Mycotoxins such as aflatoxins have pivotal health effect on humans and animals. One of the most common foodborne fungi are the aflatoxins producer [1]. Aflatoxins of *A.flavus* and *A.parasiticus* are the most widely subjected for investigation, due to that they have carcinogenic, mutagenic and teratogenic properties [2-3-4-5-34].

Several methodologies have been developed for detection of aflatoxigenicity of *A. flavus*. There are many highly specific and sensitive culture methods can used for

determination of aflatoxins in samples, which allow the determination of mycotoxins, if samples contaminated with aflatoxigenic fungi [6]. Culture based methods such as 1) Colony fluorescence, appearance of blue fluorescence of aflatoxin B, when it is subjected to ultraviolet (UV) light, produce an intense blue fluorescence [7- 8]. 2) Observing of orange color on the reverse side of the Aspergillus Differentiation Agar plates. Visualizing of orange color on reverse side of colonies, is due to the reaction of aspergillic acid molecules synthesized by *Aspergillus* species with the ferric ions from ferric citrate of medium. 3) Ammonia vapor test, color change of undersides of aflatoxin-producing colonies when exposure to ammonium hydroxide vapor [9-10-11]. In this method, the undersides colonies of aflatoxin-producing fungi, will quickly turn plum-red when the bottom of the Petri dish has been inverted over the lid containing the ammonium hydroxide [7]. In many of these methods, more specialty and experts require for detection the presence of aflatoxins. Moreover, there are some other advanced and more accurate, but rather expensive and time consuming, techniques used to determine aflatoxigenicity of *A. flavus*, such as high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC), and fluorescence polarization assay [8-12]. Each of these methods has advantages and limitations in detection of toxigenic strains of fungi.

Also molecular techniques have been introduced as powerful tools for detecting and identifying aflatoxigenic fungi [8-12]. Several research groups [13-14] have tested the possibility of applying PCR-based detection techniques, for the presence of genomic DNA by conventional PCR. Some of other researchers, used Reverse Transcription PCR (RT-PCR) or Real-time PCR to detect the expressivity of the aflatoxin biosynthetic genes. Use of polymerase chain reaction (PCR) to amplify the aflatoxigenic genes, is more easily and more accurately in compare with other methods [12]. Hussain et al., [12] developed reliable and quick method for the detection of aflatoxin producing strains in peanuts by using a molecular approach to amplify three target genes (*aflD, aflM* and *aflR*) involved in the aflatoxin biosynthesis. Attempts to decipher and determine the role of potential gene markers in the aflatoxin biosynthetic pathway began shortly after the determination of the structure of this toxin [17]. There are estimated to be 27 enzymatic steps in the aflatoxin biosynthesis. Some structural intermediates in biosynthesis of aflatoxins pathway, enzymatically identified [17-18]. At least 30 genes identified that they involve in biosynthesis of aflatoxins. These genes are found on chromosome III and clustered within a 75-kb region of the *A. flavus* and *A. parasiticus* genome. They localized around 80 kb away from telomere [1-17-18-19]. Developed multiplex PCR have been used for discrimination between toxigenic and non-toxigenic strains within the *A. flavus* group [15]. Applicability of RT-PCR (reverse transcription-PCR) technique tested to differentiate between aflatoxin-producing and aflatoxin-non-producing strains of *A.flavus* by Scherm et al., [16]. The *aflD* (nor-1) and *aflO* (omtB) are two potential gene markers play important role in aflatoxins biosynthesis, their determination give the reliable evidence of aflatoxigenicity of isolates [17-18-19-21].

Here, we used the most reliable culture methods, and conventional PCR to amplify potential gene markers (*aflD* and *aflO*) for detection the aflatoxigenicity of *A. flavus* in local isolates samples, which allowed us to investigate the prevalence the aflatoxigenicity of *A. flavus* in the local area samples.

Materials and Methods

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 that included agricultural soils cultivated with maize and different vegetables. Maize grains were collected from corn fields at Sulaimani governorate.

Fungal Isolation

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. After inoculation of media by samples, then they were incubated at 28º C. The plates have been observed after (5-7) days [22-23-24-25].

