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Molecularly Diagnostic of Aflatoxigenic *Aspergillus flavus* Isolated from Nuts

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ABSTRACT

Aflatoxigenic fungi like *Aspergillus* spp., commonly contaminants food, which has different methods to detect their presence. The early detection of food contaminants is quite significant to prevent the hazard on the human health and economic. This study aims to detect the isolation and diagnosis of the companying fungi from some nuts that are available in local markets of Babylon province (Iraq) and explore their ability to produce aflatoxins. The *Aspergillus* spp., was the most common, followed by each of the *Cladosporium* spp. and *Penicillium* spp. The results from isolation and diagnosis of this study showed that *Aspergillus flavus* was the most visible fungus in all kinds of examined nuts. The highest rate of appearance for this fungus was in pistachios, reaching 70%, whereas the rest of kinds of nuts ranged between 60-10%. The Polymerase Chain Reaction (PCR) technique was utilized for the diagnosis of *A. flavus* by using a special primer. In this study, the PCR technique was used for the detection of Aflatoxigenic *A. flavus* and ammonia chemical detection was used to compare this technique.

Key words: *Aspergillus flavus*, mycotoxin, PCR, *omtB, aflD*

INTRODUCTION

Aspergillus species has different capabilities such as physiological and phenotypic diversity, the production of enzymes and mycotoxins. Their spread associated with a high production of reproductive units with low weights makes it hang in the air (Auberger *et al*., 2008). The problem of food contamination by fungi producing toxins is one of the important problems at the present time. The FAO indicated that there is no less than 25% of the world's food contaminated with mycotoxins (Kovacs, 2004). There are some types of seeds used in the manufacture of nuts (e.g., pea nuts, pistachios, walnuts, chickpeas and pumpkin seeds, water melon seeds and cashew nuts), getting exposure to contamination by fungal mycotoxins belonging to the genus *Aspergillus* spp., during the storage (Cocker *et al*., 1984). Aflatoxin is a set of secondary metabolic compounds that are highly toxic produced by fungus *Aspergillus flavus*, which produces aflatoxin B (AFB), whereas fungus *A. parasiticus* produces both AFB and aflatoxin G (AFG). These fungi grow on grains, some field crops (i.e., wheat and barley), most of family plants, grass, oilseed and milk. There are about 18 species of aflatoxins including B1, B2, G1, G2, which are the most common types of aflatoxins, whereas aflatoxin M1, M2 contaminated milk products (IARC., 2002). Aflatoxins cause many diseases and illnesses. In actual fact, aflatoxins cause liver cancer in humans, all acute and chronic toxicity. Also, aflatoxins lead to teratogenic, embryogenic, mutagenic problems and immunosuppression. There is no drug to cure the cases of poisoning, which results from such toxins. However, these toxins do not cause the induction of the immune system in humans and animals to offset because of their small molecular weight (Carlile *et al*., 2001).

The dose plays a key role in determining the type of infection by aflatoxins, high dose exposure leads to a severe poison resulting in the direct damage, bleeding, the necrosis of liver and the blockage of bile duct, which eventually leads to death. Meanwhile, a non-lethal low dose causes immunological effects. Both high and low doses of aflatoxins often have a cumulative effect causing cancer (Adhikari *et al*., 1994). For quantification and detection of aflatoxins in food and foodstuffs, different analytical techniques such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), ultra-performance liquid chromatography/tandem mass spectrometry and Enzyme Linked Immunosorbent Assay (ELISA) have been developed (Trucksess *et al*., 1994; Whitaker *et al*., 1996; Reddy *et al*., 2001; Ventura *et al*., 2006).

In recent years, PCR has been used for the detection of aflatoxin-producing fungi in food (Shapira *et al*., 1996; Degola *et al*., 2007; Cruz and Buttner, 2008; Passone *et al*., 2010; Levin, 2012; Rodriguez *et al*., 2012). In general, these methods are highly sensitive and specific (Lievens and Thomma, 2005). More than 30 years of continued research on exploring metabolic pathways of vital synthesis of aflatoxins found that there are more than 20 common enzymes in this process. These enzymes are controlled genetically by a group of genes including (*ver-1*, *nor-1*, *omtA*) grouped within an area that accounts for about 75 kb of fungal cell genome (Trail *et al*., 1995; Yu *et al*., 2004; Woloshuk and Prieto, 1998). Jene aflr is an organizer of these genetic groupings. This gene was found in *A. flavus*, *A. parasiticus*, *A. sojae* and *A. oryza* strains of *Aspergillus* (Chung *et al*., 1998). The present study used a PCR assay targeting O-methyltransferase gene (*omt-B*), which is involved in the aflatoxin biosynthetic pathway for the detection of *A. flavus* and *nor1*. The PEPO1 and PEPO2 primers were used for the detection of *A. flavus* isolates.

MATERIALS AND METHODS

Sample collection: In the current study, ten varieties of nuts were brought to the laboratory. Five of which are non-canned (local), whereas the rest are canned (imported from different brands) including pumpkin seeds, watermelon seeds, pea nuts, walnuts, almonds, maize, sunflower seeds, pistachios and cashew nuts from the local markets in a period of 5th November, 2012 to 15th February, 2013. They were preserved in laboratory conditions for the purpose of investigating the frequency of the presence of fungi present in nuts.

