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Immunodetection and risk assessment for *Aspergillus* contamination in nuts using a highly specific monoclonal antibody.

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Abstract

Fungal contamination in nuts is unavoidable and is a major challenge to nuts safety, quality and then for human health after consumption particularly immunocompromised individuals. This study aimed to early detection of potential pathogenic *Aspergillus* in nuts to avoid the harmful effects of allergens and mycotoxins. Three different geographic locations in Basra, Iraq were screened, and a total of 28 fungal isolates were recovered from tested nuts including cashews, almonds, walnuts, pistachio and sunflower seeds. ELISA was used to identify isolated fungi based on antigenic structure *via* using a highly specific MAb for *Aspergillus*, JF5. The accuracy of ELISA was validated by ITS-rDNA sequence in comparison with NCBI database. The highest incidence of isolated fungi belongs to main four genera *Aspergillus* (78.57%), *Alternaria* (10.71%), *Cladosporium* (7.14%), followed by *Penicillium* (3.75%). The most dominant species was *A. flavus*, a potential aflatoxins producer, (28.57%). Abu was highly contaminated geographic site comparing with Zubayr and Qarmat. These findings raise awareness about contaminated nuts particularly there is no database available and highlighted the accuracy of using specific MAb for fungal identification and diagnosis. The occurrence of well-known toxigenic species such as *A. niger, A. flavus* and *A. terreus* suggested the possible risk of mycotoxin contamination of the nuts for human consumption.

Keywords: Monoclonal antibody, Fungal antigen, *Aspergillus*, ITS, Diagnosis, Nuts.

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Introduction

Nuts are widely consumed as plant origin foods due to the nutrition they contain, and are considered a source of microorganism growth, including toxigenic and pathogenic fungal species. Therefore, contamination of nuts has a significant impact in plant production, economics, human and animal health [1]. The expanded nut-products trade has a highlighted nut's role in the exposure to diseases associated with the occurrence of toxigenic fungal species and their secondary metabolites [2].

Mycotoxigenic fungi are ubiquitous environmental moulds, and pathogens of both plants and animals [3]. These pathogens are considered as natural contaminants of nuts. The most predominant mycotoxic and pathogenic fungi are in tropical environments, and mainly belong to *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* [4]. Moreover, these fungi have been reported as serious life-threatening pathogens with high rates of mortality and morbidity in immunocompromised patients [5,6]. Nut contamination can occur either by fungal colonization or their production of secondary metabolites during or after harvesting, storage and transition [7].

Mycotoxins are fungal secondary metabolites and exhibit toxic effects in human and animal health through contaminant foods or feeds consumption [8]. The presence of mycotoxins in nuts leads to a disease called mycotoxicosis that has emerged in several countries and requires development of new strategies to early prevention, also require urgent investigations to avoid human infections [9]. Since *Aspergillus* species, including *A. flavus*, *A. niger*, *A. fumigatus*, *A. terreus* and *A. nidulans*, are a major wide spread source of aflatoxins (AFTs) production [10], these pathogenic fungi are one of the biggest threat to human health. The risky aspects of these species are due to invasive aspergillosis, leading to high mortality rate in impaired immune response individuals *via* direct propagules infection [11,12], or in aflatoxicosis through of aflatoxin-tainted foods [13]. Liver cancer, reduction in protein metabolism, mutagenic, skin necrosis and fat absorption are all risky consequences of contaminated nuts [14]. For the immune system, aflatoxins significantly supress T-cell activities and macrophage phagocytosis, resulting in immunosuppression [15-17].

There is no formal information about nuts consumption in the Iraqi population comparing with other countries available by the Food and Agriculture Organization (FAO) or any of academic study. We understand the consumption of nuts in Iraq is a kind of daily habits. Based on this fact, the rate of nuts consumption is estimated to be ranged between 50 to 150 g/ person daily. Sunflower seeds the most popular kind of nuts consume daily. Generally, there is no data available about the role of contaminated nuts and infected patients after nuts

consumption. Recent studies have shown an urgent need for mycotoxigenic fungi identification globally to develop a mycotoxins prevention strategy [18].