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 grain samples. This method previously described by [26]. This step was followed by surface sterilization of the grains in NaOCL for 120 seconds, then the maize grains were washed off using dH2O. 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 28º C.

Identification Of Aspergillus Species

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 28ºC and 25ºC respectively for seven days.

After Aspergillus isolates grown on both media under the mentioned incubation conditions, 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, [27]. 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 [9-28-29-30].

Molecular Identification

As an alternative way to rapid and accurate diagnosis for our isolates, PCR-based methods recommended. Due to their high specificity and sensitivity in diagnosis, we decided to use PCR-based protocol for the detection of our isolates of *A. flavus* [2-9-31]. 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.

Extracted DNA from isolates subjected as template for PCR amplification. The PCR protocol designed to differentiate *A. flavus* from other closely related species in particular from A. parasiticus, and organisms commonly found on grains [2-9-31-32]. Multi-copy sequences of (ITS) (ITS-1, 5.8 S and ITS-2) of rDNA amplified with specific PCR assays by 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 [2-32].

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 MgCl2, 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*

Production ability of mycotoxins are vary among the Aspergillus species, therefore, there is a need for screening for their toxin production abilities [14]. All identified *A.flavus* isolates were tested for their aflatoxigenic potential.

Using Colony Fluorescence, For Detection Of Aflatoxin Producers

Coconut Milk Agar media have been used for a primary screening of aflatoxin production according to [33].Coconut constituents have effect on the fluorescent emission production in coconut culture [10]. For preparation of Coconut Milk Agar media, 100 ml of coconut's milk, purchased locally, homogenized for 5 minutes with 1000 ml of hot distilled water. Four layers of cheese cloth used for filtration of homogenate, and pH of clear filtrate adjusted to (7) with 2 N NaOH. Mixture heated till it boiled, then cooled to 50°C, then pH adjusted again to (7). The mixture was then autoclaved for 18 min at 121ºC under 1.5 bar pressure, cooled to about 40 to 45°C, and poured into sterile petridishes [8-10-33].

Aflatoxigenicity of isolates, checked by appearance of blue fluorescence of aflatoxins producer colonies when grown on coconut milk agar medium. Suitability of solid media such as potato dextrose agar and coconut milk agar have been checked by some of researchers to differentiate aflatoxigenicity of Aspergillus species [7-8-10-33]. Loop full of *A.flavus* inoculum transferred to the center of coconut milk agar plate and inoculate with it, then incubated in the dark at 28°C. After seven days of incubations, presence and absence of fluorescence ring in the agar surrounding the colonies under the UV light scored as appositive and negative results [7- 8-10].

Using Aspergillus Differentiation Media In Detection Of Aflatoxin Producers Based On Cultural Characteristics:

Aspergillus differentiation media (AFPA) is a selective and differential medium, it used for detection of aflatoxin producing Aspergillus species from food samples. [8-9-15]. In this method, after seven days of incubation at 28 °C, aflatoxigenic *A.flavus* species have

been distinguished from other Aspergillus species based on visualizing of orange color on reverse side of colonies, this is due to the reaction of aspergillic acid molecules synthesized by Aspergillus species with the ferric ions from ferric citrate of medium [9-10-15].

Detection Of Aflatoxin Producers Based On The Ammonia Vapor Test

For this method, *A.flavus* was grown on medium such as potato dextrose agar and incubated for seven days. After incubation, the dish was inverted up on the lid contain 1 or 2 drops of concentrated ammonium hydroxide solution. The undersides of aflatoxin-producing colonies quickly turn plum-red after the bottom of the Petri dish has been inverted over the lid. Essentially no color change occurs on the undersides of colonies that are not producing aflatoxins [7].

Molecular Detection Of Aflatoxigenic *A.flavus* Isolates

Recently, molecular methods have been developed to distinguish and identify aflatoxinogenic strains of *A.flavus* from other foodborne fungi [15]. Cluster genes in aflatoxin biosynthesis pathway contain structural marker genes such as (aflD nor-1, and aflO omtB) which they are responsible for coding key enzymes in the production of aflatoxins. Two primer pairs have been used for specific amplification of (aflD and aflO) genes table (1) [35]. As a system control for DNA amplification, housekeeping gene tub1 coding for β-tubulin has been chosen in the system.

