MOLECULAR TECHNIQUES FOR DETECTION OF AFLATOXIGENIC *ASPERGILLUS* FLAVUS AND DETERMINE THEIR AFLATOXIN IN PISTACHIOS.

K.I.Hassan
Professor
Dept. of Food science &Quality control. College of Agricultural Engineering Sciences. Sulaimani University
khlood.hassan@univsul.edu.iq

ABSTRACT

The better approach to control the contamination of food by aflatoxin (AF), is identifying the fungal strains responsible for producing that toxin. To estimate the presence of aflatoxigenic fungi growth and AFs in pistachios, thirty two pistachios samples (8 fresh and 24 salt roasted) were collected from Sulaimani markets (Iraq).Rapid screening method for AFs production were utilized initially based on culture methods, the positive samples confirmed by polymerase chain reaction (PCR) that were applied using DNA extracted from the isolates of *A. flavus* targeting the genes (*afR, aflP, aflD, and aflQ*). For determination of AF production of those isolates, ELISA technique was applied. The results show, contamination of 20 (62.5%) samples of pistachios with fungi, among them *A. flavus* was the predominant. Ten (71.4%) of the *A. flavus* isolates were amplified the target genes by applying PCR, indicating their ability to produce AFs. Application of ELISA technique revealed AFs detection in 21(65.6%) samples of pistachios. The mean levels of AFs determined were 6.06 and 12.08 µg/kg in fresh and salt roasted samples respectively. 19 (59.4%) of pistachio samples were exceed the maximum allowable limit set by the European union of total AFs concentration .Strong correlation obtained between AFs gene expression with AFs production. However, to avoid false negative and false positive results, applying more than one diagnostic molecular technique were suggested.

Key words: Pistachios, Aflatoxin, PCR, ElISA, *A. flavus*.

مجلة العلوم الزراعية العراقية -2023 :54(6):1773-1783 حسن

استعمال التقانات الجزيئية في الكشف عن عزلات ال *ASPERGILLUS FLAVUS* المنتجة للافلاتوكسينات مع تحديد كمية الافلاتوكسينات في الفستق.

خلود ابراهيم حسن
استاذ مساعد
قسم علوم الاغذيه والسيطرة النوعيه .كلية علوم الهندسة الزراعيه.جامعة السليمانيه

المستخلص

ان افضل طريقة للسيطرة على الاغذية الملوثه بالسموم الفطريه، هو تحديد السلالات الفطرية المسؤولة عن إنتاج تلك السموم .وللكشف عن الفطريات المنتجه للافلاتوكسين ومدئ تلوث الفستق به، تم جمع 32 عينة من الفستق (8 طازجة و 24 محمصة بالملح) من أسواق السليمانية (العراق).للكشف الاولي السريع عن العينات الموجبه (المنتجه لمادة الأفلاتوكسين) تم استخدام الاوساط الزرعية الصلبة وتم التأكيدعلى العينات الموجبه بتطبيق تفاعل البلمرة المتسلسل (PCR) باستخدام الدنا المستخلص من عزلات ال *Aspergillus flavus* باستهداف الجينات المسوؤله عن انتاج الانزيمات المشتركه في مسار التخليق الحيوي لانتاج الافلاتوكسين(afR ,aflP, aflD, and aflQ). ولتحديد كمية الافلاتوكسينات المنتجه بواسطه تلك العزلات, تم تطبيق تقانة الاليزا.اظهرت النتائج تلوث 20 (62.5%) عينه من عينات الفستق بالفطريات ,ومن بينها كانت عزلات الفطر *Aspergillus flavus* هي الاكثر شيوعا. تم تاكيد انتاج الافلاتوكسين في 10 (71.4 %) عزلة منها عند تطبيق تفاعل البلمرة المتسلسل وذلك من خلال الحصول على النواتج المتضاعفة للجينات المختارة. كما اظهر تطبيق تقنية الاليزا أنه من بين 32 عينة من الفستق تم الكشف عن الافلاتوكسين في 21 (65.6٪). وكان معدل تركيز الافلاتوكسينات فيها 6.06 و12.08 ميكروغرام / كغ لعينات الفستق الطازجة والمحمصة بالملح على التوالي. وكانت نسبه الافلاتوكسين في 19 (59.4%) من عينات الفستق أعلى من الحد الأقصى المسموح به من قبل الاتحاد الاوربي. كما تم الحصول على علاقة طردية قوية بين التعبير الجيني للافلاتوكسينات وانتاج الافلاتوكسينات ولذلك وللحصول علئ نتائج دقيقة يقترح تطبيق اكثرمن تقانةجزيئية للكشف عن الفستق الملوث بالافلاتوكسينات.

كلمات مفتاحيه:الفستق ,الافلاتوكسينات , ايلاز, تفاعل البلمرة المتسلسل, *A. flavus*.

