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Molecular Deduction of *Aspergillus flavus* Isolated from Wheat Grains in Karbala City

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Abstract: A total of 200 samples of wheat were collected during the period from the beginning of October 2019 until the end of January 2020 from government and private grain stores and local markets in Karbala Governorate to determine the percentage of infection with *Aspergillus flavus*. Molecular diagnosis of *A. flavus* was performed using polymerase chain reaction (PCR) technology using the *AFO* gene. The results showed 138 samples of the total 200 samples were infected with different fungi. 62 samples of them belong to the Species *A .flavus*. Of the 62 samples, 23 were strong production, 15 were medium production, and 24 were weak producer of aflatoxin, depending on the Ammonia vapor test.

Keywords: *Aspergillus flavus*, Ammonia, Vapor Test.

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Introduction

Wheat is an important food crop for a large number of the world population, including Iraq, as it is a food source for more than 35% of the world population (7). It is one of the major crops in the world in arid and semi-arid regions where 37% of the wheat crop grows in (1). Wheat crops are exposed to several pests, which cause material losses estimated at 360 billion dollars annually, and part of these losses is due to fungal infection, especially during the harvest, as the wheat is grown and harvested in conditions suitable for the growth of many fungi (3).

The genus *Aspergillus* is one of the common species of fungi, and it includes several species up to 200 species, most of which are found in soil, air, and food waste such as bread, jams, tissues, leather, paper, and agricultural crops and cause their spoilage (22). According to the Food and Agriculture Organization (FAO) statistic, more than 25% of the world's food is contaminated with mycotoxins (50-53 mg / kg food) and 4.5 million people in developing countries are exposed to uncontrolled quantities of these toxins (10).

Aspergillus flavus is found globally as a saprophyte in soils and causes disease for many important agriculture crops. Common hosts of the pathogen are cereal grains, the pathogen can invade seed embryos and cause infection, which decreases germination and can lead to infected seeds planted in the field. The pathogen can also discolor embryos, damage seedlings, and kill seedlings, which reduce grade and price of the grains (15).

Aspergillus flavus is also an opportunistic human and animal

pathogen, causing Aspergillosis in immunocompromised individuals (2). Aflatoxins are mycotoxins produced by the genus *Aspergillus* that grow on food grains and many other agricultural crops. The molds that are major producer of aflatoxins are *A. flavus* (4) and *A. parasiticus* (14) and can cause contamination of a wide range of agricultural products whether in the field or during the storage period (9). Aflatoxin B1 is considered as a class 1 human carcinogenic activity (5).

The colonies of this mold grow quickly on the Sabourauds agar and the barley extract (Malt extract), and the colonies are greenish yellow and smooth, as the region of the radial or vertical conoid heads disjointed relatively varies in specifications from isolation to another (6). As for conidia mounts, they shall be tampered with, thick walls with dimensions ranging from 1-2.5 mm in length and 10-20 micrometers in thickness. Only one row of vial structures is observed in newly formed vesicles doubling with the age of the vesicle (20,6). This study was conducted for molecular investigation and characterization of *Aspergillus flavus* infected wheat samples in Karbala governorate fields and stores.

Materials and Methods

Collection of Samples

Two hundred samples of wheat grains were collected during the period from the beginning of October 2019 to the end of January 2020 from governmental and private grain stores and the local markets of
Karbala Governorate, 250 g / sample were placed in sterile bags and transferred to the laboratory and keep in it until used.

Samples Culture

The direct method was used to isolate the fungi accompanying the grain samples of the study by taking (100) grams of each wheat grains sample sterilized superficially with a 3% solution of sodium hypochlorite for a period of 2 minutes. Then the samples were washed with distilled water and then taken 6 grains and dried using blotting paper, the samples were placed on the Sabouraud Dextrose Agar with the addition of 40 mg / liter of chloramphenicol (in order to prevent the growth of bacteria). Than incubated at a temperature of 28 °C for 5-7 days after incubation period the fungal isolates were purified by transferring a disk from each colony and culturing it in a new SDA dish, the process repeated several times (8).

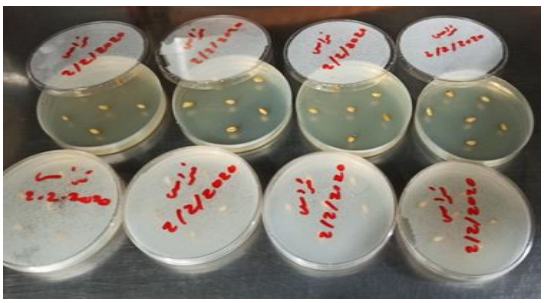

Figure (1): It shows the method of placing grains in dishes.