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		
Tub1-(F)	*tub1*	GCTTTCTGGCAAACCATCTC	1498	50
Tub1-(R)		GGTCGTTCATGTTGCTCTCA		

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 MgCl2, 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 [16].

Results

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 [9-28-30-36]. The color of the colonies was used for first identification of *A.flavus*. After seven days of incubation at 28°C on AFPA, CMA and at 25°C

on PDA, plates were observed for varieties of macroscopic characteristics such as colony color, colony diameter, colony reverse color figures 1,2 and 3.

Table 2. Morphological features used to identify A. flavus on AFPA and PDA The size of the colonies are observed after seven days of incubation on both media.

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

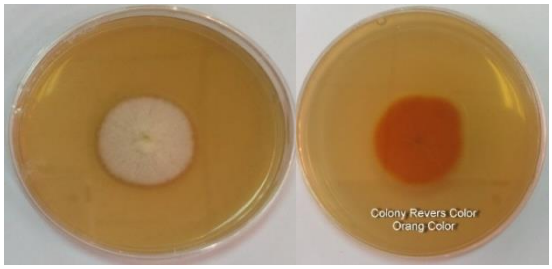


Figure 1. Colony characteristics of *A. flavus* : Name of Medium: AFPA, Colony color: White , Colony Diameter: (18) mm, Colony Reverse Color: Yellowish orange After seven days of incubation at 28°C.

Figure 2. Colony characteristics of *A. flavus*. Name of Medium: CMA. Colony color: Green. Colony Diameter: (40) mm. Colony Reverse Color: Hyaline. After seven days of incubation at 28°C.

Figure 3. 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 25°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 [30-37]. Conidial heads are typically radiate and biseriate figure 4.

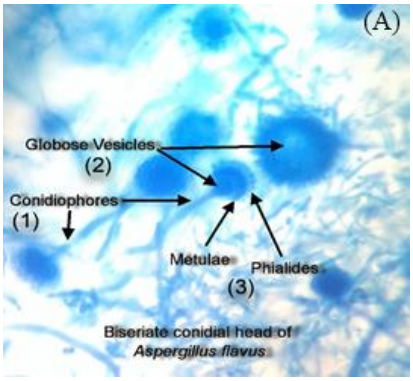


Figure 4. Biseriate conidial head of *A.flavus*: 1) Aspergillum like spore bearing conidiophore. 2) Globose vesicles. 3) Vesicles bearing (Metulae and Phialides) biseriate conidial head. Microscopic observation of the fungal isolate under 400x magnification (lactophenol cotton blue).

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 5 [2-31].As an alternative way, for rapid, high specific, sensitive diagnosis and detection of aflatoxigenic strains of *A. flavus* ,PCR based method used [2].

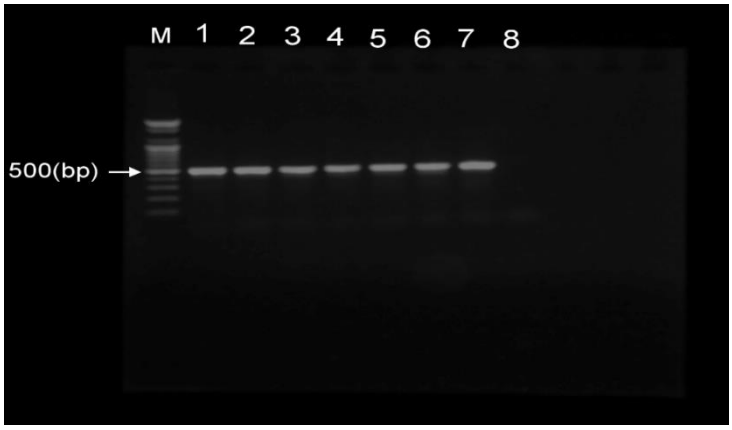


Figure 5: PCR-based detection of *A. flavus*.

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.

Cultural Methods For Aflatoxin Detection

Various analytical methods have been reported for the detection of aflatoxins. Much interest has come in developing and using cultural methods for detecting aflatoxins in fungal cultures. Screening was done by using colony fluorescence upon exposure to UV, ammonia vapor test and orange color characteristics in Aspergillus differentiation media [8-10].