Received:16/11/2021 Accepted:2/3/2022

INTRODUCTION

Pistachio (Pistacia vera L.) is one of the world's most healthy and delicious nuts, it is rich in oleic, linoleic acid, minerals (Ca, Mg, K, Na and P), vitamin B6, thiamin, as well as other bioactive molecules, such as polyphenols, phytosterols carotenoids, tocopherols, and dietary fiber, (12). Before harvest, the shells of most pistachios nuts are split, exposing the kernel of pistachio seeds to fungal infection, and this make pistachio from the nuts that are more vulnerable to fungal attacks, and the highest contribution to mycotoxin exposure in adults (21). Mycotoxins are toxic Product of certain species of mold and fungi. More than 500 various types of mycotoxins have been documented so far and their number continues to rise (5), however, only six types regularly contaminating food, these include: aflatoxins (AFs), ochratoxins, fumonisins, zearalenone, trichothecenes and patulin. Among them, AFs are considered the most significant in their abundance, toxicity and human impact (30). AFs are bisfurans that are polyketide-derived, produced primarily by some species of *Aspergillus fungi*. The six major groups of AFs include: B1, B2 with their metabolite M1 and M2 as well as G1 with G2 .Among these groups AFB1 are still the most toxic form , as they have been considered within group 1 carcinogen (strongly associated with liver cancer). It is also cause immune system suppression, infertility, malabsorption of nutrients (37). Food contamination with AFs is considered as a global problem, which became exacerbate in developing countries, due to faulty storage conditions which induce fungi growth and AFs production (3). The most popular aflatoxogenic fungi species are some sections of the Aspergillus genus including 16 species of Flavi , three species of Nidulantes and two species of Ochraceorosei . The more aflatoxigenic species within section Flavi are *Aspergillu flavus* (34).However there are also variability between the strains of *A. flavus* in their ability to produce AFs and only 40–50% of these strains were aflatoxigenic (11), which cause huge economic losses and make a great need to detect them. Some studies still used the classical methods to detect AFs producer strains such as Ultraviolet light (UV) test which based on the fluorescence that produced by a substance under UV light and yellow pigmentation as well as ammonia vapor test (50; 1), however these methods are just qualitative, and cannot identify critical contamination points of food which is important for risk modelers. Hence fast and reliable methods have been developed for identification of aflatoxogenic fungi, include applying wide varieties of biosensors such as surface plasmon resonance biosensors (35) and quartz crystal microbalance (23) or applying PCR-based techniques targeting various genes involved in the process of aflatoxinB1 biosynthesis pathway from acetyl-coenzyme A. The DNA sequences of these genes have been published, which can be used as a as a valid marker to check AFs production (11;24). Some studies targets three of these genes (*aflP, aflD, aflR*), in applying PCR for the detection of aflatoxigenic *A.flavus* (26;44) others select seven genes (*aflQ, aflD, aflP, aflM, aflO, aflS and aflR*) for the same purpose. (1; 15) .The structural gene *aflD* (nor-1) encodes to the enzyme that catalyzes the conversion norsolorinic acid with NADH to averantin, whereas, *aflP* gene is responsible of conversion of sterigmatocystin to O-methylsterigmatocystin. The *aflQ* gene is direct the last step of AFs biosynthesis, in conversion of O-methyl sterigmatocystin to AFB1. The *aflR* gene differ from the other genes in that it regulates the function of the other genes that involved in the AFs biosynthesis (52).The serious harmful impact of AFs on human health and their huge economic losses worldwide, has led many countries to establish a maximum tolerable level (MRLs) of mycotoxins in food. Accordingly, robust, feasible and reliable analytical methods are needed to monitor AFs at concentration level below than that MRLs established. Recently many of these techniques applied for this purpose such as High performance liquid chromatography (8), lateral flow immunoassays, cytometric bead arrays (53) near and mid infrared spectroscopy, colour imaging and fluorescence spectroscopy (47).Ultra-High Performance Liquid Chromatography combined with electrospray quadrupole, Time of Flight Mass Spectrometry

(45) and electrochemical immunosensor which was used for determination of aflatoxin B1in pistachio by Kaminiaris et al., (22). However the majority of these new techniques are require skilled operators, requiring much time and need high-cost equipment as well as considerable sample pre-treatmen (49). Hence for rapid, accurate and cost effective analysis of AFs, ELISA technique is preferred (2).Many studies still used ELISA for detection of aflatoxinB1in food and feed samples (54, 48), it was also used for determination of AFs in pistachio (13 ; 40). The purpose of this research was to scrutinize the aflatoxigenic ability of some *A.flavus* strains isolated from pistachio nuts using PCR technique targeting some AFs synthesis pathway genes (*aflP, aflQ, aflD*, and *aflR*). This study also intended to evaluate the relation of the presence of these genes in *A.flavus* strains isolated from pistachios with their ability in aflatoxin production which determined by ELISA.

MATERIALS AND METHODS

Sample collection: Thirty two of pistachios samples were collected in the frame of the current study from some sales points in Sulamani market in Kurdistan region of Iraq, which mainly imported from Iran and turkey, during June -September 2021. Sampling was performed according to the sampling protocol of European regulation methods for the control of mycotoxin in food.(Commission Regulation Number 401/2006 (14). The pistachios samples were transported to the laboratory in polystyrene boxes with a cooling gel where the analysis must done properly. Each of the 32 collected samples were thoroughly mixed and divided into two units; each unit contained 100 g of kernels, where one unit is tested for mycoflora identification and another for AFs determination. Pistachios kernels were surface sterilized with 3% sodium-hypo-chloride (NaOCl) for one minutes and then dried after being rinsed three times with distilled water.

Isolation of fungal species from pistachios samples: Ten grams of each pistachio samples was cultured on the PDA (Potato Dextrose Agar) containing 100 mg/l of chloramphenicol to suppress the growth of bacteria. The plates were incubated for 5-7 days at 25 -27°C. Then they were cultured several times to get pure cultures .The species of fungi was identified by examining the main characteristics of fungal colonies developed on culture medium such as colony color, size, appearance with morphology of conidia and scale of sclerotia (50).

Screening of aflatoxin production using Yellow pigmentation and ammonia vapor tests: Screening of AFs production was performed by growing the *A.flavus* isolates at 27 °C for 5-7 days on the medium of coconut extract agar. The presence of an orange-yellow staining, that observed on the backside of the agar plates after in the incubation period suggests the ability of the isolate to produce AFs in yellow pigment production test. By inverting the petri dishes over 3 drops of 28–30% ammonium hydroxide in ammonia vapor test, the yellow pigments turn to plum-red colour in colonies , that have been subjected to vaporized hydroxide vapor, indicating their ability to produce AFs. (43).

