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Identification of *Aspergillus flavus* isolated from stored nuts in local markets of Baghdad (Iraq), and quantification of nuts aflatoxins using ELISA method

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ABSTRACT

Stored nuts in the markets are naturally infected by different fungal species such as *Aspergillus*. The present study was carried out to evaluate the occurrence of toxicogenic *A. flavus* strains on nuts in Iraq. A total of 112 nuts samples including hazelnuts, pistachio, peanut, and walnut with typical symptoms of dark and green discolored lesions on kernels were collected from various markets in Baghdad. Strains of *Aspergillus* spp. isolated from nuts seeds and their morphological characterization was based on MEA, PDA, and CYA. The identification of *A. flavus* isolates were confirmed molecularly using primers T1/T2. A total of 25 fungal isolates belonged to *Aspergillus* species that were identified as *A. niger* (10), *A. flavus* (10), and *A. japonicus* (5). In molecular analysis, sequences of partial *β-tubulin* gene were blasted in GenBank to confirm morphological identification of *A. flavus* isolates. Aflatoxins (AFs) contamination of ten infected samples with *A. flavus* was evaluated using ELISA method. Natural occurrence of AFs could be detected in all tested samples, ranging from 6.50–74.48 µg/kg. Our results completed the previously data about genetic potential of AFs production of *A. flavus* strains in Iraq and revealed in infected nuts also can be one of the great concern in Iraq.

1. Introduction

Aspergillus flavus is one of the important mycotoxigenic species that its infections can occur in the field, during postharvest and storage (Nagur et al., 2014). Injured seeds are usually readily infect by different toxigenic fungi especially *A. flavus* (Reddy et al., 2009). The pathogen can also damage seedlings and reduces the price of grains (Perenicova et al., 2001; Nagur et al., 2014). Aflatoxins are mycotoxins produced by *A. parasiticus* and *A. flavus* that can contaminate damaged nuts during storage (Juan et al., 2008). Many studies showed four Aflatoxins (B1, B2, G1 and G2) produced by *A. parasiticus* and *A. flavus* (Bennett and Klich, 2003; FAO 2003; Tanaka 2007). Aflatoxin B1 (AFB1) is the most important occurring toxic compound that can reduce productive efficiency (Shim et al., 2007). AFB1 is the most toxic to animals, causing harmful effects including carcinogenic, mutagenic, and oesophageal cancer (Li et al., 2001; CAST, 2003; FAO, 2003; Ardic et al., 2008). The problem is more in tropical and sub-tropical part of the world where there is no proper place to keep the grains dry for a long time till consumption (Reddy et

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al., 2009). In different countries, to control the health of animals and humans, aflatoxins effect on humans and animal health regularly evaluated. The European Union has a maximum allowed level of 20 ng/g for aflatoxins total (AFT) in animal feed. However, in Iraq as the sub-tropical country, maximum level of AFT is not investigated. So, the accurate information about maximum level of AFs in Iraqian nuts is necessary to prevent their effects. Several studies have demonstrated that AFs are frequently present in different nuts including Iran and Turkish as the most important neighbour countries of Iraq (Rasti et al., 2000; Rahimi *et al*., 2007; Ozgur et al., 2016; Gholami–Shabani et al., 2017). However Rifaie and Al-Maqtoofi (2016) studied the aflatoxins contamination of nuts in Basrah province, Iraq and reported *A. flavus* as the most important mycotoxinogenic fungi in nuts. But unfortunately, until today, not enough attempts have been made to identify members of the *Aspergillus* spp. associated with nuts in Iraq. In general, these studies were conducted to gain more information on the diversity of *Aspergillus* species in nuts, morphological and molecular characteristics of the isolated species, and their potential in producing mycotoxins. Therefor this study was aimed to (1) isolate and identify *Aspergillus flavus*, and (2) determine natural occurrence of AFs in stored nuts in local markets of Iraq.

2. Materials and Methods

2.1. Isolation and identification of Aspergillus flavus

One-hundred and twelve nut samples with infected kernels by fungi were collected from commercial markets in Baghdad, Iraq in summer 2016. Parts of the kernels tissues with typical symptoms of dark and green discolored lesions on kernels were placed on potato dextrose agar (PDA) containing rose bengal at a concentration of 50 ppm (Doster and Michailides 1994; 1995). All plates were incubated at 28◦C temperature for seven days. All the fungi isolated from kernels were purified through single spore technique. Water agar (WA), malt extract agar (MEA), potato dextrose agar (PDA), and Czapek's yeast agar (CYA) were used for identification of *Aspergillus* spp. For identification of *Aspergillus* species, morphological characters including the conidiophores and conidial heads characteristics and colonies pigmentations were recorded (Klich, 2006).

2.2. DNA extraction

PDA medium was used to grow *A. flavus* isolates to produce mycelium for DNA extraction. All of the *A. flavus* isolates were grown on PDA with sterile dialysis membranes (Lui et al., 2000). All Petri dishes were incubated until all membrane surface covered with *Aspergillus* colony. Frozen mycelial mats were grounded with a mortar and pestle to fine powder in liquid nitrogen.

DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen, Germany) according to the manufacturers' protocol. The presence of DNA was determined by 0.8% agarose gel. A constant voltage of 90 and 400 mA was applied across the gel for 90 min and visualized under UV light by ethidium bromide (EtBr) staining (Chehri and Hasani, 2017; Chehri and Satter, 2018).

2.3. PCR amplification

DNA samples were subjected to PCR amplification. The partial *β-tubulin* gene was amplified using primers T1 (5'-AAC ATG CGT GAG ATT GTA AGT-3') and T2 (5'-TAG TGA CCC TTG GCC CAG TTG-3') (O'Donnell and Cigelnik, 1997). DNA amplification was performed with an initial denaturation of 1 min at 94°C followed by 39 cycles of 30 sec. at 94°C, 30 sec. at 58°C and 1 min at 72°C, and a final extension of 5 min at 72°C (1).

2.4. DNA sequencing

The sequences of partial *β-tubulin* gene was amplified and purified using Quiagen columns according to the manufacturer's instructions. The purified DNA samples were kept at -20°C until sequencing. The purified DNA samples were kept at -20°C until sequencing. The purified PCR products were sent to a service provider. Bio Edit was used in order to edit the sequence files (Tamura et al. 2007). In order to assess the relationships between the major taxa, ambiguous parts of the *β-tubulin* gene were removed from further analysis. To identify all isolates of *A. flavus*. The *β-tubulin* sequences were compared with other available *A. flavus* sequences in GenBank using Basic Local Alignment Search Tools (BLAST).

2.5. Enzyme-linked immunosorbent assay (ELISA) analysis

According to the protocol of the manufacturer, aflatoxins content in the samples was analyzed using the Quantitative Aflatoxins Test Kit (Neogen Technical Services, USA) (Chehri and Hasani, 2017).

3. RESULTS

3.1. Isolation and identification of Aspergillus species

A total of 112 nuts samples including hazelnuts, pistachio nuts, peanuts, and walnuts with typical symptoms of dark and green discoloration on kernels were collected from various markets in Baghdad of Iraq, 2016 (Doster and Michailides, 1994; 1995). All samples were transferred to laboratory of mycology in Department of Biology, Faculty of Sciences, Razi University. A total of 25 *Aspergillus* isolates comprising 11 isolates from pistachio, six isolates from hazelnuts, four isolates from walnut, and four isolates from peanut were isolated from moldy kernels collected from different markets in Baghdad.

Three species of *Aspergillus* including *A. flavus, A. niger*, and *A. japonicus* were identified using morphological features. From these, *A. flavus* (10) and *A. niger* (10) were the most frequent species (Table 1).

Most of the isolates showed dark black powdery and dark brown aerial mycelium in *A. niger* and *A. japonicus* members, respectively. Colonies colours of *A. flavus* were deep green to olive green in colour. Colonies always floccose centrally while on CYA, fluffy, wrinkles colonies were formed. Reverse uncoloured to yellow and exudates were rarely present. *A. flavus* produced dark brown to black shiny sclerotia in oval shape, deep green colour of conidial heads. Conidia were globose to elongate in shape with rough surfaces. Some isolates were biseriate on CYA and almost all isolates were uniseriate on MEA.

3.2. PCR amplification and data analysis of β-tubulin gene

A single band approximately 640-bp was successfully amplified from all the 10 isolates using T1 and T2 primers (Figure 1). All the sequences from 10 isolates were aligned and blasted in Gen Bank (NCBI). Percentage of sequence similarity of *A. flavus* blasted in gene bank confirmed the morphological identification.

3.3. Enzyme-linked immune sorbent assay (ELISA) analysis

Our results showed that ten infected samples with *A. flavus* were found positive for AFs contamination ranging from 6.50–74.48 µg/kg (Figure 2). Results in this research showed, 30% (3 out of 10) of all samples infected to *A. flavus* had contamination levels higher than the maximum level of AFs Quantitative Test kit (range of quantitation 5-50 µg/kg) and 70% (7 out of 10) of analyzed samples had contamination levels lower than the minimum level of AFs Quantitative Test kit (Figure 2).

Table 1. Frequency of Aspergillus species isolated from stored nuts in local markets of Baghdad, Iraq.

Host	*A. flavus*	*A. niger*	*A. japonicus*
Pistachio	5	4	2
Hazelnuts	2	2	2
Walnut	1	3	0
peanut	2	1	1
Total	10	10	5

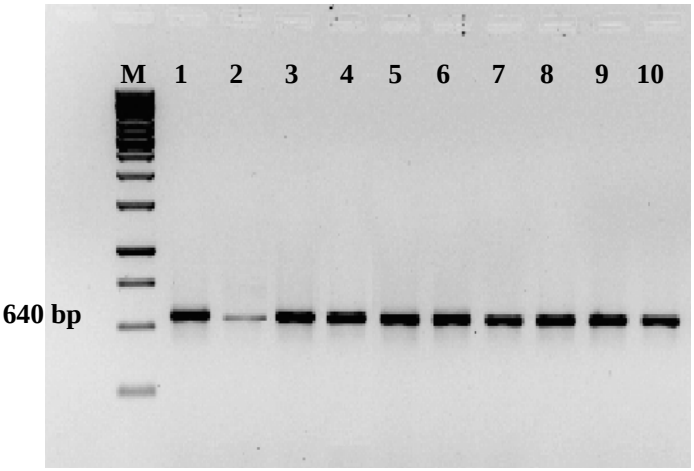

Fig 1. PCR amplification products of the *β-tubulin* gene from 10 isolates belonged to *A spergillus flavus* associated with nuts in Iraq (Ladder= DNA size marker of marker of one kb).