Detection Of Aflatoxin Producers Based On Colony Fluorescence

Aflatoxigenicity of isolates, checked by appearance of blue fluorescence of aflatoxins producer colonies when grown on coconut milk agar medium (CMA) figure 6. Aflatoxins produce an intense blue fluorescence visible at approximately (450 nm), when exposed to long-wavelength (365 nm) ultraviolet (UV) light [7-8].

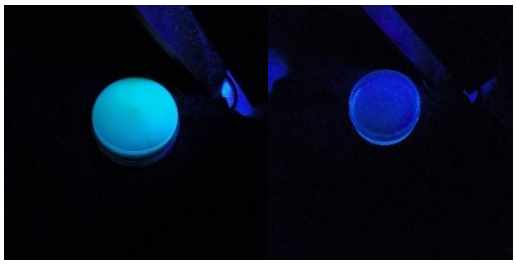


Figure 6 Colony fluorescence detection of aflatoxin: Coconut milk agar plate inoculated with loop full of *A.flavus* colonies and then incubated for 7 days at 28°C. A) CMA plate with aflatoxin producing fungal colonies showing fluorescence on UV exposure. B) CMA plate with non-aflatoxin producing fungal colonies showing no fluorescence can be detected on UV exposure.

Detection Of Aflatoxin Producers Based On Reverse Color Change On AFPA

Aspergillus differentiation media (AFPA) is a selective and differential medium which it used for detection of aflatoxin producing Aspergillus species. As it can be seen from

figure 7, in this method, after seven days of incubation at 28 °C, aflatoxigenic *A. flavus* species distinguished from other Aspergillus species based on visualizing of orange color on reverse side of colonies, this is due to the reaction of aspergillic acid molecules synthesized by Aspergillus species with the ferric ions from ferric citrate of medium [9-10-15].

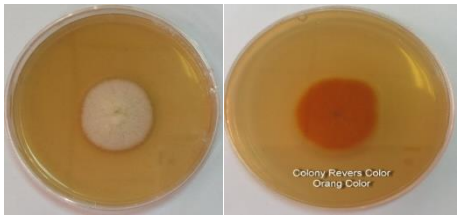
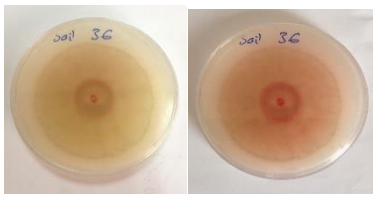


Figure 7. *A. flavus* on AFPA, after 7 days incubation at 28ºC, with characteristic orange color on reverse side of plate.

Ammonia Vapor Test For Rapid Detection Of Aflatoxigenic Aspergillus:

Differentiation of aflatoxigenic and non-aflatoxigenic Aspergillus by using ammonia vapor test was one of the applicable and rapid technique for this purpose [10]. In this method, the undersides of aflatoxin-producing colonies quickly turn plum-red after the bottom of the petridish has been inverted over the lid containing the ammonium hydroxide. Essentially no color change occurs on the undersides of colonies that are not producing aflatoxins figure 8 [7].



(a) **(b)**

Figure 8. A.flavus was grown PDA incubated for 7 days at 25 ºC. Aflatoxin-producing colonies quickly turn plum-red after the bottom of the petri dish has been inverted over the lid containing the ammonium hydroxide. (A)Aflatoxigenic fungal isolate before exposed to ammonium vapor. (B)Aflatoxigenic fungal isolate producing pink color when exposed to ammonium vapor.

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 two structural gene markers (aflD, and aflO). As a system control for DNA amplification, housekeeping gene tub1 coding for β-tubulin has been chosen in the system. PCR results revealed that all toxigenic isolates of *A. flavus* included in this study contained both potential gene markers (aflD and aflO) figure 9. Non DNA templates running for each primers have been used to detect there were no any contamination in our PCR results.