DNA Extraction from *A.flavus* strains

to isolate fungal genomic DNA ,The hyphae have been used., that harvested from the pure fungal strains freshly growing on potato dextrose broth using a Cetyl trimethyl ammonium bromide (CTAB) based extraction method with some modification as described by Hassan *et.al.*, (17). After centrifugation of the PDA broth, the precepts were freeze-dried with liquid nitrogen and ground to fine powder with a mortar and pestle. A 900 µl of 2% CTAB ((l.0 M Tris /HCl, (pH 7.5); 1% (w/v); 5 M NaCl; 0.5 M EDTA and 1% (v/v) pre-warmed to 60°C was added to the powder directly , mixed and incubated at the same degree for one hour. The extracts were cooled prior to the addition of 900 µl phenol: chloroform (1:1) with mixing for 90 sec then the phases were separated by centrifugation at 10,000 rpm for 10 mints. A 500 µl of the supernatant was transferred to a fresh centrifuge tube ,then 750 µl of chloroform was added on it with mixing for 10 minute then again centrifuged at 10,000 rpm for 10 mints and the aqueous collected into a sterile micro tube. The nucleic acids were precipitated by adding 250 µl of 7.5M ammonium acetate with 1 ml of cold isopropanol to the aqueous and the mixture kept on ice for an hour. Finally the tube was centrifuged at 10,000 rpm for 10

mints, the supernatant was removed. The dried pellets were re suspended in 100 µl distilled water.

Application of PCR Reaction

For detection of aflatoxogenic species of *A. flavus*, PCR reaction was performed using the extracted DNA from all isolates of *A. flavus* and the primers (Table 1) that targeting the regulatory gene (*afR*) with the structural genes that encode for the enzymes responsible of the last steps of aflatoxin biosynthesis pathway (*aflP, aflD*, and *aflQ*) . All of the targets genes were amplified by separate reactions following optimization of annealing temperature (56 -62) °C. The PCR reactions were actually carried out in a total volume of 25 µL with 100 ng of DNA template, 80 mM MgCl2, and 10X PCR buffer containing 50 mM KCl, 1 mM dNTP, 1 U of taq polymerase, and 0.3 pmol of forward and reverse primers; then the volume was completed by distilled water. To amplify DNA, the following programming was used: 3 min at 93°C (1 cycle), 30sec at 93°C, 30sec at (58-61) °C and 1min at 72°C (35 cycles) with five min at 72°C (1 cycle). A ten µL aliquot of PCR products were separated with a 1.2 % agarose gel electrophoresis that stained by adding ethidium bromide, and visualizes using gel Imaging System under UV light.

Table 1. Nucleotide sequence of the primers used in the current study that target the genes that used in the production of aflatoxin (*aflQ, aflD, aflP* and *aflR*).

Genename	Sequence	Amplicon size (bp)	References
***aflQ* (Ord-*1*)**	***F* (5′-TTA AGG CAG CGG AAT ACA AG-3′)**		**(1);(16)**
	***R*(5′-GAC GCC CAA AGC CGA ACA CAA A-3′)**	**719**	
aflD* (*Nor-1)	***F5–ACC GCT ACG CCG GCG CTC TCG GCA C-3***		
	R 5′-GTT GGC CGC CAG CTT CGA CAC TCC G-3	**399**	**(1), (6)**
***aflP*(*Omt*-1)**	**F 5′- GGC CCG GTT CCC TGG CTC CTA AGC-3**		
	R 5′ - CGCCCCAGTGAGACCCTTCCTCG-′.	**1034**	**(1), (6)**
aflR* (*apa-2)	**F5′ -TATCTCCCCCCGGGCATCTCCCGG-3′ R5′-**		**(1),(27)**
	CCGTCAGACAGCCACTGGACACGG-3	**1254**	

Quantitative determination of AFs by ELISA: AF extraction was carried out by grinding 5 g of Pistachios samples and mixed with 30 mL of water/methanol (25:75) and shacked for 15 min before centrifugation at 10000 RPM for 12 min. Then five mL of the supernatant were transferred to a 15-mL glass tube of it was evaporated. The dried residue was reconstituted with 0.25 mL of 94:6 water/methanol mixtures and centrifuged for 10 min at 17000 RPM, at 4 °C; the supernatant was used directly after that for the analysis. The technique was validated in pistachio matrix, according to Commission Regulation (EC) No 401/2006 (14). Three pistachios (blank samples) were spiked with AFs at three concentration levels. The spiking experiments were performed three times. Validation parameters, such as specificity ,accuracy, LOD, and linearity were determined. For determination of AFs in the pistachios samples, NEOGENE total AF ELISA test kit (AOAC- R1050901) was used. A sufficient number of dilution strips were inserted into a micro well holder. For each sample, one dilution well was used (0, 5.0, 15.0, and 50.0 ppb). The same number of antibody-coated micro well strips was inserted into a micro well holder. Then 100 µl of each standard or sample was added into the micro-titer plate ,then 200 µl enzyme conjugate were added and mixed carefully. Then, 100 µl of the contents from the dilution well was transferred into the antibody-coated well to initiate the reaction, incubated for 15 min at room temperature then, the contents of the wells were discarded and the wells were washed to remove any unbound toxin. After that 100 µl of substrate was added to each well and mixed gently for 5 min incubation at room temperature ,as the color changes to yellow, 100 µl of stop solution was used to stop the reaction . Finally, absorbance was measured by the ELISA reader at 450 nm ,within 30 min of the addition of stop solution.

RESULTS and DISCUSSION

Morphological characterization.

Of the 32 samples of pistachio (8 fresh samples and 24 salt roasted samples), fungi were detected in almost 20 (62.5%) samples (4 fresh samples and 16 salt roasted). Fungal colonies with green to yellow color with white edge appeared surrounding pistachio nuts after 3-5 days culture on PDA are regarded as *A.*

flavus (found in 14 samples) Colonies with a darker green color are regarded as *A. parasiticus* (found in 8 sample), whereas the dark black powdery colonies were identified as *A niger(*found in 4 sample), other characters of these colonies take in consideration in identification of these strains(50) .These characters of fungi also described by Hossain et.al,(20)).From these isolates *A. flavus* was the predominant isolate (70%). The prevalence of toxigenic fungi in pistachios may due to their rich contains of fatty acid, which act as basic material from which acetyl-CoA is made. to obtain an acetyl-CoA(oxidation of fatty acid produce Acetyl-CoA) that in turn act as a signaling molecule for AFs production. Moreover among nut tree, pistachio is more vulnerable to contamination with fungi, because the shells of most pistachio nuts cleft or split naturally prior to harvest, and sometimes the hull that covering the shell attached to it, so that it splits with the shell, exposing the kernel to fungi. There are many other reasons of high incidence of pistachios contamination with fungi especially the salt roasted one, such as insufficient condition of handling and storage (high temperature and humidity).As Iraq is among the countries that import pistachio mainly from Iran and Turkey which add importing related challenges such as longer time needed for shipments and insufficient quality control laboratories on border lines. Hence, in order to preserve the quality of pistachios, they should be kept in appropriate (low) moisture levels throughout marketing and storage.