The samples sterilized with 3% of sodium hypo chloride and then planted on Petri dishes containing PDA incubated under 25°C for 5 days (Pitt and Hocking, 1997). Next, they were cultured for several ties to get pure cultures and were diagnosed (Klich, 2002; Domsch *et al*., 2003). Finally, calculated appearance and frequency ratios of isolated fungi follow Eq. 1 and 2:

$$\text{Ratio of appearance (\%)} = \frac{\text{No. of samples that the fungus appeare on it}}{\text{Total No. of samples}} \times 100 \quad (1)$$

$$\text{Ratio of appearance (\%)} = \frac{\text{No. of fungus isolates}}{\text{Total No. of isolates}} \times 100 \quad (2)$$

In a study of phenotypic variation of *A. flavus* isolates, it was cut a part from the adage of colony and cultured on PDA, CZA, SDA, MEA and then incubated for 7 days at 25°C. After that, the culture characters and the nature and color of colonies were studied.

Molecular identification of *Aspergillus flavus*: A kit was used for extraction and purification of DNA and processed from Promega Corporation, which includes solutions, namely DNA rehydration solution, protein precipitation solution, cell lysis solution, nuclei lysis solution, RNase solution. Another solutions were also used in DNA extraction such as Ethylene Diamine Tetra Acetic-acid (EDTA), isopropanol, lyticase and ethanol (70%).

DNA extraction: According to Anderson (2008) and Ciardo *et al*. (2010), DNA was extracted by taking *A. flavus* colony growing on PDA at 25±2°C for 7 days in Eppendroff tubes. Next, 393 μL EDTA and 7.5 μL (20 g L^{-1}) lyticase were added to each tube and mixed by a vortex for 5 min. The tubes were then incubated in a water bath for one hour at 37°C. After that, the tubes were centrifuged in a speed (1400 rpm) for 2 min and the supernatant was moved to new Eppendroff tubes. Later, 200 μL of nuclei lysis was added to these tubes and mixed by a vortex and the 70 μL of protein precipitation was added and mixed in the tubes but in ice for 5 min and then centrifuged at 13000-14000 rpm for 3 min by micropipette. The supernatant was moved to new Eppendroff tubes containing 300 μL Isopropanol and mixed gently and then centrifuged at 13000-14000 rpm for 2 min. Next, the supernatant was removed and the tubes were dried. After that, 70% of ethanol was added to each tube and mixed gently and then the tubes were centrifuged at 13000-14000 rpm for 2 min, the ethanol was removed and the tubes were dried for 10-15 min. About 50 μL of DNA rehydration solution was also added to the tubes and then 1.5 μL of RNase was added to purify DNA and mixed by a vortex for 1 sec. Later, it was incubated at 37°C for 15 min. The tubes were then centrifuged at 13000-14000 rpm for 5 sec and incubated at 65°C for 1 h. Finally, the genomic DNA was checked by agarose gel electrophoresis and restored at -20°C for further use. For molecular identification of *A. flavus*, the PCR technique and the primer PEPO1 CGACGTCTACAAGCCTTCTGGAAA, PEPO2 CAGCAGACCGTCATTGTTCTTGTC (~200 bp were used and master mix Tag (R) Green was equipped by Promega company.

PCR amplification: The PCR reactions were carried out in a total reaction volume of 25 μL (12.5 μL master mix, 5 μL DNA, 1.5 μL forward primer, 1.5 μL reverse primer; the volume was completed to 25 μL by free water). Then, the tubes were mixed by microcentrifuge and the following programmer was used to amplify DNA 5 min at 94°C (1 cycle), 1 min at 94°C, 1 min at 59°C and 1 min at 72°C (36 cycles) and 5 min at 72°C (1 cycle). A 10 μL aliquot of PCR products were separated with a 1.5% agarose gel stained with ethidium bromide (0.5 μL) and the Electrophoresis was done, at 100 V. The agarose was examined under UV light with the wave length (320 nm) by using UV transilluminator and photographic by digital camera.

Chemical detection of Aflatoxigenic *Aspergillus flavus*: This test was done by using ammonia (Saito and Machida, 1999).

Molecular detection of Aflatoxigenic *Aspergillus flavus*: For molecular identification of *A. flavus*, the PCR technique and the (*aflD*) primers NOR1-F-R (NOR1-F ACC GCT ACG CCG GCA CTC TCG GCA C and NOR1-R GTT GGC CGC CAG CTT CGA CAC TCC G (400 bp)) and OmtBII-F-R (OmtBII-F ATG TGC TTG GGI TGC TGTG G, OmtBII-R GGA TGT GGT YAT GCG ATT GAG (611 bp) were used.

PCR amplification: The PCR reactions were carried out in a total reaction volume of 25 μL (12.5 μL master mix, 5 μL DNA, 1.5 μL forward primer, 1.5 μL reverse primer; the volume was completed to 25 μL by free water). Then, the tubes were mixed by Microcentrifuge. The following programme was used to amplify DNA. For Nor primer, it was 10 min at 94°C (1 cycle), 1 min at 94°C, 1 min at 65°C, 2 min at 72°C (33 cycles) and 5 min at 72°C (1 cycle). For *OmtBII-* primer, it was 5 min at 94°C (1 cycle), 1 min at 94°C, 2 min at 79°C and 2 min at 72°C (33 cycles) and 10 min at 72° (1 cycle). The electrophoresis was then done (Sambrook and Russell, 2001).