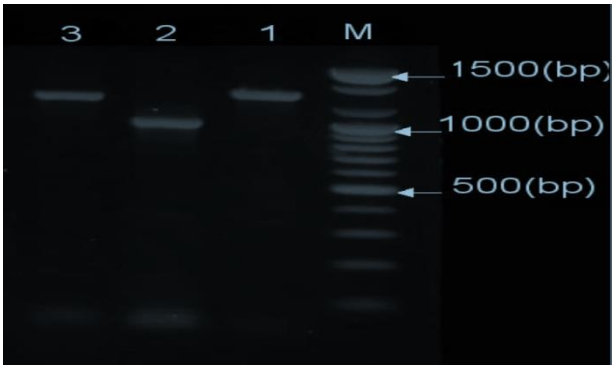


Figure 9. PCR-based detection of aflatoxigenic *A. flavus.*

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 tub1primer 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.

Discussion

Require time for accurate detection of toxicogenic fungi, is a one of the most critical point, it should take in consideration during search for incidence of toxigenic strains among the isolates. Take the advantages of classical methods in preliminary determination of toxigenic strain allow us to select the most suitable approaches in diagnosis of aflatoxigenic fungi. Culture methods such as colony fluorescence upon exposure to UV light, ammonia vapor test and colony appearance on aspergillus differentiation media, are examples of classical methods, they used for determination of toxigenic strains of our isolates. Furthermore to corroborate of our results, two potential gene markers (aflD and aflO) which they involved in the aflatoxin biosynthesis pathway have been detected by conventional PCR to convince of aflatoxigenicity of our results.

Morphological Identification Of The Isolates:

The description features reported by [9-28-29-30-37] have been used for morphological identification of isolates. A. fluvus is morphologically characterized by yellow green woolly or granular colonies on PDA its colonies are flat, with radial grooves [37]. Differences in colony diameter of *A. flavus* have been observed. Colony diameter on AFPA was smaller in compared to its diameter on PDA, and this is due to the difference in nutrient ingredients in between AFPA and PDA media.

Seriation and microscopical features like metulae and phialides used as a primary keys for differentiation among *A. flavus* and A. parasiticus. Rodrigues et al. [9], reported that A. parasiticus is mostly observed having uniserate conidial head. However, *A. flavus* shows biseriate conidial head. Figure 4.

Molecular Identification Of The *A. Flavus:*

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) [2-5-9-31-38]. Seven out of eighteen isolates in this study were identified by specific PCR protocol. Extracted DNA from isolates subjected as template for PCR amplification. The PCR

protocol designed to differentiate *A. flavus*, from other closely related species in particular from A. parasiticus, and organisms commonly found on grains [2-9-31-32]. However, for further and accurate discrimination between those species that they show high degrees of DNA relatedness with *A. flavus* such as A. oryzae, the mentioned protocol was not sufficient for differentiation among *A. flavus* and *A. oryzae* [2-9-38]. Took the advantage of differences in genes involved in aflatoxins biosynthesis pathway between *A. flavus* and *A. oryzae*, allow differentiate them accurately [38].To discriminate between *A. flavus* and A. oryzae, marker genes in aflatoxins biosynthesis such as (aflD, and aflO) have been detected in all seven isolates.

Molecular methods widely applied in the identification of a large number of Aspergillus species with different levels of success. Complex regions of rDNA and structural aflatoxin genes reported as a markers for identification of *A. flavus* [9]. Al-Wathiqi et al., [31] 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 relate fungal species [39-40]. El Khoury et al., [41] designed gene-specific primers for the aflatoxin biosynthesis genes. In order to distinguish between *A. flavus* and *A. parasiticus*.

Detection Of Aflatoixgenic *A. Flavus*:

Three different cultural based methods and conventional PCR, were used in order rapidly differentiate between aflatoxigenic and non aflatoxigenic of Aspergillus species. [9-10-11].

Detection Of Aflatoixgenic *A. Flavus* By Using The Culture Based Method Colony Fluorescence

Nair et al., [8], Davis et al.,[33] describe the method, in which colony of aflatoxigenic strains produced blue fluorescence on the reverse side of the Coconut Milk Agar medium when exposed to UV light. [10]. In our results, we observed that Coconut Milk Agar medium showed an increase in the number of fungal colonies exhibiting blue fluorescence zones. This can be explained by the enhancement of aflatoxin production and detection using UV light in the case of coconut supplemented agar media [33]. Aflatoxigenicity of *A. flavus* on coconut milk agar (CMA) media reported by Yazdani et al., [10], as a result appearance of fluorescence ring around the colonies. Treatment by enhancer such as cyclodextrins agents will substantially improve the fluorescence emission of aflatoxins Bl and G1[7].Yazdani et al., [10] showed that the cyclodextrins test did not have enough sensitivity for detection of aflatoxins.