To our knowledge, there is no study has used the combination of highly specific monoclonal antibody (MAb) JF5 and molecular technique Internal Transcribed Spacer (ITS) for diagnosis *Aspergillus* species isolated from contaminated nuts for human consumption. This work aimed to screen nuts and determine the potential fungal contamination using immunodiagnostic test to provide an epidemiology view of opportunistic pathogens that can be associated with mycoses infections due opportunistic pathogens. The study is focused on three geographical areas that close to borders and have the largest portion of population in Basrah, South of Iraq.

Material and Methods

Sample collection

Five kinds of unroasted and unsalted nuts including cashew, almonds, walnuts, pistachio and sunflower seeds were purchased by laboratory staff (1 kg) each type between April to May 2017 from three different geographic local markets in Abu Al Kasib, Qarmat Ali and Zubayr within Basrah province, Iraq (Figure 1). The first three types of nuts were unshelled whereas pistachio nuts were split-shells and sunflower seeds were in-shell. Nuts samples were immediately transferred to the laboratory as soon as purchased, for processing on arrival by mycological culture and molecular treating as illustrated in (Figure 2).

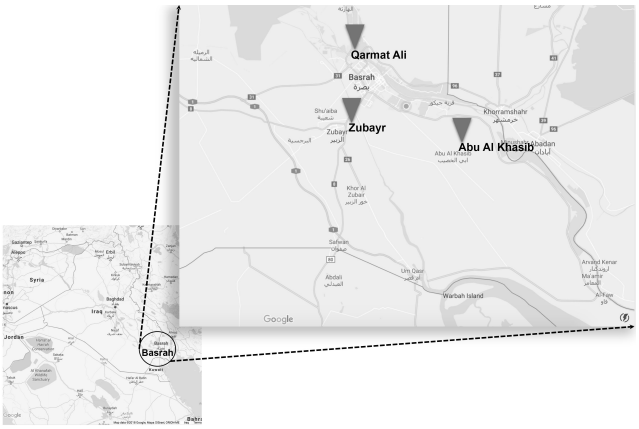

Figure 1. *Map of Basrah governor, Iraq shows three of nut samples collection geographical areas used in this study.*

Fungal isolation from nuts

To study the mycotoxigenic fungi of the nuts samples, 90 seeds of each kind of nuts were placed on petri dishes and then superficially sterilized by flooding with 5% sodium hypochlorite solution freshly prepared for 5 min at room temperature followed by washing three times with sterile H_2O. Washed samples were then blotted on sterile Whatman filter paper sheets to dry prior cultivation. Four seeds of each nuts kind at three replicates were then placed on the surface of potato dextrose agar (PDA) (LAB098; Lab M Limited, UK) containing 0.1 mg/ml of the broad-spectrum antibiotic Rifampicin (P17278; Ajanta Pharma, India). The plates were then incubated at 25°C and daily monitored for fungal growth recovery. A sample was considered infected when fungal growth was detected. Subsequently, the recovered isolates were purified by sub-culturing on PDA with rifampicin until axenic cultures were generated.