RESULTS AND DISCUSSION

Isolation and diagnosis of fungi associated with a variety of nuts studied: In this study, 404 isolates were obtained belonging to 6 genera of filamentous fungi as well as yeasts (Table 1). The number of isolates *Aspergillus* spp., was the highest isolates 142, followed by the isolates of *Cladosporium* spp. and *Penicillium* spp. This result is similar to that of a study conducted by (Nyirahakizimana *et al*., 2013). It was found that the genus *Aspergillus* spp., was the major fungus that affects nuts and seeds. In this study, the results of isolation and diagnosis indicated that the *A. niger* and *A. flavus* were the most fungi presence in all classes of nuts (Table 2). The highest presence rate of *A. flavus* was 80% in peanut, whereas, in pistachios, sunflower, almond and pumpkin seeds, the ratio was 60% of each type and the ratio differ from 50-10%. In the rest type of nuts, the highest percentage of *A. niger* presence was 70% in peanut and 60% in walnut. In sunflower seeds and peanut, the highest rate of appearance of *Penicillium* spp., was 90 and 80%, respectively. Meanwhile, in nuts, in cashew nuts and pumpkin seeds, the presence rate of *Cladosporium* spp., reached at 70%. In the rest of the items, the rate was in range (30-60%).

Table 1: Number of isolates of fungal species isolated from some types of nuts

Type of isolates	No. of isolates
Cladosporium spp.	86
Penicillium spp.	76
A. niger	62
A. flavus	55
Alternaria spp.	43
Rhizopus spp.	34
A. fumigatus	11
A. candidus	8
A. terreus	6
Bipolaris	2
Yeasts	21
Total	404

Table 2: Appearing ratios of fungi isolated from different types of nuts

	Appearing ratios of fungi (%)									
Type of nut	*A. flavus*	*A. niger*	*Penicillium* spp.	*Cladosporium* spp.	*Alternaria* spp.	*Rhizopus* spp.	*A. fumigatus*	*A. terreus*	*A. candidus*	Yeast
Peanut	80	70	90	50	30	10	10	0	10	20
Pistachio	60	30	60	30	20	50	0	10	20	10
Sun flower	60	10	90	60	30	10	20	0	0	40
Almond	60	50	70	60	50	0	10	10	10	10
Cashew	30	50	50	70	60	20	0	10	0	20
Walnut	50	60	80	70	40	20	30	0	0	20
Pumpkin seeds	60	50	40	70	40	0	10	10	10	20
Corn	10	40	40	30	30	70	0	0	0	0
Watermelon seeds	10	40	50	50	20	10	0	0	20	40
Hazel nut	10	20	30	30	10	60	20	0	0	0

Table 3: Percentage frequency of fungi isolated from different types of nuts

	Frequency of fungi isolates (%)									
Type of nuts	*A. flavus*	*A. niger*	*Penicillium* spp.	*Cladosporium* spp.	*Alternaria* spp.	*Rhizopus* spp.	*A. fumigatus*	*A. terreus*	*A. candidus*	Yeast
Peanut	62.5	50.00	68.75	37.50	25.00	6.25	6.25	0.00	6.25	12.50
Pistachios	50.0	43.75	50.00	35.29	17.64	37.50	0.00	5.88	17.64	5.88
Sunflower	37.5	6.25	68.75	60.00	17.64	12.50	12.50	0.00	0.00	23.50
Almond	42.8	33.30	47.60	59.09	31.80	0.00	4.76	4.76	4.50	4.50
Cashew	17.3	34.70	26.08	52.17	39.13	13.04	0.00	8.69	8.69	17.39
Walnut	35.0	40.00	45.00	68.40	31.57	10.00	20.00	0.00	0.00	10.52
Pumpkin seeds	42.1	42.10	36.80	61.10	22.20	0.00	5.26	5.26	5.50	11.10
Corn	7.1	50.00	28.50	25.00	25.00	50.00	0.00	0.00	0.00	0.00
Watermelon seeds	5.2	26.30	21.50	47.36	15.70	10.52	0.00	0.00	10.52	26.30
Hazel nut	5.8	11.70	29.40	25.00	10.00	58.80	11.76	0.00	0.00	0.00

With regard to frequency ratios (Table 3), the highest frequency ratio of the *A. flavus* was 62.5% in peanut, whereas, in the rest of the items, the ratio of frequency ranged from 50-5.2%. The frequency ratio of *A. niger* was 50% in each of peanut and corn. Meanwhile, in the rest of the items, it ranged from 43.75-6.25%. The *Penicillium* spp., was more frequency in sunflower seeds and peanut with 68.75%, respectively and the highest frequency recorded in fungus *Cladosporium* spp., was 68.4% in walnut.