Identification of AFs Producing Isolates by Yellow pigmentation and ammonia vapor tests.: To identify AFs producing isolates of *A. flavus*, culture methods were utilized initially, based on yellow pigmentation and vapor ammonia tests. Among the 14 isolates, 12 (85.7%) isolates were classified as potentially AFs producer based on these tests, which are still used as inexpensive assays for detection of aflatoxigenic fungi in developing countries (1). Both tests based on the yellow dyes that occur due to the anthraquinone intermediates which are produced along the AFs biosynthesis pathway (42). However in ammonia vapor test ,the yellow pigments change to plum-red in highly aflatoxigenic isolates and to pink color in moderately aflatoxigenic isolates after they exposed to ammonium hydroxide vapor. Because these two tests are based on different mechanisms, combining them increase the sensitivity of the assays for detection of aflatoxigenic isolates. In general cultural methods have been frequently employed to detect the generation of AFs. in contaminated food, but it is not possible to rely entirely on culture methods only for this purpose, and more molecular reliable methods need to reduce reduced false positives for aflatoxigenicity and confirm the results. Nevertheless, culture methods remain most effective for first screening of great numbers of Aspergilla's isolates in a short time with limited resources. (38).

Identification of Aflatoxin-producing fungi using PCR: Currently the identification of aflatoxigenic fungi has become increasingly dependent on molecular characterization such as PCR-based assays, due to its specificity, accuracy and even without the need for isolating pure cultures. In order to identify of the AFs-producing *A.flavus* strains isolated from pistachios samples , PCR applied using DNA extracted from the 12 isolates of *A flavus* ,with four sets of the primers targeting the structural genes (*aflP, aflQ,* and *aflD*) and the regulatory gene (*aflR*) that involved in the production of AFs in *A. flavus* . The results show the presence of specific amplified fragments (399, 719, 1034 and 1254 pb) which represent the target genes (*aflD, aflQ, aflR* and *aflP* respectively) in 10 (71.4%) isolates of A. flavus (Figure 1) even one isolate show the absence of just one of the amplified product (*aflP*).These results indicate the potential of AFs-producing abilities by these isolates. However two (16.6 %) of these isolates did not show any amplified product of the target genes, indicating their inability to produce AFs . Other studies also found more diverse pattern in the *A.flavus* isolates' ability to generate AFs by applying PCR technique targeting the genes involved in AFs biosynthesis pathway (44; 15) or by applying Real time PCR targeting the same genes in other studies (1,39) .Molecular techniques using species specificprimer by PCR also used for identification of other species (29, 33,51)

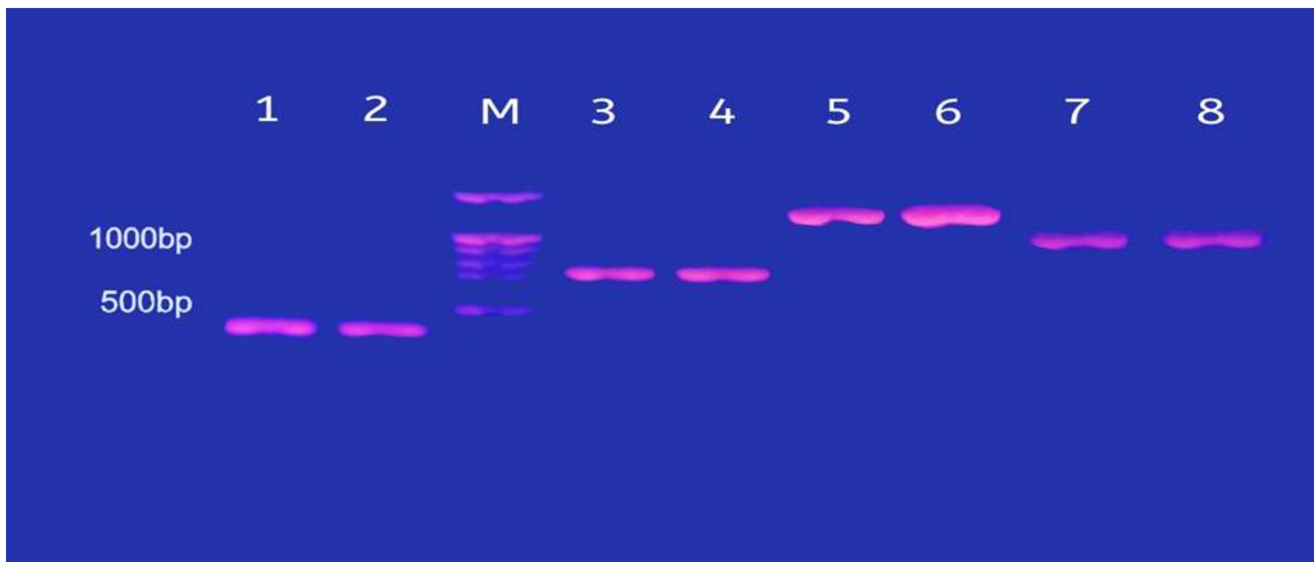

Figure 1. Detection of *aflD, aflQ,* and *aflDP* genes amplified by PCR with separated primer set, using genomic DNA extracted from *A.flavus* . The amplified PCR products were analyzed by 1.2% agarose gel electrophoresis: lane1and 2, *aflD* (399 bp), line M: 1 kb DNA ladder, lane;3and 4 : *aflQ* (719 bp), lane 5 and 6 *aflR* (1254 pb) and lane 7and 8: *aflP* (1034 bp),