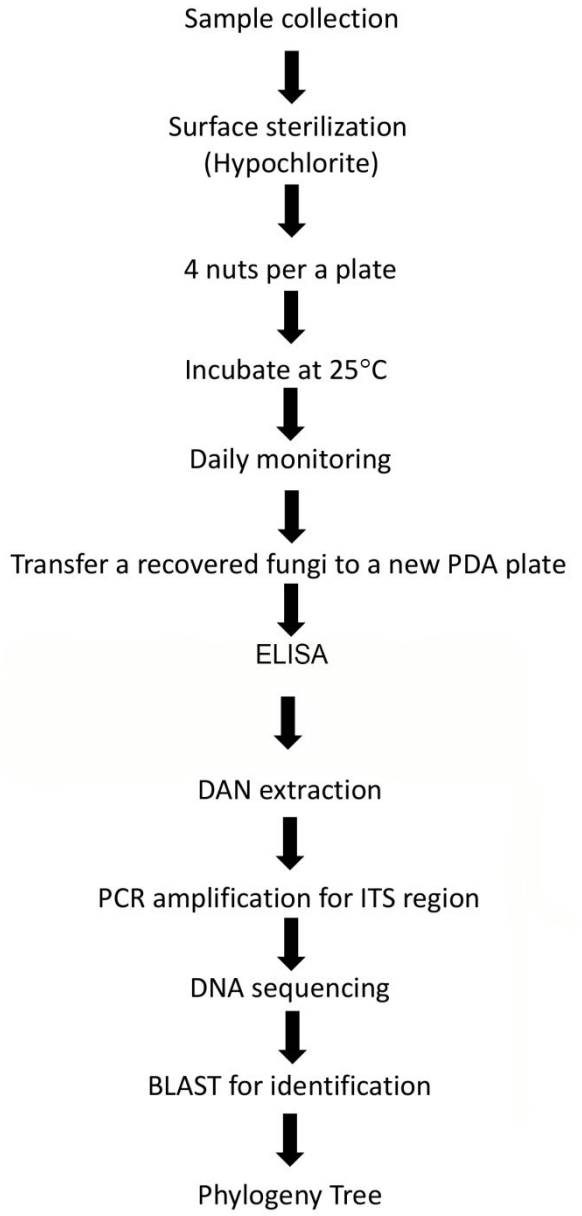

Figure 2. *Schematic diagram showing isolation and identification work-flow of potentially mycotoxigenic fungi recovered from contaminated nuts.*

A reference code was allocated to each of the 28 isolates recovered. The isolates were then maintained as PDA slopes at 25°C before DNA extraction for molecular identification of fungi as described below.

For *Aspergillus* antigen detection, recovered fungi were grown on PDA slopes and surface washing containing water-soluble antigens prepared as described in [19]. Fifty µl volumes were then used to coat the wells of microtitre plates. After incubating overnight at 4°C, wells were washed four times with PBST (PBS containing tween-20, 0.05% (v/v)), once each with PBS and distilled H_2O and then air-dried in a laminar flow hood. Wells containing immobilized antigens were blocked for 10 min with 100 µl of blocking solution 1% (wt/vol) BSA (Sigma A-7030) in PBS. Diluted primary MAb JF5 (mouse IgG3 specific to *Aspergillus* and *Penicillium* spp.) [20] in 10% FBS/PBS (1:1000) was conjugated with HRP, following manufacture instructions from Lightning-LinkTM HRP Conjugation Kit (Expedeon, UK, #701-0001), and then was added into the wells for 1 h incubation. Bound antibody was visualised by incubating wells with TMB (T2885; Sigma) substrate solution for 30 min. The reactions were stopped by the addition of 3 M H_2SO_4 and absorbance values were determined at 450 nm. Wells were given four 5 min rinses with PBST between incubations and a final rinse with PBS before addition of the substrate solution. Working volumes were 50 µl per well and control wells were incubated with PBS. All incubation steps were performed at 25°C in sealed plastic bags. The threshold for detection of the antigen in ELISA was determined from control means. Consequently, absorbance values>0.100 were considered as positive for the detection of antigen.

Identification of fungi by analysis of the ITS regions of the rRNA-encoding gene unit

The recovered fungi were grown on PDA for 5 d at 25°C and then 3 × 5 mm agar plugs taken from the leading edge of PDA cultures, inoculated into 250 ml conical flasks containing 100 ml of potato dextrose broth (PDB), and incubated at 25°C for 48 h. Fungal biomass were collected by filtration through a sterile Whatman filter paper and snapped in liquid N_2 (LN_2). Collected mycelia were ground in a mortar with LN_2 until a fine powder was produced and then transferred into a clean 1.5 ml microcentrifuge. Genomic DNA extraction was conducted using hexadecyltrimethylammonium bromide (CTAB) method as described in [21]. Briefly, 500 µl of 2X hexadecyltrimethylammonium bromide (CTAB) was added, incubated for 30 min at 65°C with occasional shaking. An equal volume of chloroform:isoamyl:alcohol (24:1:1) was added and shaken for 30 min at room temperature. The samples were then centrifuged at 14000 Xg for 10 min.