These results are consistent with those of the study conducted by Abdel-Gawad and Zohri (1993), who found that fungus *A. flavus* appeared in all nuts types (i.e., pistachio nuts, cashew nuts, hazelnuts and almonds). Another study on the class pistachio (soft and dry) indicated that the *A. flavus* was able to contaminate (48%) samples of soft pistachios and (35%) of the dry (Mahoney and Molyneux, 1998). Another study on samples of pistachios and chickpeas conducted in Algeria indicated that more fungi that were visible in samples of pistachios was *Penicillium* (38%), followed by *A. niger* (30%) and *A. flavus* (22%) and the most important contaminated fungi of chickpeas were *A. flavus*, *A. niger*, *A. nidulans*, *A. ochraceas* and *Penicillium* spp. (Ahmad and Singh, 1991). In a study conducted in Brazil, the results showed that *A. niger* was the most contaminated fungus in Brazilian pistachios *A. flavus* (Freire *et al*., 1999). A study carried out by Kenjo *et al*. (2007) in Japan also found that the *A. niger* and *A. flavus*. were the most visible and frequency fungi in almonds. This depends on environmental conditions of temperature and function of hydrogen as well as the nature of the material appropriate food for the growth of pathogens.

Ababutain (2013) stated that the temperature and relative humidity were important factors affecting the growth of *A. niger*. Nawar (2008) also mentioned that the growth of fungus increased due to relative humidity increase, whereas, low humidity inhibited the growth of fungus and the optimum temperature for the growth of fungus *A. niger* is 30°C. This causes a spread of crop infection with *Aspergillus* spp., especially the types *A. flavus* and *A. niger* may return to simple nutrition requirements as well as their ability to produce a highly large number of asexual reproductive units (conides) and that high susceptibility to withstand critical environmental conditions and possession of a multi-enzyme system enabled them to exploit different food sources.

Determination of culture characters for *Aspergillus flavus*: Isolates of *A. flavus* were cultured on four types of culture media (PDA, SDA, MEA and Czapecks agar) (Fig. 1, Table 4). The result showed that the color of colony changed when the culture media component changed. This result is consistent with the findings of Klich (2006) and Okuda *et al*. (2000) that the isolation to develop the agricultural circles showed multiple contrasting different components of the culture media and environmental factors, light, acidic and the degree of occupation.

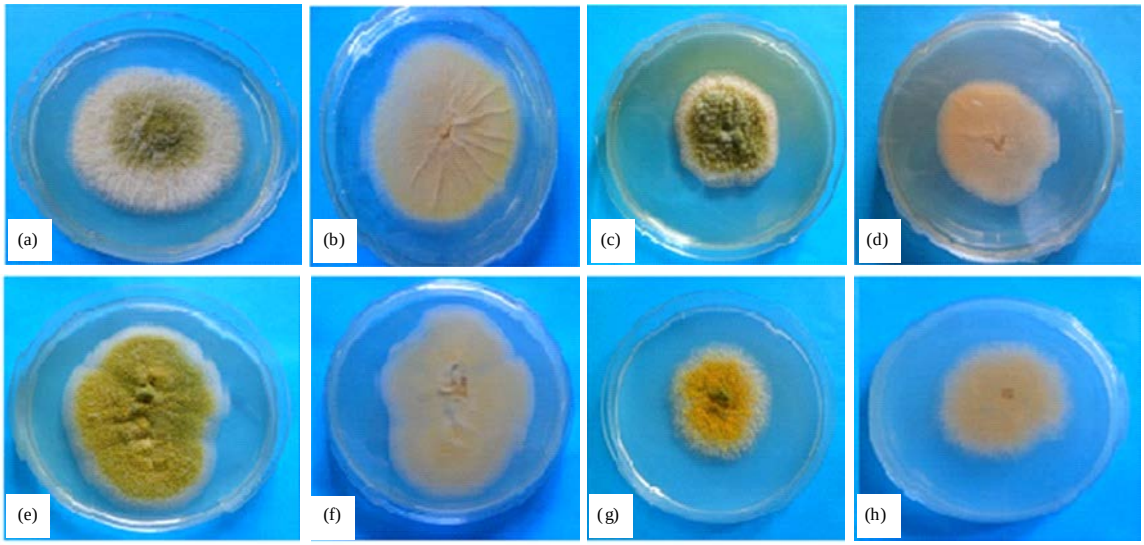

Fig. 1(a-h): Culture characters of *A. flavus* on different culture media, (a-b) Front and behind colony of *A. flavus* on SDA, (c-d) Front and behind colony of *A. flavus* on MEA, (e-f) Front and behind colony of *A. flavus* on PDA and (g-h) Front and behind colony of *A. flavus* on CZA

Table 4: Phenotypic features of *A. flavus*

Culture media	Colony color	Colony edge	Colony elevation	Mycelium growth
PDA	Green yellowish	Rough	Elevated	Dense growth
SDA	Green	Rough	Elevated	Dense growth
MEA	Deep green	Rough	Elevated	Dense growth
CZA	Yellow	Rough	Elevated	Medium growth

PDA: Potato dextrose agar, SDA: Sabouraud dextrose agar, MEA: Malt extract agar, CZA: Czapek dox agar

