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Phenotypic and molecular diagnosis using polymerase chain reaction (PCR) technology for some aflatoxin-producing fungi isolated from food

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Abstract---The study was conducted in the laboratories of the College of Education for Pure Sciences for the academic year 2021-2022 on fungi that produce aflatoxin toxin that were isolated from some foodstuffs traded in the local markets in the city of Karbala and were diagnosed molecularly using a technique Polymerase chain reaction (PCR) which successfully doubled NS8 and NS1 with prefixes (SSU-) The same primers were used to determine the sequence of nitrogenous bases of rRNA belonging to the International Center for Information Technology Genbank. National Center of Biotechnology Information (NCBI) it has been deposited in a database (fungi biogenic). The results of isolation, which were collected from imported and local foodstuffs, were contaminated with fungi, and that all isolated fungi were producing aflatoxin toxin. This was inferred by using coconut medium with ammonia solution. hf ifheehehai eifenhihehidnhf eht dna Penicillium oxalicum, Penicillium expansum, Aspergillus flavus, Cladosporium Uredinicola, Aspergillus sydowii, Aspergillus Oryzae, Aspergillus Tamarii, Aspergillus nomius, Alternaria triticina, Penicillium griseofulvum, Aspergillus austwicki, Aspergillus flavus, Cladosporium cladosporioides, Actinomucor elegans, Penicillium brevicompatum, Trichophyton mentagrophytes, Gibberella intermedia, Debaryomyces hansenii, Aspergillus caespitosus, Aspergillus versicolor. All of these isolates have been recorded in the World Gene Bank, and serial numbers have been given to them MT03303, AF033479, MZ35787, JN088229, LC094427, MH66405, MT25485, MK45036, JX418360, MF03465, OM72177, MW51015, MF47593, MT503291, MT50329, MQN6937, MQ55893, MQN6937. The results

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showed that there is a similarity of 99 - 100% between these isolates and the global isolates.

***Keywords*---**PCR, Phenotypic, aflatoxin.

Introduction

Mycotoxins are one of the most important secondary metabolic compounds produced by some types of fungi (moulds), which can affect the health of humans, animals and plants (creppy, 2002). Contamination of food, grains, stored products and feed with fungi and mycotoxins is one of the problems that threaten many developing countries, especially countries that lack adequate storage conditions and is a source of very great concern. This prompted these countries to search for healthy food sources to achieve their food security (Makun. et al., 2010). Mycotoxins have great effects on public health, as people consume mycotoxins in small quantities in their food without the appearance of clear pathological effects, the danger of which increases when ingested in high concentrations, which leads to serious health problems. Mycotoxins lead to nephrotoxicity, immunosuppression, teratogenicity of fetuses, and congenital malformations, and can cause acute and chronic effects in humans and animals, ranging from death or disruption of the central nervous system, heart, blood vessels, pulmonary systems, and liver cancers (Zbynovska, 2016). According to the Food and Agriculture Organization of the United Nations (FAO), approximately 25% of world crop production is contaminated with mycotoxin compounds (Park, et al 2009). The agricultural crops that are most affected before and after harvest by the growth of fungi and mycotoxins are field pistachios, corn, cotton seeds, rice, nuts, grains and fruits, in addition to contamination of animal products such as meat, eggs and milk that are contaminated through nutrition (Rachaputi et al 2002).

Aflatoxins are one of the most dangerous types of mycotoxins carcinogenic to the liver, and history has recorded many accidents that occurred due to aflatoxins, which are mainly produced by the fungi *Aspergillus parasiticus*. *Aspergillus flavus* (Rashid *et al* 2008). Since the discovery of aflatoxins in 1960, more than 18 types of aflatoxins have been found, including (B1, B2, G1, G2) that are produced by the genus Aspergillus, where *A. flavus* produces aflatoxin B1, B2 While *A. Parasiticus* produces the four main types of aflatoxins in addition to the presence of M1, M2, which are secondary metabolites of B1 and B2 toxins.(Carlson *et al* 2002). It is estimated that approximately 4.5 million people in developing countries are constantly exposed to uncontrolled amounts of aflatoxins that affect the human immune system (Wiliams 2004). An example of the great health effects of mycotoxins is what happened in Kenya in July 2004 due to contamination of maize, which led to the death of 125 people out of 317 (Lewis *et al* 2005) . In view of the importance of the subject and its danger to human health and its direct relationship with food, we highlighted in this study some foods available in the local markets in Karbala Governorate to find out the extent of their contamination with fungi that produce aflatoxin toxin. The study focused on the following :