The supernatant was collected into clean tubes followed by adding 500 µl of chilled isopropanol and incubation on ice for 5 min. Next, samples were centrifuged at 14000 Xg for 10 min and the pellet was drained and resuspended in 500 µl in nuclease-free-water. The DNA was re-precipitated by adding 0.1 vol of sodium acetate, 3 M (pH 5.2) and 2 vol of absolute ethanol and incubated for 10 min at 20°C. After centrifugation at 14000 Xg for 20 min, the purified genomic DNA was recovered and washed with 400 µl of ethanol, 70% (v/v), and then was dried for 5 min in a vacuum rotary desiccator and resuspended in 25 µl of nuclease-free water (Sigma) containing RNAse A, 10 µg/ml. A NanoDrop spectrophotometer (Thermo Scientific) was used to quantify DNA concentration.

PCR and gel electrophoresis

Sequencing of the ITS1-5.8S-ITS2 region of the rRNA-encoding gene unit technique was used for recovered mycotoxigenic fungi identification at molecular level according to procedures described elsewhere [5], using the primers ITS1ext (5'-GTAACAAGGTTTCCGTAGGTG-3') and ITS4 ext (5'-TTCTTTTCCTCCGCTTATTGATATGC-3'). The primer sequences are based on those described by White et al. [22]. The ITS1-5.8S-ITS2 region was amplified by polymerase chain reaction (PCR) procedure using PT-100 thermocycler as follows. The PCR mixture was carried out using a gDNA template approximately 10-25 ng, 25 µl Red Taq Master Mix (VWR), 2.5 µl of each primer at 0.5 picomoles (pmol), and the final volume of reaction was adjusted to 50 µl with Nuclease free water or MQ water. The 37 cycles of PCR conditions after a first initial denaturation step at 95°C for 5 min were followed: 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min, and final extension at 72°C for 10 min.

To confirm amplification and the gDNA band size, the PCR products were subjected to electrophoresis in 1.5% agarose gel (ApplyChem) using a 1X tris-borate EDTA buffer (TBE) (0.09 M tris-borate, 2 mM EDTA) with the addition of ethidium bromide 4 µl of a 0.5 µg/ml stock per 100 ml of agarose. The amplified DNA band size was determined using the 1 Kb plus size marker (Invitrogen). DNA bands were visualized and imaged under UV light using UV lamp (Spectroline). After DNA fragments were cut from gel, amplified bands were purified using PCR DNA clean kit (Thermo Fisher) according to the manufacturer's instructions.

Nucleotide sequence accession numbers

Newly determined ITS sequences were submitted to GenBank and the ITS accession numbers MH237623 to MH237650 were obtained. Species designations of recovered fungi are shown in Table 1.

Species identification by DNA Sequence analysis and phylogenetic tree

The purified PCR products were submitted to Macrogen Korea for sequencing. Subsequently, the recovered fungal sequences data were compared with database of the GenBank species by depositing in the National Center for Biotechnology Information (NCBI) databases by using NCBI BLAST® (https://blast.ncbi.nlm.nih.gov). According to Leonard et al. [23], a serial of bioinformatics tools were used for basic editing and analysis to establish the phylogenetic tree including SeaView Version 4.5.4 (http://doua.prabi.fr/software/seaview) (Manolo Gouy, Laboratoire de Biométrie et Biologie Evolutive CNRS/ Université de Lyon "Licensed under the GNU General Public Licence"), FigTree Version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) (Andrew Rambaut Institute

of Evolutionary Biology, University of Edinburgh), REFGENE bioinformatics tool (http://richardslab.exeter.ac.uk/refgen.html) and TREENAMER bioinformatics tool (http://richardslab.exeter.ac.uk/treenamer.html.

Results

Isolation and Frequencies of fungal contaminants

In this study, a total of 60 samples of unroasted and unsalted nuts, in triplicate (20 samples each replicate) were tested. Nuts were examined to detect the percentage of potential mycotoxigenic fungal contamination. Based on the geographical distribution, nut samples from Abu Al Kasib (42.85%) were the most contaminated commodities by fungal species, followed by Zubayr (39.28%) and Garmat Ali (17.85%) (Figures 3A and 3B).

A total of 28 fungal species were recovered from nuts that surface-sterilized. The incidence of *Aspergillus* species was dominant (71.42%). Both *Penicillium* spp. and *Alternaria* spp. were recovered at the same rate (10.71%), compared with the lowest contamination of all commodities by *Cladosporium* spp. (7.14%) (Figure 3C).