Molecular identification of *Aspergillus flavus* by PCR: Forty isolates of fungus pure *A. flavus* were obtained and undergone all of these isolates to molecular diagnoses by PCR technology by using a specialist primer (PEPO1) and the opposite one (PEPO2). *Aspergillus flavus* diagnosis aims to amplify the target area (1st exon). Logotheti *et al*. (2009) discovered that only 30 isolates of the *A. flavus* have shown polymerization in size 200 base pairs while ten remaining isolates did not show that bands (Fig. 2). It is clear from the results that the genotype showed Aspergillopepsin PEPO area using a pair primer (PEPO1/PEPO2), which gave the outputs of polymerization 200 pairs based portal, which removed all forms of ambiguity surrounding the cases overlap phenotypic between type *A. flavus* and the nearest species nearby. That dated back to the same sex and showed recipes color of colonies that were similar to fungi *A. parasiticus*, *A. oryzae* and *A. nomius*. That is considered an important step to determine the ownership of fungal isolates that show the same appearance of the colony chromatography (Geiser *et al*., 2000; Rodrigues *et al*., 2007).

Ability of *Aspergillus flavus* to produce aflatoxins by using ammonia: The results of chemical detection of aflatoxin production by amid coconut and ammonia showed the ability of some *A. flavus* isolates to produce aflatoxin (Table 5). There was a distinct change of the bases of colonies to the red color in various degrees (Fig. 3). This gradient in color may be due to the ability of different isolates to produce aflatoxins (Saito and Machida, 1999) because it made clear that the

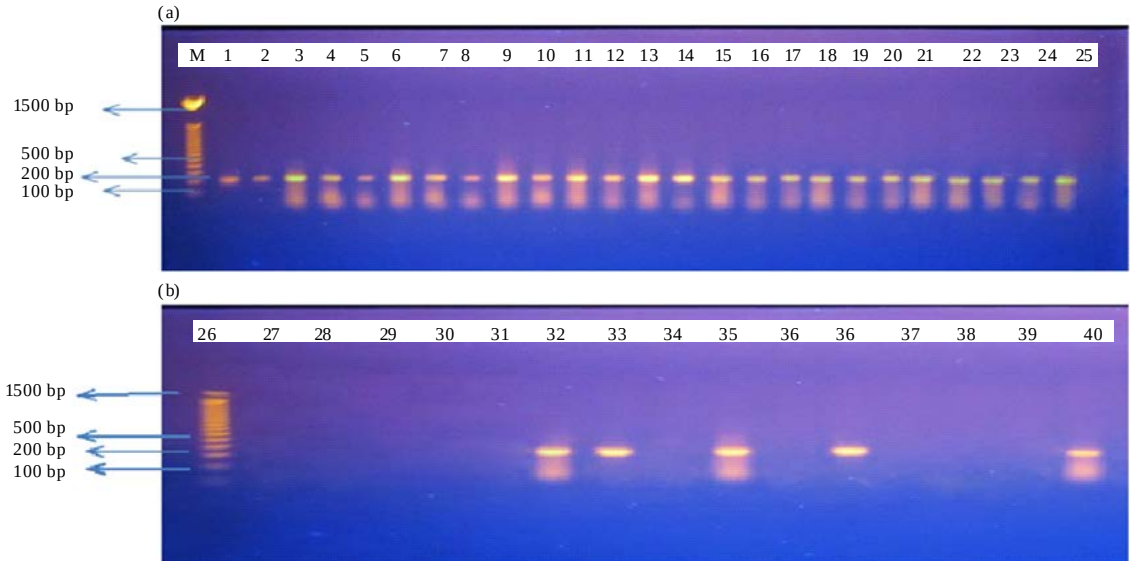

Fig. 2(a-b): Polymerization product by using specialist primer PEPO1-PEPO2 for 40 isolates of *A. flavus* on 1.5% agarose

Table 5: Ability of *A. flavus* on the production of aflatoxin by using ammonia

Source of isolates	No. of isolates	Ratio of isolates capable of aflatoxin production
Peanut	4	50.0
Pistachio	3	66.5
Almond	3	66.6
Walnut	3	66.6
Sun flower seeds	5	60.0
Pumpkin seeds	6	66.6
Cashew	3	0.0
Water melon seeds	1	100.0
Hazel Any	1	0.0
Corn	1	100.0

degree of red color was back to the produced quantities of aflatoxins, Isolation of a dark red color indicated its ability to produce higher quantities of aflatoxins than the isolates that their base of colonies in bright red or pink color. This result is comparable to a study conducted in Italy by Gallo *et al*. (2012) found that the ratio of *A. flavus* isolates producing aflatoxin was 55%.