Identification and Characterization of the *Aspergillus flavus* Conventional Methods of Identification and Characterization of the fungi

Dishes were checked after 5 days of culturing for the purpose of diagnosing growing colonies on the culture medium the classification keys mentioned (18,19) was followed diagnosing isolates of the morphological and microscopic morphological properties in terms of shape colony, its color and texture, type of yarn spinning, conidian heads, the shape and color of the conidian heads, and their dimensions were studied. For the purpose of its accurate diagnosis, it was examined under a light microscope using the glass slide culture technique (13). Slices were prepared ready for microscopy, as a drop of distilled water was placed in the center of the slide, and a small part was transported by means of a sterile loop to this drop, then the cover was placed with little pressure by the fingers. The percentage of occurrence and frequency of each fungal species was calculated based on the two equations (25).

$$\text{Percentage of appearance} = \frac{\text{The number of samples which the species appeared}}{\text{Total number of samples}} \times 100 \quad \text{... (1)}$$

$$\text{Percentage of frequency} = \frac{\text{Number of single species isolates}}{\text{The total number of all isolates}} \times 100 \quad \text{… (2)}$$

Ammonia vapor test

The Ammonia Vapor test, based on (24) was adopted to test the ability of fungal isolates isolated from grain sources to produce aflatoxin, as these isolates were activated 5 days before the experiment was conducted by planting them on SDA medium, after which part of the vaccine was transferred, The fungi from the edge of the isolation colony to be tested placed in the center of the coconut extract agar (CEA) dish, then the dishes were incubated at a temperature of 25 ° C for a period of 7 days, after which the ability of the fungus isolates to produce aflatoxins was detected by using a 20% ammonia solution by placing filter papers saturated with an ammonia solution. In the cover of the plate containing the isolate of the growing fungus with medium (CEA), then the plates were incubated inverted for a period of (24-48 h.) at a temperature of 25 ° C. A change in the color of the colony bases from transparent color to red indicates that the isolate is able to produce aflatoxins.

Molecular Methods Identification and Characterization of *A. flavus*

In this study, real time (PCR) method was used for fungus isolate diagnosis by AFO gene primer sequence of *A. flavus*, as follows:

Preparation of agarose gel at 1% concentration

1- 50 ml of TBE X1 were taken and placed in a flask.
2- 0.5 g of agarose powder was weighed and added to the solution.
3- The solution was boiled by using a microwave, until the powder dissolved.
4- 2 µl of ethidium bromide was added (0.5 µg / ml) to the agarose solution.

5- The solution was left until cool to a temperature of 50 - 60 ° C.

DNA extraction

Fungal DNA was extracted using the TRIzolR LS Reagent following the protocol provided by the manufacturer (Trizol LS Reagent, 2012).

PCR primer and PCR technique

The specificity of the primers for the identification of *Aspergillus* species was evaluated by PCR, using genomic DNA isolated from various strains isolated in pure cultures, and identified by microscopic analysis and colonies characteristics. Isolates designated as *A .flavus* were tested with the primer AFOF20:5'CACATTCAAGCCAGAT TACG-3; /AFOR24: 5'-GCT TAG GGTTGTTCATACGAGCAC-3'; described by Cornea et al. (23), based on DNA polymerase II gene from *A.flavus*. The amplicons obtained had the predicted size for 5 isolates considered as *A.flavus* (679 bp) (Figure 5). Five isolates were designated also as *A.flavus*.

Electrophoresis

The samples were electrically extracted using agarose gel to ensure the presence of DNA, 7 µl of DNA sample was taken and mixed with 3 µl of loading buffer TBE (X1) and carried through the agarose gel at a concentration of 1% for 30 min / 70 volts. At UV transilluminator, the integrity of the DNA was determined, when a single beam was observed rather than a smear band that indication of broken DNA.

Results and discussion

Collection and isolation of *Aspergillus flavus* by Conventional Methods

The results of this study showed that 142 samples out of a total of 200 samples were infected with various fungi. 62 samples belonged to the genus *A.flavus* as shown in the table (1). The results of Ammonia vapour test showed that 23 samples were strong producer, 15 samples were medium producer, and 24 samples were weak producer of aflatoxins, as shown in the figures (2) and (4). Species of the genus *Aspergillus* were diagnosed based on morphological characteristics of colony included the size, arrangement of *Aspergillus* head, the color of conidia, the growth rate, and microscopic examination by using cotton blue stain. *A. flavus* was identified based on diagnostic keys, mentioned by (19, 18).

The results of the microscopic examination shows that this isolate has a divided fungal yarn from which protruding transparent, tapered end conidia carriers with thick walls range in length from (900-1100) µm and thickness from (10-13) µm ending in oval-shaped vesicles with a diameter of (22.1) µm and thickness of (5.8-6.1) µm, completely covered in one row of the structures range in length from (22.1-23.9) µm and thickness from (5.8-6.1) µm, each carrying a series of spherical conides up to 4 µm in diameter, these characteristics completely match the taxonomic characteristics of *A. flavus* mentioned by (18,19).