1- Isolation of fungi from some local foods
2- Testing its ability to produce aflatoxin toxin

3- Molecular diagnosis by PCR technique of isolated

Materials and Working Methods

1- Sample Collection:
46 samples were collected from different food sources, namely nuts, legumes, fruits, vegetables, gyps, tomato paste, pasta, noodles, rice, millet, indomie, biscuits, bread and sesame randomly from the local markets in Karbala governorate, with three samples for each food item and weighing 3 g for each sample for the purpose of obtaining On isolates of aflatoxin-producing fungi

2- Culture Media: (Middle Of Potato Acar Extract And Dextrose A- (PDA Potato dextrose agar)
The medium was prepared according to the manufacturer's instructions by dissolving 39 g of the medium in 1 liter of distilled water, then sterilized with an autoclave device at a temperature of 121 °C and at a pressure of 1 atmosphere for 20 minutes, then cooled to 45 °C, then added to it the antibiotic amoxicillin at a concentration of 250 mg / l The medium was used for the purpose of isolating and diagnosing fungi .

B- Cocconut Extract Agar (CEA)
The medium was prepared according to the method laid down by Dianese and Lin (1976), by taking 100 grams of shredded coconut and adding 300 ml of distilled water to it and heated for 20 minutes, after which it was watered | |

The distilled water was heated for 20 minutes, then it was filtered using sterile gauze, 2% of the agar was added to the filtrate, and the volume was completed to 300 ml of distilled water. Then the medium was sterilized. The medium was used to test the ability of fungi to produce toxins. 4- Isolation and identification of fungi accompanying foodstuffs: The food samples mentioned in paragraph (1) were transferred to the fungi laboratory in the Department of Life Sciences - College of Education - University - Karbala and cut into small pieces 5 mm and superficially sterilized with sodium hypochlorite solution at a concentration of 2% for two minutes after which they were washed with sterile distilled water and then placed on leaves Filtered to get rid of the water, and then it was planted in plastic dishes (8 cm in diameter) containing the medium of PDA by placing four pieces of food at a distance of 3 cm from the edge of the dish and a fifth piece in the middle of the dish. 25 + 2 heat for seven days (Al-Saadoun et al., 2011) After the end of the incubation period, the fungi isolates were purified by transferring a disc diameter of 5 mm from each colony and planted on the same culture medium, and the process was repeated several times to obtain pure isolates of the fungi, after that the percentage of Each mushroom according to the following equation.

Percentage of appearance

The percentage of the appearance of fungal species in the studied samples was calculated using the following equation (Booth *et al.*, 1988). The number of samples in which one species or genus appeared Percentage of appearance (%) = 100 number of samples the college Frequency percentage The percentage of the frequency of each isolated fungal species was calculated for samples taken from

the study sites using the following equation. (Rajasinghe *et al.*, 2009) The number of isolates of the species the one Percentage Frequency (%) = 100 X the total number For isolates of all kinds Detection of aflatoxins using a solution and intentionally.

Detection Of Aflatoxins Using A Solution Ammonia

The ability of the fungi isolates to produce aflatoxin was detected using the coconut medium prepared in paragraph (B) poured into plates with a diameter of 8 cm, then three replicates were inoculated with discs of the fungus isolates grown on PDA medium with a diameter of 5 mm and a week old in the center of the plate and the process was repeated on All studied isolates were then incubated at a temperature of 25°C for a week. The isolates capable of producing mycotoxins were detected using a 10% ammonia solution by using filter papers saturated with the solution in the cover of the dish, then the dishes were incubated in an upside down manner for 4 days at a temperature of 25°C. A change in the color of the colonies from transparent to orange or red indicates the ability of the isolates to produce toxins (Lin and Dianese, 1976).