The reason of selecting these four genes (*aflD, aflP, aflQ* and *aflR*) in the current study for identification of AFs producing fungi, was due to the fact that the probability of the production of any toxin can be predicted according to the presence of the main genes that responsible of producing the enzymes that direct the biosynthesis of that toxin. AFs biosynthesis pathway involves at least 27 enzymatic reactions for conversion of acetyl-coenzyme A to aflatoxin B1 according many intermediate substances include: Acetic acid, polyketide, norsolorinic acid, averantin, arerufunin, averufin, hydroxyl-versicolorone, versicinal hemiacetal acetic acid, versicolorinB, then A, sterigmatocystin-o-methylsterigmatocystin, aflatoxin B1 (52). The genes that are responsible of coding of these enzymes are grouped together in a cluster and their expression is arranged by two genes (*aflS* and *aflR*) (30). Since AFs start to accumulate rapidly, it appears that the sequences of these genes are highly conserved, hence; it is reliable to apply PCR technique using primers targeting their sequences for identification of aflatoxinogenic isolates. However selecting just one gene, or just the regulatory gene(aflR) was inadequate and cannot differentiate between aflatoxogenic and non aflatoxogenic strains as example the aflR gene regulate both aflatoxin B1 and sterigmatocytin production hence analyzing of this gene for aflatoxin B1 marker may be misleading **.**Hence more than one gene (*aflD, aflP, aflQ*)were selected in this study to idetified aflatoxogenic strain, especially that encode for the enzymes responsible of the last steps of aflatoxin production Pathway (24 ,25) as well as the regulatory gene(aflR). It's worth to mention here, that false negative results may be obtained due to mutations that occur within the primers' targeted binding site of AFs biosynthetic genes. Moreover the presence of some substances in food may inhibit the PCR, such as fats, polysaccharide and phenolic compounds, by reducing the purity of the extracted DNA, also lead to false negative results (18, 10).On the other hand, false positives results may arise due to the fact that PCR technique targeting specific genes cannot detect the mutation in the other genes (not targeted) that involved in AFs biosynthesis pathway, neither can detect their absence in those isolates. Hence the detection of the expression of the four target genes in some strains of *A flavus* using PCR ,even it is good indication of its aflatoxic activity ,but it is still not evidence if these strains were AFs producers or not. So, to differentiate toxigenic from a toxigenic isolates of *A flavus* remains to be confirmed by other techniques for reducing the false positives and negative results.

Determination of AFs by ELISA technique.

For determination of AFs in pistachio samples, ELISA technique selected in this study due to its rapidity, accuracy, sensitivity, cost-

effective, and easy to use. Among the 32 samples of pistachio AFs were detected in 21(65.6%) (Four of fresh samples and 17 in salt-roasted samples) with limit of detection of 2ppm. Ostadrahimi et al.,(36) found that the incidence of AFs contamination was 2.3% in the fresh pistachios but was more higher (55.17%) in the salt-roasted ones . In a big survey study conducted using 3181commercial raw pistachio samples in Iran, AFs were detected in 23.4% of them (30) .Whereas the incidence of contamination with AFs in pistachios samples in Turkey was 14.6% (19). The Variability between these studies, in the incidence contamination of pistachios is due to variation in geographical area, moisture, temperature, and hygienic conditions. As well as sampling which plays a decisive role in measuring of mycotoxin levels, due to that mycotoxigenic fungi do not grow regular on the food, hence the contamination in food samples is not homogeneous. The mean levels of AFs determined in pistachios samples in this study was 6.06 and 12.08 µg/kg in fresh and roasted samples respectively. Depending on European Food Safety Authority, 59.4% of pistachio samples were above the MTLs for total AFs concentration which is 10 µg/kg pistachio (42). High levels of AFs (13.45 µg/kg) in pistachios samples also found by Riba et.al (40) with the mean levels of 0.48 µg/kg in the fresh samples and 22.02 µg/ kg in the salt-roasted pistachio samples. Detecting higher values of contamination with AFs in pistachio samples (which is imported mainly from Iran and Turkey) may attributed to their poor harvest condition, transportation and handling for fresh samples and improper packaging and poor storage condition (high temperatures and moisture) and long-term storage for the salt-roasted samples). Determining higher levels of AFs in the roasted samples of pistachios might be due to that in roasting procedure probably destroy the fungi but their toxins (AFs) are resistance to heat, but in contrary their rate increase especially with long term storage in bad conditions in markets and stores. The reason of focusing on AFs in this study is due to their high toxicity compared with the other kinds of toxin ,especially AFB1, as one of its primary derivatives is aflatoxin-exo-8,9-epoxide . which reacts with cellular macromolecules including DNA, forming a genotoxic DNA adduct Which is strongly associated with the carcinogenic effect of aflatoxin B1, or by the effects of AFB1 on cell function through the induction of oxidative stress (7). Moreover, it induce various metabolic, and cell structure disruptions (9).

The relation between the expression of AFs genes and the formation of AFs: The results of this study Show that most positive samples of AFs production which were analyzed by ELISA were positive for AFs genes expression (*aflD, aflQ, aflR* and *aflP*) by PCR and strong correlation recorded between them, Mahmoud et.al.,(32) also found a positive correlation between AFs production and PCR amplification of aflatoxogenic genes (*aflD* and *aflQ*).Other studies have reported a correlation between AF production and gene expression using real time PCR targeting 6 genes (*aflD, aflQ, , aflP, aflO, aflS and aflR*) (10,18).This indicates that Induction of AFs genes can be utilized as a reliable marker for AFs monitoring. (15). However in the present study, it was noted that two strains produced detectable AFs (determined by ELISA) but were negative for the PCR products , due to the conditions in salt-roasted pistachio samples are unfavorable for fungi growth ,therefore they may be eradicated, but the produced toxins will probably remain.. On the other hand three samples of pistachio show no AFs production although they are positive for the PCR products due to the variability between the ability of the *A. flavus* isolates in producing AFs and only 40–50% of these strains were aflatoxigenic (10), as well as the conditions that enhance the growth of aflatoxigenic fungi are not necessarily suitable to the formation AFs (28). Pakshir et.al,(38) also recorded such results .Hence to assess toxicological risks associated with contaminated food aflatoxigenic strains should be detected as well as estimation of the levels of AFs they produced (46, 31). This method may improve in the future using high performance liquid chromatography (4, 32).