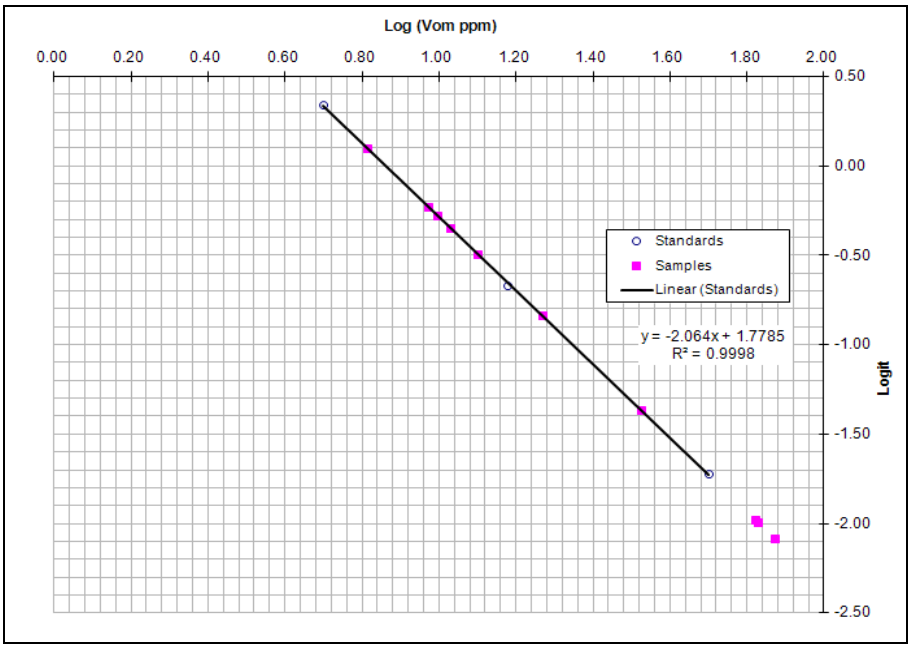

Fig 2. Concentrations of aflatoxins recovered from nuts in Iraq by AFs Quantitative Test kit (range of quantitation 5-50 µg/kg).

4. Discussion

Previous studies showed *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. were the most important toxigenic fungi isolated from stored crops especially nuts kernel (Reddy et al., 2009; Zorzete et al., 2011). The existing of *Penicillium* spp., and *Fusarium* spp. in dried figs and nuts kernels in Iraqian markets were reported by Saadullah and Abdullah (2015), and Chehri and Sattar (2018), respectively. Our results based on morphological and molecular studies showed the *A. flavus,* also was one of the most prevalent *Aspergillus* spp. associated with moldy kernels in different markets in Baghdad, Iraq. Our results are in harmony with reported by Abdullah et al. (2009) and Hussein and Saadullah (2018) that revealed great incidence of *A. niger* and *A. flavus* in medicinal plant and cereals, respectively, in Iraq.

Use of molecular marker using molecular analysis by the partial *β-tubulin* gene sequencing in this study separates all *A. flavus* isolates and confirmed morphological studies. All *A. flavus* isolates produced amplicon size of about 640 bp for T1 and T2 primer pair. Our results were in agreement with the finding reported by Sheila et al. (2018) and Radwan et al. (2014) who tested the same primers to distinguish of *A. flavus* from other *Aspergillus* spp.

Natural occurrence of AFs could be detected in all infected samples with *A. flavus*. Results in this research showed, 30% of all samples infected to *A. flavus* had contamination levels higher than the maximum level of AFs Quantitative Test kit (range of quantitation 5-50 µg/kg) that were in agreement with the finding reported by Sheila et al. (2018), Nagur et al. (2014), and Rahimi et al. (2007). The presence of AFs in different nuts in Iraq can cause serious toxicity and illness in human and animals. These results showed that AFs might be the most life-threatening chemotype in nuts in Iraq, which is in agreement with previous studies in Iraq (Rifaie and Al-Maqtoofi, 2016). The results obtained in this study completed the previously data about genetic potential of AFs production of *A. flavus* strains in Iraq and revealed infected nuts also can be one of the great concern in Iraq. Our studies, also revealed the high frequency of AFs contamination in nuts in Iraq which can be used as a guide for better management strategies towards reduction of mycotoxin contamination.

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Conflicts of interest

There are no conflicts of interest.

Refereces

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