Diagnostics Of The Isolated Fungi

First: Phenotypic diagnosis of aflatoxin-producing fungi isolated from food The aflatoxin-producing fungal isolates that were isolated from foodstuffs were diagnosed based on the external appearance of the colonies after purification on PDA medium based on the taxonomic keys approved for each of (Moubasher, 1993; Neill, 1936).

Secondly: Diagnosis of aflatoxin-producing fungi by PCR technique

A - DNA extraction methods FavorPrep TM DNA genome DNA extraction mini kit

1- Grind 50 mg wet weight.
2- Add 400 µL of FAPG1 Buffer and 8 µL of RNase A solution (50 mg/mL) and vigorously vortex. Incubate the mixture at room temperature for 2 min and then at 65 °C for 10 ~ 20 min and inverted 2-3 times during the incubation.
3- Add 130 µl of FAPG2 Buffer to the mixture. Vortex to mix well and incubate the mixture on ice for 5 min
4- Place the filter column in the group tube and transfer the entire mixture from the previous step to the filter column. Centrifuge the filter column at a maximum speed of 14,000 rpm for 3 min.
5- Transfer the clarified lysate (supernatant) from the collection tube to a new microcentrifuge tube (not provided). Discard
6- Use of filter shaft and group tube. The lysate volume measurement is shown
7- Add 1.5 volume of FAPG3 buffer (add ethanol) to the clarified lysate and mix well by tubes. Be sure to add ethanol (96~100%) to FAPG3 Buffer when first use.
8- Place the FAPG column into a new collection tube and carefully transfer up to 750 µl of the sample mixture to the FAPG column. Centrifuge at a maximum speed of 14,000 rpm for one minute. Discard the flow and place the FAPG

column back into the collection tube. Repeat step 7 with the rest of the sample mixture.

9- Add 400 µl of W1 Buffer (ethanol added) to the FAPG column. Centrifuge at a maximum speed of 14,000 rpm for 30 seconds. Discard the flow and place the FAPG column back into the collection tube. Be sure to add ethanol (96~100%) to the W1 Buffer on first use.

10- Add 650 µL of wash buffer (ethanol added) to the FAPG column. Centrifuge at a maximum speed of 14,000 rpm) for 30 seconds. Discard the flow and place the FAPG column back into the collection tube. Be sure to add ethanol (96~100%) to Wash Buffer when first use. Repeat step 9 for another wash. Centrifuge at max speed 14,000 rpm for an additional 3 min to completely dry the FAPG column. An important step! This step will avoid the remaining liquid to inhibit subsequent enzymatic reactions

11- Combine the FAPG column with the elution tube, add 100 µl of pre-heated elution solution to the center of the membrane in the FAPG column. Place the FAPG column for 1 minute at room temperature. Important step! For an effective rinse, make sure the Elution Buffer is distributed over the center of the film and is completely absorbed.

12- Centrifuge at maximum speed of 14,000 rpm for 1 minute to extract purified DNA.

B - DNA concentration determination:

Gel electrophoresis for DNA quality analysis:- The agarose solution was prepared by dissolving 1 g of agarose powder in 100 ml of 1x TBE in a (100) ml beaker, melting the agarose in a hot mold until the solution became clear. The agarose solution was cooled to about (50-550 °C), rotating the flask occasionally to cool evenly. Red dye (3 µl) was added to the warm gel and the ends of the casting tray were sealed with two layers of tape. The combs were placed in the gel casting tray. The dissolved agarose solution was poured into the casting tray. The agarose was allowed to solidify at room temperature, the comb was carefully pulled out and the tape removed. The gel was placed on an electrophoresis chamber which was filled with TBE buffer (1x). DNA samples (5 µl) were mixed with DNA loading solution (3 µl) and loaded into wells of an agarose gel. Agarose gel electrophoresis was completed at 70 V, 65 A for one hour. The DNA was observed by viewing it under UVtrans illumination.