Figure 3D shows the percentage of unroasted and unsalted five nut samples that were naturally contaminated by fungi. Cashews were found to be the most significantly contaminated nuts (42.85%), with potential mycotoxigenic fungal species of *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium*, while the lowest level of contamination was found in both almonds and pistachios (10.71%). Walnuts and sunflower seeds were in the middle (17.85%).

Immunodetection of fungal antigens

ELISA test using highly specific MAb, JF5, to *Aspergillus* and related fungi belong to certain *Penicillium* species was used to identify recovered axenic fungal cultures from nuts *via* tracking crude antigen extracts. The MAb was shown to be specific for their target species (Table 1). Total of 28 fungal isolates tested by ELISA, up to 71% reacted with MAb JF5 specific for *Aspergillus*. The remaining fungi were unrelated species non-reactive with MAb JF5. In addition, MAb cross-react with related with antigens from *Penicillium griseofulvum*.

Identification of fungal species

The result of DNA sequencing analysis of ITS region for 28 fungal isolates confirmed the specificity test of ELISA outcomes. MAb JF5 successfully detect strains of *A. flavus* prevalence was (28.57%), followed by *A. niger* (17.85%). *A. nidulans* and *A. ruber* were less frequent at (3.57%). An undefined *Aspergillus* was isolated (10.71%) at species level after NCBI submission. Other fungal species recovered from tested commodities include: *Alternaria alternata*, *Alternaria infectoria*, *Cladosporium herbarum*, *Cladosporium limoniforme* and *Penicillium griseofulvum* (Table 1 and Figure 4).

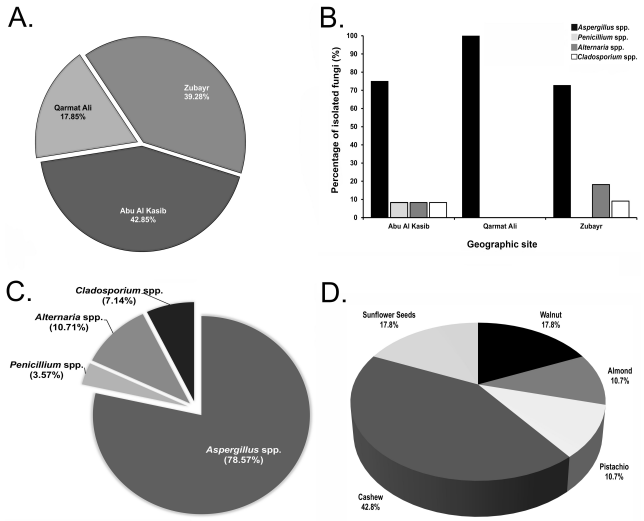

Figure 3. *Frequency of recovered fungi from tested nuts based on the ITS1-5.8S-ITS2 region of the rRNA-encoding gene unit identification. (A) distribution of fungal species based on geographical location, (B) frequency of fungal genus within three collection sites, (C) percentage of recovered moulds, (D) percentage of contaminated five types of nuts.*

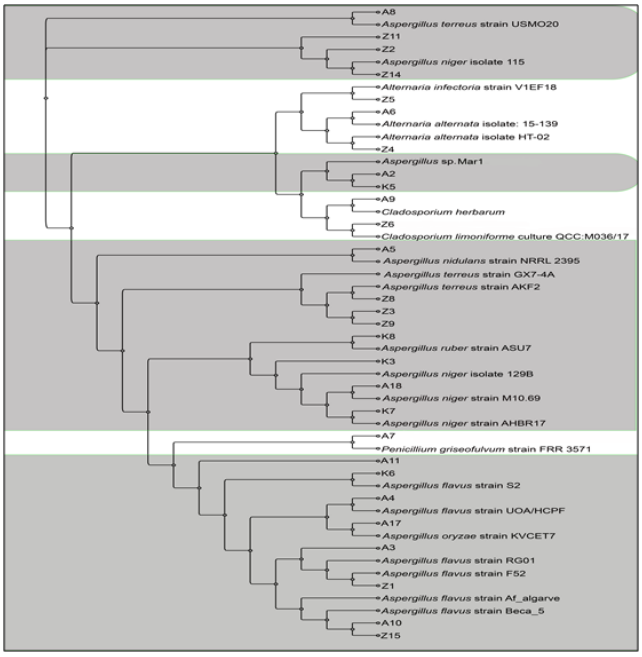

Figure 4. *Phylogram generated from maximum likelihood analysis of recovered fungi from nut samples based on combined the ITS1-5.8S-ITS2 region of the rRNA-encoding gene sequenced data compare with NCBI strains.*