Detection of aflatoxin production by PCR: Polymerase Chain Reaction (PCR) technique results showed the existence of a gene (*OmtB*) responsible for the production of aflatoxins in 12 isolates of the fungus *A. flavus* from a total of 30 isolates where it was noted that the location of the gene appeared at the nitrogenous base (611 bp) when the primer OmtIIB (F-R) was used. This result is similar to the findings of Rahimi *et al*. (2008). In their study, 75 isolates could be isolated and 46 of them only produced aflatoxin when the same primer was used. This study also found the existence of a gene (*aflD*) in 9 isolates at the nitrogenous bace 400 bp (Fig. 4 and 5). This result is also comparable to a study conducted in Iran by Erami *et al*. (2007) because the same primer NOR1 (FR) was used, which was responsible for gene (*aflD*) and found that 7 of 14 isolates could produce aflatoxins. Similarly, a study conducted in India by Priyanka *et al*. (2012) also used the same primer which was responsible for gene (*aflD*) and got 60 isolates producing aflatoxins from a total

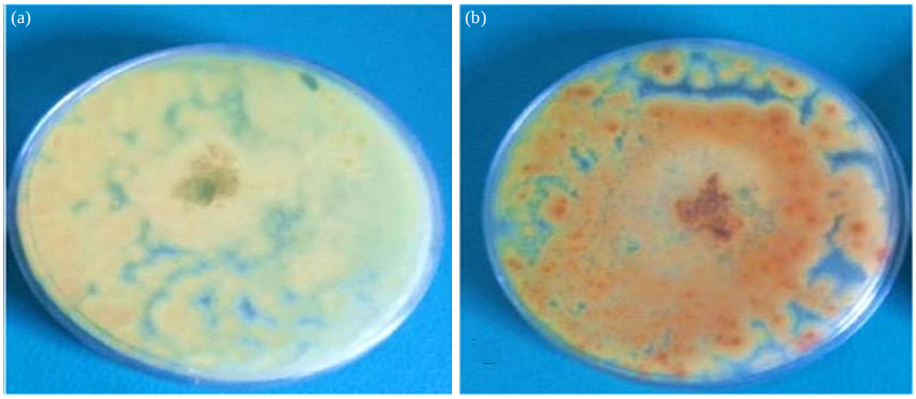

Fig. 3(a-b): Detection ability of isolates to produce aflatoxins, (a) Non aflatoxin production isolates and (b) Aflatoxin production isolates

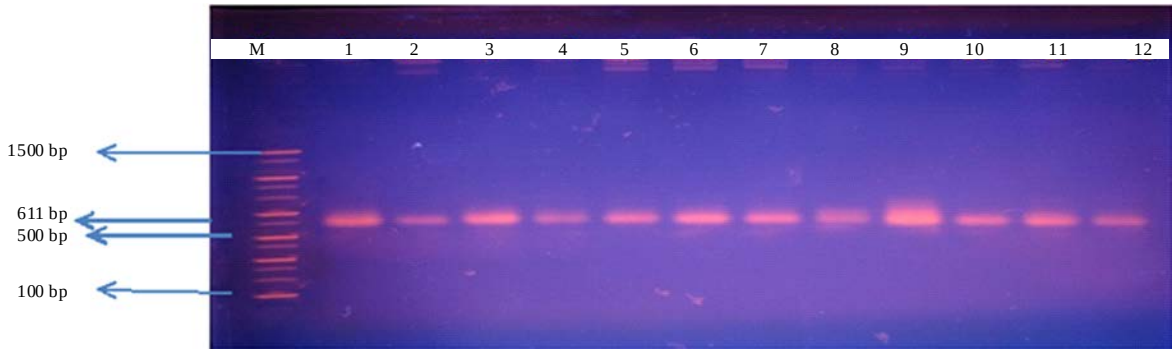

Fig. 4: Detection of aflatoxin production by using the primer (OmtB (F-R)) in *A. flavus*

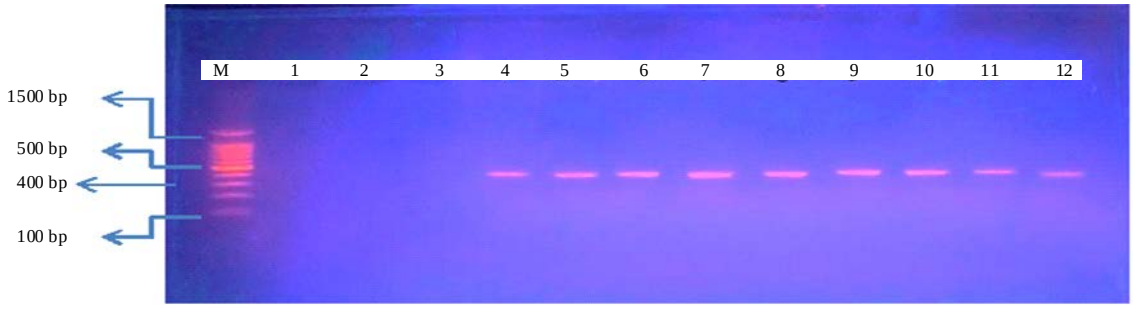

Fig. 5: Detection of aflatoxin production by using the primer (NOR (E-R)) in *A. flavus*

of 89 isolates of *A. flavus*. The findings of these studies agree with results of previous studies on chain of DNA sequence of the gene *Omt* appropriate design for starters special for diagnosis of fungi producing aflatoxins (Scherm *et al*., 2005). The results of these study are also similar to those of previous studies on chain of DNA sequence of the gene *Omt* appropriate design special primer for diagnosis of fungi producing aflatoxin (Scherm *et al*., 2005; Rahimi *et al*., 2008; Rodrigues *et al*., 2009).