Detection Of Aflatoixgenic *A. Flavus* By Using The Culture Based Method Aspergillus Differentiation Media

Aflatoxigenic *A. flavus* can produce yellowish orange color on the reverse side of the colonies, when they have been grown on the (AFPA) medium. (AFPA) is a selective and differential medium for *A. flavus* and *A. parasiticus*. It was used for the rapid detection of aflatoxigenic species. The coloration is due to the reaction of aspergillic acid molecules synthesized by Aspergillus species with the ferric ions from ferric citrate of medium [8-9]. Both *A. flavus* and *A. parasiticus* are capable to produce potent aflatoxins. Some of

researchers stated that, only aflatoxins B1 and B2, synthesized by *A. flavus*, whereas *A. parasiticus* has ability to produce aflatoxins B1, B2, G1 and G2 [1-18-42]. Our results shows that this medium (AFPA) is suitable for primary detection of aflatoxigenic *A. flavus*.

Detection Of Aflatoixgenic *A. Flavus* By Using The Culture Based Method (Ammonia Vapor Test)

Novel method has been introduced by Saito and Machida, [43], as a rapid and sensitive identification way to differentiate aflatoxin producing and nonproducing strains of *A. flavus*. Plum red color was observed when the (PDA) culture of aflatoxigenic *A. flavus* was inverted over the lid containing the ammonium hydroxide. This change in color is due to anthraquinone intermediates compounds of aflatoxins biosynthesis which act as pH indicator dyes, and are more visible when they have turned red at alkaline pH [1]. Essentially no color change occurs on the undersides of the colonies that are not producing aflatoxins [11-44]. Yazdani et al., [10] detected the production of toxin by TLC and HPLC while negative results of ammonium vapor test for some of the isolates have been observed. Based on that, they concluded that, the ammonium hydroxide test was an unreliable test for detection of aflatoxins. In contrast regarding to the our results, we demonstrated that ammonium vapor test can be used as a method for primary screening for detection aflatoxigenic of *A. flavus*.

Detection Of Aflatoixgenic *A. Flavus* By Using Molecular Method (Conventional PCR)

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 [12-14- 45]. Several PCR based systems have been developed to discriminate between aflatoxin producing and non-producing *A. flavus* strains.

Hussain et al., [12], Degola et al., [14], and Criseo et al., [15], 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., [46] 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., [47] have used real-time PCR to monitor the expression of an aflatoxin biosynthetic gene of *A. flavus* in wheat. Houshyarfard et al., [48] 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 nonproducing strains of *A. flavus* [14-16-47].

Among the 27 genes involved in aflatoxin biosynthesis only two structural key genes (aflD, and aflO) have been used in this study as a target molecular markers to detect the aflatoxigenic *A. flavus*. Scherm et al. [16] studied 13 isolates of *A. flavus* and found consistency of (aflD and aflO) 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) [18]. (aflO,and aflP) are necessary for final formalities of aflatoxins biosynthesis [19-21]. 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 and aflO) genes visualized at (990, 1333) respectively in figure 9. Rodrigues et al., [9], Scherm et al. [16], Latha et al., [46] reported that the expression profile of the two genes (aflD and aflO) were consistently correlated with a strain's ability to produce aflatoxins. Degola et al., [14] shows that one strain that apparently transcribed all the relevant genes (aflD and aflO) but did not produce aflatoxin in the medium.

According to the Houshyarfard et al., [48], biosynthesis of aflatoxins depend on the several factors. Firstly, presence of certain genes. Secondly, the genes should be intact. It means that any changes for instance deletions or insertions within the gene regions or regions flanking the gene will influence the aflatoxin's biosynthesis. 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., [14] 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 [14]. 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.

Conclusions

In this study, it is concluded that seven isolates of *A. flavus* were able to confirm the aflatoxin production by using culture methods and amplifying the two target genes (aflD and aflO) as these genes are considered as indicators of aflatoxin production.

Conflict Of Interest

The authors declare they have no conflict of interest.

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