Table 1. *Results of ELISA detection and identification of fungi using ITS sequencing.*

Location	Isolate code	Source	Predicted fungal genus	ELISA Absorbance	Identification based on	GeneBank
				Ab at 450nm	ITS sequencing	Accession no.
Abu Al Kasib	A2	Walnut	*Aspergillus*	0.943	*Aspergillus* sp. Mar1	MH237623
Abu Al Kasib	A3	Walnut	*Aspergillus*	1.211	*Aspergillus flavus*	MH237624
Abu Al Kasib	A4	Sunflower Seeds	*Aspergillus*	0.974	*Aspergillus oryzae*	MH237625
Abu Al Kasib	A5	Sunflower Seeds	*Aspergillus*	1.065	*Aspergillus nidulans*	MH237626
Abu Al Kasib	A6	Sunflower Seeds	*Alternaria*	0.001	*Alternaria alternata*	MH237627
Abu Al Kasib	A7	Cashew	*Penicillium*	0.627	*Penicillium griseofulvum*	MH237628
Abu Al Kasib	A8	Cashew	*Aspergillus*	1.203	*Aspergillus flavus*	MH237629
Abu Al Kasib	A9	Cashew	*Cladosporium*	0.002	*Cladosporium herbarum*	MH237630
Abu Al Kasib	A10	Cashew	*Aspergillus*	1.192	*Aspergillus flavus*	MH237631
Abu Al Kasib	A11	Pistachio	*Aspergillus*	1.253	*Aspergillus flavus*	MH237632
Abu Al Kasib	A17	Amond	*Aspergillus*	1.099	*Aspergillus flavus*	MH237633
Abu Al Kasib	A18	Cashew	*Aspergillus*	1.381	*Aspergillus niger*	MH237634
Qarmat Ali	K3	Walnut	*Aspergillus*	1.27	*Aspergillus niger*	MH237635
Qarmat Ali	K5	Cashew	*Aspergillus*	1.001	*Aspergillus* sp. Mar2	MH237636
Qarmat Ali	K6	Cashew	*Aspergillus*	1.089	*Aspergillus flavus*	MH237637
Qarmat Ali	K7	Pistachio	*Aspergillus*	1.209	*Aspergillus niger*	MH237638
Qarmat Ali	K8	Sunflower Seeds	*Aspergillus*	0.554	*Aspergillus ruber*	MH237639
Zubayr	Z1	Cashew	*Aspergillus*	1.019	*Aspergillus flavus*	MH237640
Zubayr	Z2	Cashew	*Aspergillus*	1.256	*Aspergillus niger*	MH237641
Zubayr	Z3	Cashew	*Aspergillus*	1.046	*Aspergillus terreus*	MH237642
Zubayr	Z4	Cashew	*Alternaria*	0.002	*Alternaria alternata*	MH237643
Zubayr	Z5	Cashew	*Alternaria*	0.001	*Alternaria infectoria*	MH237644
Zubayr	Z6	Sunflower Seeds	*Cladosporium*	0.002	*Cladosporium limoniforme*	MH237645
Zubayr	Z8	Almond	*Aspergillus*	1.28	*Aspergillus terreus*	MH237646
Zubayr	Z9	Almond	*Aspergillus*	1.262	*Aspergillus terreus*	MH237647
Zubayr	Z11	Pistachio	*Aspergillus*	1.067	*Aspergillus terreus*	MH237648
Zubayr	Z14	Walnuts	*Aspergillus*	1.195	*Aspergillus niger*	MH237649
Zubayr	Z15	Walnuts	*Aspergillus*	1.037	*Aspergillus flavus*	MH237650

Discussion

Mycotoxins have great impact on the human health, animal production and countries economy [24]. Similar to the other crop plants, nuts are susceptible to the growth of mycotoxin producing fungi and contain significant amount of different mycotoxins, most prominently, aflatoxins produced by *Aspergillus* species [25]. These toxigenic fungi are ubiquitous and have a particular importance as nuts contaminants of human consumption. In addition to the reduction of nutrient in contaminated nuts, the activity of fungal propagules growth and mycotoxin production indicates a potential increase risk in developing pulmonary mycotoxicosis and aspergillosis, particularly in immunocompromised patients [26]. The most common *Aspergillus* species associated with aflatoxins production is *A. flavus* [27]. This species is widely detected in food and feeds stuffs [28].