CONCLUSION

To identify AF producing strains of *A. flavus* isolated from pistachio, PCR was applied successfully targeting the structural genes

(*aflD, aflP, aflQ*) with the regulatory gene(*aflR*) that encode for the enzymes that involved in the last steps of AF biosynthesis pathway , which considered as a rapid ,accurate and reliable technique for discrimination between AF-producing and non-producing strains ,however, challenges will remain in terms of detection limits, as well as the presence of false positive or negative results. Hence they should be confirmed by other techniques, such as ELISA, which applied for monitoring AF content of pistachios with limit of detection of 2ppm. Strong correlation obtained between AFs production which were analyzed by ELISA with AFs genes expression which analyzed by PCR indicating the need to combine the two diagnostic molecular methods for detection of contamination of pistachio with AFs, which could be very useful of their application on other kinds of food.

REFERENCES

1. Abd El-Aziz, A. R.M., S.M. Shehata, S.M Hisham and A.A Alobathani.2021. Molecular profile of aflatoxigenic and non-aflatoxigenic isolates of Aspergillus flavus isolated from stored maize .Saudi Journal of Biological Sciences. 28(1)1383–1391. DOI: 10.1016/j.sjbs.2020.11.073

2. Agriopoulou ,S., E. Stamatelopoulou and T. Varzakas . 2020.Advances in occurrence, importance, and mycotoxin control strategies: Prevention and detoxification in foods. Foods.9 (2) 10-33.
DOI: 10.3390 /foods9020137

3.Al-Jaal, B., S. Salama, N. Al-Qasmi and M. Jaganjac, 2019.Mycotoxin contamination of food and feed in the Gulf Cooperation Council countries and its detection. Toxicon . 171(21) 43–50. DOI: 10.1016/j.toxicon.2019.10.003

4.AL-Zubaidy ,A.M.A., K. I. Hassan, and B. S. Jabbari. 2016 Analysis of naturally occurring penolic compounds of the genera Clinopodium L.,Hymenocrater fish & c. A. Mey. and Melissa L. Iraqi Journal of Agricultural Sciences 47(1) 343-384. https://doi .org/10. 36103/ijas.v47i1.641

5. Anfossi, L., C. Giovannoli and C. Baggiani.2016. Mycotoxin detection. Curr. Opin. Biotechnol. 37(1) 120–126. DOI: 10.1016/j.copbio.2015.11.005

6. Al-Shuhaib,M.B., A.H. Albakri, S.H. Alwan, N.B. Almandil, S.AbdulAzeez and J. Borgio, 2018. Optimal PCR primers for rapid and accurate detection of *Aspergillus flavus* isolates. Microbial Pathogenesis. 116(6) 351-355. https://doi.org/10.1016/j.micpath.2018.01.049

7. Ayala, A., M.F. Munoz and S. Arguelles. 2014. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondi aldehyde and 4-hydroxy-2-nonenal. Oxid. Med. Cell. Longev, 38(3) 36-43. Doi: 10.1155/2014/360438

8. Baazeem, A., E .Garcia-Cela , A. Medina and N. Magan .2021. Interacting abiotic factors affect growth and aflatoxin b1 production profiles of *Aspergillus flavus* strains on pistachio-based matrices and pistachio nuts. Front. Microbiol. 11(6) 24-37. https://doi.org/10.3389/fmicb.2020.624007

9. Benkerroum, N. 2019. Retrospective and prospective look at aflatoxin research and development from a practical standpoint. Int. J. Environ. Res. Public Health.16 (19):3633. doi: 10.3390/ijerph16193633

10. Burns, M., Wiseman, G., Knight,A., Bramley, P., Foster, L. Rollinson, S. Damant, A. and S.Primrose.2016.Measurement issues associated with quantitative molecular biology analysis of complex food matrices for the detection of food fraud. Analyst.141(`1) 45–61. https://doi.org/10.1039/C5AN01392E

11. Davari, E., M. Mohsenzadeh, G. Mohammadi, and R. Rezaeian-Doloei, 2015. Characterization of aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* strain isolates from animal feedstuffs in Northeastern Iran. Iranian Journal of Veterinary Research. 16 (2) 150–155. DOI.PMCID: PMC4827679

12.D'Evoli, L., Lucarini, M., Gabrielli, P., Aguzzi, A. and G.L., Boccia .2015. Nutritional value of Italian pistachios from Bronte (*Pistacia vera, L.*), their nutrients, bioactive compounds and antioxidant activity. Food Nutr Sci. 101(6) 1267-1276. doi: 10.4236/fns.2015.614132

13.Dini, A., P. Khazaeli, A. Roohbakhsh, A.Madadlou, M. Pourenamdari, L. Setoodeh, A. Askarian, N. Doraki, and H.Moradi. 2013. Aflatoxin contamination level in Iran's pistachio nut during the years 2009–2011.