C - Preparing the primer

Primers were lyophilized, dissolved in free ddH2O to give a final concentration of 100 µL/µL as stock solution and kept at -20 to prepare a concentration of 10 µL/µL as suspension primer, 10 µL of stock solution in 90 µL of ddH2O free water to reach volume Final 100 µl. used in the study Table 3: The sequence of prefixes used in this study.

Results and Discuss

First: phenotypic diagnosis of fungi isolated from food Fungal isolates isolated from food_during the study The results of isolating and purifying fungi from the

tested foodstuffs showed that there were 21 types of fungi during this study belonging to 8 genera of fungi that were isolated from the food. The fungi *Cladosporium* represented by only two species, the rest of the genera appeared with one species for each of them.
The following table shows the aflatoxin-producing fungi isolated from foodstuffs.

Table of the percentage frequency types of aflatoxin-producing fungi isolated from food

Percentag	Nambar of isolates	Type of fungi	hت
h%4.76	1	*Penicillium oxalicum*	1
%4.76	1	*Penicillium expansum*	2
%14.29	3	*Aspergillus flavus*	3
%4.76	1	*Cladosporium uredinicola*	4
%4.76	1	*Aspergillus sydowii*	5
%4.76	1	*Aspergillus oryzae*	6
%4.76	1	*Aspergillus tamarii*	7
%4.76	1	*Aspergillus nomius*	8
%4.76	1	*Alternaria triticina*	9
%4.76	1	*Penicillium griseofluvum*	10
%4.76	1	*Aspergillus austwicki*	h11
%4.76	1	*Aspergillus flavus*	12
%4.76	1	*Cladosporium cladosporioides*	13
%4.76	1	*Actinomucor elegans*	14
%4.76	1	*Penicillium brevicompatum*	15
%4.76	1	*Gibberella intermedia*	16
%4.67	1	*Debaryomyces hansenii*	17
%4.67	1	*Aspergillus caespitosus*	18
%4.76	1	*Aspergillus versicolor*	19

Secondly ; Molecular diagnosis of aflatoxin-producing fungi DNA extraction and Polymerase chain reaction All the fungi isolated in this study contained a single bundle of extracted DNA, whose purity was measured by using the Spectrometer under wavelengths 260 and 280 The results of the analysis of the ribosomal RNA 18S gene for the SSU region are positive for all fungi, and the same primers were used to determine the sequence of nitrogenous bases for all fungi to be diagnosed, which were deposited in the Genbank database of the International Center for Biotechnology Information National Center of Biotechnology Information (NCBI) The results showed that all fungi interacted with their genomes with the initiator and matched with their counterparts by 99-100%. It is the first recording of these species from Iraq, according to what is shown in the registration information and the study of convergence and similarities between the recorded fungi. The first isolate was determined *Penicillium oxalicum* serial code MT03303, second isolate *Penicillium* expansim serial code AF033479, third isolate *Aspergillus flavus* serial code MZ35787, fourth isolate *Cladosporium Uredinicola* serial code JN088229,

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fifth isolate *Aspergillus Sydowi*, sixth isolate Aspergillus flavus sequence code MZ35787, fourth isolate *Cladosporium Uredinicola* serial code JN088229, fifth isolate Aspergillus *Sydowi*, sixth isolate LCy094427, code AsperH. nomius sequence code MK45036, isolate ninth *Alternaria triticina*, sequence JX418360, isolate 10 Penicillium griseofulvum sequence code MF03465, isolate 11 *Aspergillus austwickii* sequence code OM72177, isolate twelfth *Aspergillus flavus* sequence code MW5471059dos and isolate 4th isolate MW54759dosio MF29 sequence 3 The fifteenth isolate *Penicillium brevicompatum* serial code MT155892 The sixteenth isolate *Trichophyton mentagrophytes* Serial code MT155892 sequence MN99993, isolate seventeenth *Gibberella intermemedia* serial code HQ44324, isolate eighteenth *Debaryomyces hansenii* serial code MN37191, isolate nineteenth *Aspergillus caespitosus* serial code KU866669, isolate twentieth *Aspergillus flavus* serial code MK64522, sequence 45 code MT Aspercolorg4