Additionally, aflatoxins have reported as a group 1 human carcinogen by the International Agency for Research on Cancer [14]. They also have bioaccumulation property leading to teratogenic [29].

Since aflatoxins are serious immunosuppressive mycotoxins, consumption of contaminated nuts with aflatoxins results in dysregulation of the immune cells: proliferating,

differentiating, and the communication network between cellular and humoral components is disrupted. In addition, mycotoxin-induced immunosuppression may lead to impaired T-lymphocytes and B-cells, and specifically to antibody production. Significant effect of these toxins has been reported mainly through the suppression of phagocytosis activity of innate immune cells, including macrophages and neutrophils [30-32]. Due to the defects in human immune responses after consuming mycotoxin contaminated commodities, the susceptibility to opportunistic fungal infections such aspergillosis is increased [33].

Extensive research on mycotoxins contamination production, detection, distribution, and their medical effects in agriculture products, including nuts, have been done worldwide such the US, Brazil, India, Nigeria, and others. The main goal of this study was to evaluate the mycotoxigenic contamination in nuts for human consumption, and then better understand their distribution. To the best of our knowledge, this study has demonstrated, for the first time, the detection and distribution of toxigenic fungi using this molecular technique, ITS-rDNA, in nuts intended for human consumption within three different local geographic areas in the south part of Iraq, Basra.

In this study, a total of 28 different potential mycotoxigenic fungi were recovered from nut samples, which include shelled cashews, almonds, walnuts, half-shelled pistachios, and unshelled sunflower seeds. These commodities were selected in this study as they are the most popular nut products consume by both competent and immune-impaired individuals. All isolated species were filamentous fungi with no observation to yeast moulds. These isolates were potential toxigenic species belong to main four genera that well known as mycotoxins producers and human pathogens including *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium*. Previous studies have reported similar observation of fungal contamination in tested commodities [34,35]. On the other hand, yeasts have been isolated from contaminated nuts at a very low percentage [36,37].

The results of this study show that the highest rate of nut contamination came from *Aspergillus* species (78.57%) followed by *Alternaria*, *Cladosporium* and *Penicillium*.

Significantly, *A. flavus* was the most prevalent contaminant species in all tested nut samples including cashews, walnuts, sunflower seeds, almond and pistachios in accordance with [38]. This species is ubiquitous and has capability to produce one or more of the carcinogenic aflatoxins that causing aflatoxicosis after human consumption, in particular by immunocompromised patients [27,39]. The International Agency for Research on Cancer has identified aflatoxin as group 1, highly carcinogenic product, to human [14].

The percentage of nut contamination by fungal propagules varies based on nut type. The analysis test of fungal recovery showed that cashews were the most loaded nuts with fungal contamination (42.85%), compared with the other samples. Importantly, the incidence of *A. flavus* was the most commonly identified species in cashews [40], this finding is similar to our observation. Shells of nuts are the first physical barrier that preventing microbial invasion that explains the susceptibility of unshelled nut samples to fungal contamination [41]. In addition to the matrix structure of cashew, the moisture and nutrient contains including carbohydrates, proteins and lipids that encourage fungal propagules growth, penetration and mycotoxin production [42].

Based on geographic location, purchased nuts from Abu Al Kasib were the most contaminated samples followed by Zubayr and lastly Qarmat Ali. The 100% accuracy of the immunodiagnosis test using MAb JF5 ELISA, confirmed by using ITS sequencing PCR analysis of recovered isolates, demonstrates its robustness in detecting potentially toxigenic *Aspergillus* species in contaminated nuts. Around two third of axenic isolates were *Aspergillus* species [20]. Many factors have influenced toxigenic fungi for mycotoxin products, for example, storage conditions and ways of transport. High level of humidity during storage can stimulate mycotoxin production [2].

Acknowledgement

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