Food Control, 30(2) 540–544. DOI: 10.1016 /j.foodcont.2012.08.012
14. European Commission. 2006 ,Commission regulation (EC) No. 401/2006 . Methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Union .6 (70) 12–34
15. Fakruddin, M., C. Abhijit, N. H. Md, and M. A. Monzur, 2015. Characterization of aflatoxin producing Aspergillus flavus from food and feed samples. Springerplus Journal. 4(1) 159-173
16.Gurbuz,Y.,A. Hussein,and E. Ozkose. 2019.Determination of AFLD and AFLQ genes responsible for aflatoxin formation in some livestock concentrated feeds. PakVet J.39(2) 163-168.
DOI: 10.29261/pakvetj/2019.057
17. Hassan,K.I., A.M. Al-Zubaidy and B.S. Jabbari .2015. Assessment of taxonomic relationship between some taxa of clinopodium, hymenocrater. and melissa of Lamiaceae in Iraqi Kurdistan Region using RAPD Marker. Diyala journal for pure science.12 (2) 59-71
18. Hassan, K. I. and, S.K. HamaSaleh. 2016. A rapid methods for PCR based on detection of *Salmonela Spp*. and *Staphylococcus aureus* in spiked and naturally contaminated food. Iraqi Journal of Agricultural Sciences. 47 (Special Issue), 66-73
19. Hepsag, F., O.Golge and B. Kabak .2014 .Quantitation of aflatoxins in pistachios and groundnuts using HPLC-FLD method. Food Control 38 (2) 75-81.https://doi.org /10.1016/j.foodcont.2013.10.005
20. Hossain, Md.N., A. Talukder, F. Afroze, M. Rahim, S.Begum, Z.Haque, and M.M. Ahmed. 2018. Identification of aflatoxigenic fungi and detection of their aflatoxin in red chilli (*Capsicum annuum*) samples using direct cultural method and HPLC. Advances in microbiology. 8(1)42-53.
DOI: 10.4236 /aim.2018.81004
21.Kabak, B. 2021.Aflatoxins in foodstuffs: Occurrence and risk assessment in Turkey. Journal of Food Composition and Analysis . 96 (5) 23-34. https://doi.org/10.1016/j. jfca.2020.103734
22. Kaminiaris,M.D. S.Mavrikou, M.Georgiadou, G.Paivana, D.I.Tsitsigiannis, and S. Kintzios. 2020. An Impedance based electrochemical immunosensor for aflatoxin B1 monitoring in pistachio matrices. Chemosensors , 8(14) 121-129. DOI: 10.3390 /chemosensors8040121
23..Kizis, D., A. E.Vichou and P. I. Natskoulis. 2021. Recent advances in mycotoxin analysis and detection of mycotoxigenic fungi in grapes and derived products. sustainability, 13(5), 25-37.
https://doi.org/10.3390/su13052537
24. Kolawole,O., J. Meneelya , A. Petchkongkaew and C. Elliott. 2021. A review of mycotoxin biosynthetic pathways: associated genes and their expressions under the influence of climatic factors. Fungal Biology Reviews. 37(18) 8-26.https://doi.org/10.1016/j.fbr.2021.04.003
25. Liao, J., Z. He, Y. Xia, Y. Lei and B. Liao. 2020. A review on biosynthesis and genetic regulation of aflatoxin production by major Aspergillus fungi. Oil Crop Science. 5 (4) 166–173. https://doi.org/10.1016/j. ocsci.2020.11.001
26. Mahmoud, M.A., 2015. Detection of *Aspergillus flavus* in stored peanuts using realtime PCR and the expression of aflatoxin genes in toxigenic and atoxigenic A.flavus isolates. Foodborne Pathog Dis. 12(4) 289–296. DOI: 10.1089/fpd.2014.1854
27. Mangal M.,F. Khan, S. Bansal and H. Oberoi. 2016.Validation of PCR based detection system for aflatoxin producing molds. Indian J Exp Biol. 54(4) 472-476.PMID: 29466626
28. Mannaa, M., and K.D.Kim. 2017. Influence of temperature and water activity on deleterious fungi and mycotoxin production during grain storage. Mycobiology. 45(4) 240–254. DOI: 10.5941/MYCO. 2017.45.4.240
29. Mikaeel, F. B. and A. T. M. Al-Saeed 2020. Serological and molecular diagnosis of *toxoplasma gondii* among ewes and horses in duhok province-iraq. Iraqi journal of agricultural sciences, 51(4), 1212–1219. https://doi.org/10.36103/ijas.v51i4.1100
30. Mitchell, N.J., E. Bowers, C. Hurburgh and F.Wu, 2016. Potential economic losses to the USA corn industry from aflatoxin contamination. Food Addit. Contam. Part A Chem Anal Control Expo Risk Assess .33(2) 540–550.
DOI: 10.1080/19440049.2016. 1138545

31. Montibus, M., L. Pinson-Gadais, F. Richard-Forget, C. Barreau and N. Ponts. 2013. Coupling of transcriptional response to oxidative stress and secondary metabolism regulation in filamentous fungi. Crit. Rev. Microbiol. 41(3) 295-308. DOI: 10.3109/ 1040841X.2013.829416

32.Mohamed, M. A. and E. I. Al – Shamary , 2022 .Isolation and identification of aflatoxin B1 producing fungi from stored wheat in some silos of Baghdad. Iraqi Journal of Agricultural Sciences, 53(6) 1427–1436 https://doi.org/10.36103/ijas.v53i6.1659

33. Nadhom, B. N. 2018. Study of molecular composition of virulance bacteria isolated from bovine mastitis with biofilm production, Iraqi Journal of Agricultural Sciences, 49(5):840-846. https://doi.org/10.36103/ijas.v49i5.44

34. Mohammed , M. F., D. S. Hassawi and N. K. Ibraheem .2023. Extraction, fractionation, and seperatione of cynaroside and scolymoside from *Cynara scolymus* L. grown in Iraq. Iraqi Journal of Agricultural Sciences, 54(2) 421–430. https://doi.org/10.36103/ijas.v54i2.1716

34. Niessen, L., J. Bechtner, S. Fodil, , M.H. Taniwaki and R.F. Vogel, 2018. LAMP-based group specific detection of aflatoxin producers within Aspergillus section Flavi in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection. Int. J. Food Microbiol. 266(12) 241–250. DOI: 10.1016/j.ijfoodmicro.2017.12.013

35. Oliveira, I.S., A.G.D.S.Junior, C.A.S. De Andrade and M.D.L Oliveira. 2019, Biosensors for early detection of fungi spoilage and toxigenic and mycotoxins in food .Current Opinion in Food Science. 29(15) 64-79. https://doi.org/10.1016/j.cofs.2019.08.004