CONCLUSION

In this study *Aspergillus* has the highest level of appearance among the other genus of fungal isolates in the nuts. The isolates of *Aspergillus flavus* revealed different agricultural features on different culture media, most of its isolates have the ability of aflatoxins production. The study also shows that the diagnosis of aflatoxigenic *Aspergillus flavus* by using PCR is more efficient than ammonium, which is done by using gene *omtB* and *aflD* that are responsible for the production of aflatoxins.

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REFERENCES

Ababutain, I.M., 2013. Aeromycoflora of some eastern provinces of Saudi Arabia. Indoor Built Environ., 22: 388-394.

Abdel-Gawad, K.M. and A.A. Zohri, 1993. Fungal flora and mycotoxins of six kinds of nut seeds for human consumption in Saudi Arabia. Mycopathologia, 124: 55-64.

Adhikari, M., G. Ramjee and P. Berjak, 1994. Aflatoxin, kwashiorkor and morbidity. Natural Toxins, 2: 1-3.

Ahmad, S.K. and P.L. Singh, 1991. Mycofloral changes and aflatoxin contamination in stored chickpea seeds. Food Addit. Contam., 8: 723-730.

Anderson, T.H., 2008. Assessment of DNA contents of soil fungi. Landbauforschung-vTI Agric. For. Res., 58: 19-28.

Auberger, J., C. Lass-Florl, J. Clausen, R. Bellmann, W. Buzina, G. Gastl and D. Nachbaur, 2008. First case of breakthrough pulmonary *Aspergillus niveus* infection in a patient after allogeneic hematopoietic stem cell transplantation. Diagn. Microbiol. Infect. Dis., 62: 336-339.

Carlile, M.J., S.C. Watkinson and G.W. Gooday, 2001. The Fungi. 2nd Edn., Academic Press, San Diego, USA., ISBN-13: 978-0127384467, Pages: 608.

Chung, K.T., T.Y. Wong, C.I. Wei, Y.W. Huang and Y. Lin, 1998. Tannins and human health: A review. Crit. Rev. Food Sci. Nutr., 38: 421-464.

Ciardo, D.E., K. Lucke, A. Imhof, G.V. Bloemberg and E.C. Bottger, 2010. Systematic internal transcribed spacer sequence analysis for identification of clinical mold isolates in diagnostic mycology: A 5-year study. J. Clin. Microbiol., 48: 2809-2813.

Cocker, R.D., B.D. Jones, M.J. Nagler, G.A. Gillman, A.J. Wellbridge and S. Panigrahi, 1984. Mycotoxin training manual. Tropical Development and Research Institute Oversee Development Administration, London, pp: 300.

Cruz, P. and M.P. Buttner, 2008. Development and evaluation of a real-time quantitative PCR assay for *Aspergillus flavus*. Mycologia, 100: 683-690.

Degola, F., E. Berni, C. Dall'Asta, E. Spotti, R. Marchelli, I. Ferrero and F.M. Restivo, 2007. A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. J. Applied Microbiol., 103: 409-417.

Domsch, K.H., W. Gams and T.H. Anderson, 2003. Compendium of Soil Fungi. Academic Press, London, Pages: 894.

Erami, M., S.J. Hashemi, S.A. Pourbakhsh, S. Shahsavandi, S. Mohammadi, A.H. Shooshtari and Z. Jahanshiri, 2007. Application of PCR on detection of aflatoxinogenic fungi. Arch. Razi Inst., 62: 95-100.

Freire, F.C.O., Z. Kozakiewicz, R. Russell and M. Paterson, 1999. Mycoflora and mycotoxins of Brazilian cashew kernels. Mycopathologia, 145: 95-103.

Gallo, A., G. Stea, P. Battilani, A.F. Logrieco and G. Perrone, 2012. Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy. Phytopathologia Mediterranea, 51: 198-206.

Geiser, D.M., J.W. Dorner, B.W. Horn and J.W. Taylor, 2000. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. Fungal Genet. Biol., 31: 169-179.

IARC., 2002. Summaries and Evaluations: Aflatoxins. IARC Press, Lyon, pp: 17.

Kenjo, T., Y. Ishide, K. Aoyama and M. Ichinoe, 2007. [Fungal population and distribution of aflatoxigenic fungi in commercial almond powder products]. Shokuhin Eiseigaku Zasshi, 48: 90-96, (In Japanese).

Klich, M.A., 2002. Identification of Common *Aspergillus* Species. Utrecth: Centralbureau voor Schimmecultures, The Netherlands.

Klich, M.A., 2006. Identification of clinically relevant aspergilli. Med. Mycol., 44: 127-131.

Kovacs, M., 2004. [Nutritional health aspects of mycotoxins]. Orvosi Hetilap, 145: 1739-1746, (In Hungarian).

Levin, R.E., 2012. PCR detection of aflatoxin producing fungi and its limitations. Int. J. Food Microbiol., 156: 1-6.

Lievens, B. and B.P.H.J. Thomma, 2005. Recent developments in pathogen detection arrays: Implications for fungal plant pathogens and use in practice. Phytopathology, 95: 1374-1380.

Logotheti, M., A. Kotsovili-Tseleni, G. Arsenis and N.I. Legakis, 2009. Multiplex PCR for the discrimination of *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*. J. Microbiol. Methods, 76: 209-211.