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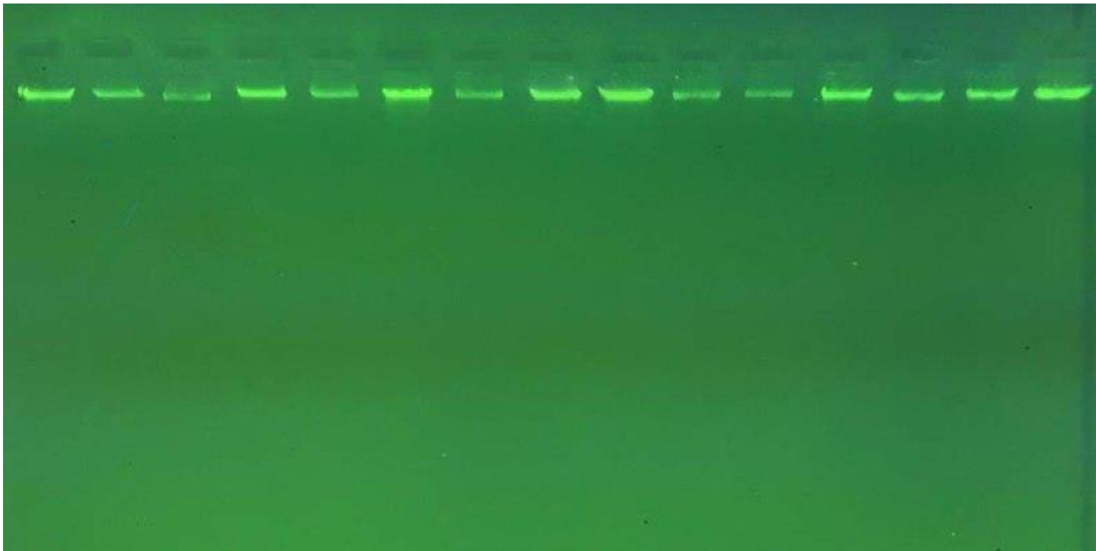

Figure (1) Gel electrophoresis to extract genomic DNA 1% agarose gel in one hour SiZer 100bp DNA Markers (Kappa/USA)

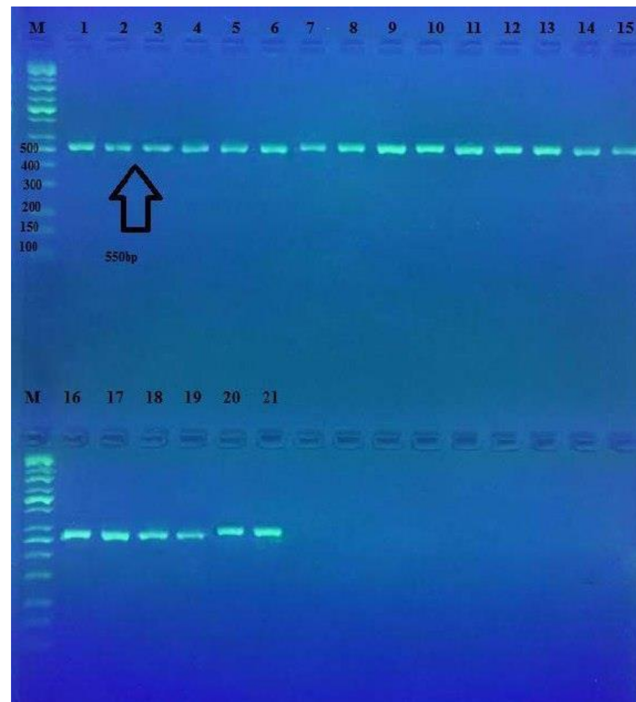

Figure (2) PCR product size range. The product was electrophoresis on 1.5% agarose at 5 V/cm2. 1x TBE buffer for 1:30 h. M: DNA ladder (100).

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