36. Ostadrahimi, A., Ashrafnejad,F., Kazemi,A., Sargheini,N., Mahdavi,R., Farshchian, M..and S. Mahluji.2014. Aflatoxin in raw and salt-roasted nuts (pistachios, peanuts and walnuts) sold in markets of Tabriz, Iran Jundishapur J Microbiol.7(1)74-86. DOI: 10.5812/jjm.8674

37. Ostry, V., F. Malir, J. Toman, and Y. Gross. 2017. Mycotoxins as human carcinogens the IARC Monographs classification. Mycotoxin Research, 33(1), 65-73. DOI: 10.1016/j.ijfoodmicro.2017.12.013

38.Pakshir,K., Z. Mirshekari, H. Nouraei, Z. Zareshahrabadi, K. Zomorodian, H. Khodadadi, and A. Hadaegh. 2020. Mycotoxins detection and fungal contamination in black and green tea by HPLC-based method. Journal of Toxicology.202(7) 24-31. https://doi.org/10.1155/2020/2456210

39. Rao, K.R., A.V. Vipin and G. Venkateswaran. 2020. Molecular profile of nonaflatoxigenic phenotype in native strains of *Aspergillus flavus*. Arch. Microbiol. 202(5) 1143-1155. DOI: 10.1007/s00203-020-01822-1

40. Riba,A., A.Matmoura1, I.S. Mokrane , F.Mathieu, and A.Sabaou . 2013. Investigations on aflatoxigenic fungi and aflatoxins contamination in some nuts sampled in Algeria. Afr. J. Microbiol. Res.7(42) 4974-4980. DOI: 10.5897/AJMR2013.5867

41. Rezaei,M.,F.Karimi,M. Parviz, A.Behzadi,M. Javadzadeh and P.Mohammad. 2014. An empirical study on aflatoxin occurrence in nuts consumed in Tehran, Iran 2013. Health. 14(6) 649–653. DOI: 10.4236/health.2014.68084

42. Schrenk,D.,M. Bignami,L., Bodin,J.K. Chipman, J. del Mazo and B. Grasl-Kraupp. 2020. Risk assessment of aflatoxins in food. EFSA Journal. 18(3):6040. DOI 10.2903/j.efsa.2020.6040

43.Shier,W.T., Y. Lao, T. Steele and H.K Abbas.2005.Yellow pigments used in rapid identification of aflatoxin-producing Aspergillus strains are anthraquinones associated with the aflatoxin biosynthetic pathway.Bioorganic Chemistry. 33(6) 426-438. doi: 10.1016/j.bioorg.2005.09.002

44. Sohrabi N.and M. Taghizadeh, 2018. Molecular identification of aflatoxigenic Aspergillus species in feedstuff samples. Curr Med Mycol. 4(2) 1-6. DOI: 10.18502 /cmm.4.2.66

45.Silva, A.S., C. Brites , A.V. Pouca , J. Barbosa and A. Freitas.2019.UHPLC-ToF-MS method for determination of multi-mycotoxins in maize: Development and validation. Current Research in Food Science. 1(5) 1-7. DOI: 10.1016/j.crfs.2019.07.001

46. Tao, F., Yao, H., Zhu, F., Hruska, Z., Liu, Y., Rajasekaran, K. and D.Bhatnagar.2019. A rapid and nondestructive method for

simultaneous determination of aflatoxigenic fungus and aflatoxin contamination on corn kernels. Journal of Agricultural and Food Chemistry. 967 (18) 5230-5239. https://doi.org/10.1021/acs.jafc.9b01044

47. Wu,Q., L. Xie, , and H.Xu, 2018.Determination of toxigenic fungi and aflatoxins in nuts and dried fruits using imaging and spectroscopic techniques, Food Chemistry. 252(12) 228-242. DOI: 10.1016 /j.foodchem.2018.01.076

48. Xue, Z., L. Long, Y. Chen, M. Chen and Y. Cheng, 2021.A nanozyme-linked immunosorbent assay based on metal–organic frameworks (MOFs) for sensitive detection of aflatoxin B1,Food Chemistry, 13(12) 338-341.DOI: 10.1016/j.foodchem.2020.128039

49.Xue, Z., Y.Zhang, W. Yu, J. Zhang, J.Wang, F.Wan, Y. Kim,Y.Liu and X. Kou.2019. Recent vances in aflatoxin B1 detection based on nanotechnology and nanomaterials-A review,Analytica Chimica Acta.106 (9) 1-27. DOI: 10.1016/j.aca. 2019.04.032

50.Yabe, K., M.Yushita, Y. Yamamoto and M. Kushiro, 2020. Detection of aflatoxigenic fungi in imported raw nuts using the dichlorvos-ammonia (DV-AM) method . JSM Mycotoxins.70(1)7- 13

51. Yassin, H. Y., A. K. Melconian, and S. S. Mahmood. 2022. Prevalence of exfoliative toxin genes among clinical isolates of *Staphylococcus aureus* in Iraq, Iraqi Journal of Agricultural Sciences, 53(2) 465–470 https://doi.org/10.36103/ijas.v53i2.1554

52.Yu J. 2012.Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. Toxins (Basel). 4(11) 1024-1057. DOI: 10.3390 /toxins4111024

53.Zhang ,L., Xiao-Wen Dou, ,Cheng Zhang, Antonio F. Logrieco and Mei-Hua Yang 1, 2018 .A Review of Current Methods for Analysis of Mycotoxins in Herbal Medicines .Toxins .10(2) 65-76. DOI: 10.3390/toxins 10020065

54. Zhan,S., J.Hu, Y. Li, X. Huang and Y. Xiong. 2021. Direct competitive ELISA enhanced by dynamic light scattering for the ultrasensitive detection of aflatoxin B1 in corn samples, Food Chemistry, 16(12)342-351. DOI: 10.1016/j.foodchem.2020.128327