Mahoney, N. and R.J. Molyneux, 1998. Contamination of tree nuts by aflatoxigenic fungi: Aflatoxin content of closed-shell pistachios. J. Agric. Food Chem., 46: 1906-1909.

Nawar, S.L., 2008. Prevention and control of fungi contaminated stored pistachio nuts imported to Saudi Arabia. Saudi J. Biol. Sci., 15: 105-112.

Nyirahakizimana, H., L. Mwamburi, J. Wakhisi, C. Mutegi, M. Christie and J. Wagacha, 2013. Occurrence of *Aspergillus* species and aflatoxin contamination in raw and roasted peanuts from formal and informal markets in Eldoret and Kericho Towns, Kenya. Adv. Microbiol., 3: 333-342.

Okuda, T.M., A. Klich, K.A. Seifert and K. Ando, 2000. Media and Incubation Effects on Morphological Characteristic of *Penicillium* and *Aspergillus*. In: Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification, Samson, R.A. and J.l. Pitt (Eds.). Harwood Academic Publisher, Amsterdam, Netherland, pp: 83-99.

Passone, M.A., L.C. Rosso, A. Ciancio and M. Etcheverry, 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of *nor*-1 gene and effects of storage conditions on aflatoxin production. Int. J. Food Microbiol., 138: 276-281.

Pitt, J.I. and A.D. Hocking, 1997. Fungi and Food Spoilage. 2nd Edn., Springer, USA., ISBN-13: 978-0412554605, Pages: 593.

Priyanka, S.R., M.V. Ramana, K. Balakrishna, H.S. Murali and H.V. Batra, 2012. A novel non radioactive PCR-DNA probe for the detection of aflatoxin producing *Aspergillus* species from major food crops grown in India. Adv. Microbiol., 2: 577-586.

Rahimi, P., B. Sharifnabi and M. Bahar, 2008. Detection of aflatoxin in *Aspergillus* species isolated from pistachio in Iran. J. Phytopathol., 156: 15-20.

Reddy, S.V., D.K. Mayi, M.U. Reddy, K. Thirumala-Devi and D.V.R. Reddy, 2001. Aflatoxins B_1 in different grades of chillies (*Capsicum annum* L.) in India as determined by indirect competitive-ELISA. Food Addit. Contam., 18: 553-558.

Rodrigues, P., C. Soares, Z. Kozakiewicz, R.R.M. Paterson, N. Lima and A. Venancio, 2007. Identification and Characterization of *Aspergillus flavus* and Aflatoxins. In: Communicating Current Research and Educational Topics and Trends in Applied Microbiology, Mendez-Vilas, A. (Ed.). FORMATEX, Braga, Portugal, ISBN: 9788461194223, pp: 527-534.

Rodrigues, P., A. Venancio, Z. Kozakiewicz and N. Lima, 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* isolated from Portuguese almonds. Int. J. Food Microbiol., 129: 187-193.

Rodriguez, A., M. Rodriguez, M.I. Luque, A. Martin and J.J. Cordoba, 2012. Real-time pcr assays for detection and quantification of aflatoxin-producing molds in foods. Food Microbiol., 31: 89-99.

Saito, M. and S. Machida, 1999. A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor. Mycoscience, 40: 205-208.

Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., USA., ISBN-13: 9780879695774, Pages: 2344.

Scherm, B., M. Palomba, D. Serra, A. Marcello and Q. Migheli, 2005. Detection of transcripts of the aflatoxin genes *aflD*, *aflO* and *aflP* by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. Int. J. Food Microbiol., 98: 201-210.

Shapira, R., N. Paster, O. Eyal, M. Menasherov, A. Mett and R. Salomon, 1996. Detection of aflatoxigenic molds in grains by PCR. Applied Environ. Microbiol., 62: 3270-3273.

Trail, F., N. Mahanti and J. Linz, 1995. Molecular biology of aflatoxin biosynthesis. Microbiology, 141: 755-765.

Trucksess, M.W., M.E. Stack, S. Nesheim, R.H. Albert and T.R. Romer, 1994. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1 and G_2 in corn, almonds, Brazil nuts, peanuts and pistachio nuts: Collaborative study. J. AOAC Int., 77: 1512-1521.

Ventura, M., D. Guillen, I. Anaya, F. Broto-Puig, J.L. Lliberia, M. Agut and L. Comellas, 2006. Ultra-performance liquid chromatography/tandem mass spectrometry for the simultaneous analysis of aflatoxins B1, G1, B2, G2 and ochratoxin A in beer. Rapid Commun. Mass Spectrometry, 20: 3199-3204.

Whitaker, T., W. Horwitz, R. Albert and S. Nesheim, 1996. Variability associated with analytical methods used to measure aflatoxin in agricultural commodities. J. AOAC Int., 79: 476-485.

Woloshuk, C.P. and R. Prieto, 1998. Genetic organization and function of the aflatoxin B1 biosynthetic genes. FEMS Microbiol. Lett., 160: 169-176.

Yu, J., P.K. Chang, K.C. Ehrlich, J.W. Cary and D. Bhatnagar *et al*., 2004. Clustered pathway genes in aflatoxin biosynthesis. Applied Environ. Microbiol., 70: 1253-1262.