Identification of toxin producing ability of *Aspergillus flavus* isolates using Ammonia vapour test

Based on the cultural and physiological characteristics, Sixty-two

isolates were identified as *Aspergillus flavus* from wheat grain samples using ataxonomic Colonies of *A. flavus* appeared radially yellowish green, producing conids formations and conidia spherical or nearly spherical (spherical) structures figure (2).

Table (1): Shown numbers and percentage of *A. flavus* isolates.

Wheat grains	Different types of fungi	*Aspergillus flavus*	Isolates producing aflatoxins		
			Strong	Medium	weak
200	138	62	23	15	24
%100	%69	%31	%11.5	%7.5	%12

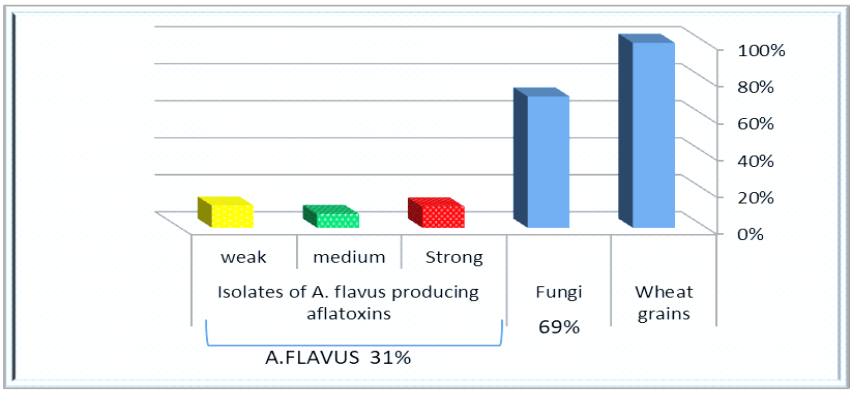

Figure (2): The ability of *A. flavus* isolates to produce aflatoxins from wheat grains of samples.

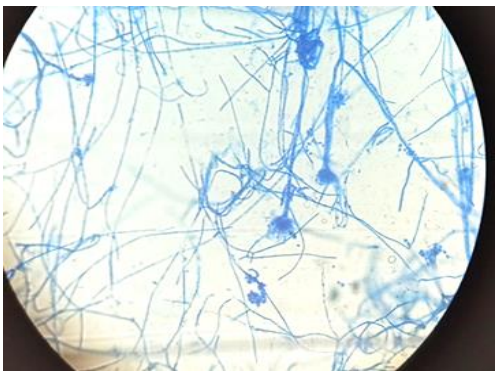

Figure (3A): Colonies of *A. flavus* grow on plate of the SDA medium after 7day incubation at28°C.

Figure (3B): *A. flavus* under light microscope fixed with lactophenol cotton blue.

The results of the microscopic examination shows that this isolate has a divided fungal yarn from which protruding transparent, tapered end conidia carriers with thick walls range in length from (900-1100) µm and thickness from (10-13) µm ending in oval-shaped vesicles with a diameter of (22.1) µm and thickness of (5.8-6.1) µm, completely covered in one row of the structures range in length from (22.1-23.9) µm and thickness from

(5.8-6.1) µm, each carrying a series of spherical conides up to 4 µm in diameter, these characteristics completely match the taxonomic characteristics of A. flavus mentioned by (18,19).

Identification of toxin producing ability of *Aspergillus flavus* isolates using Ammonia vapour test

Based on the cultural and physiological characteristics, Sixty-two isolates were identified as Aspergillus flavus from wheat grain samples using a taxonomic key and species descriptions by (12). These isolates exhibited variable toxin producing ability. In similar study by Hussein *et al*., (11) it was found that isolates *of A. flavus* produced aflatoxins at variable rates when cultured under same conditions (10). In previous study showed, on exposure of A. flavus cultures with ammonia vapour leads to varied degree of colour change, ranges from plum red, red and pink to cream. The colour of culture is correlated with the aflatoxin concentration estimated by ELISA as plum red being highly toxic (> 2000 ppb), red as toxic (501–2000 ppb), pink as moderate toxic (21–500 ppb) and cream colour as least toxic /nontoxic (< 20 ppb)isolates (16). The results of of present study of culture color showed the highest percentage of Sixty-two of *A.flavus* isolates (11.5% strong) cultures turning to plum red colour, (7.5% medium) turning to red colour, and (12%) pink colour on exposing to ammonia vapour as (Table 1) and Figure (5). This is in consistent with the findings of (16).

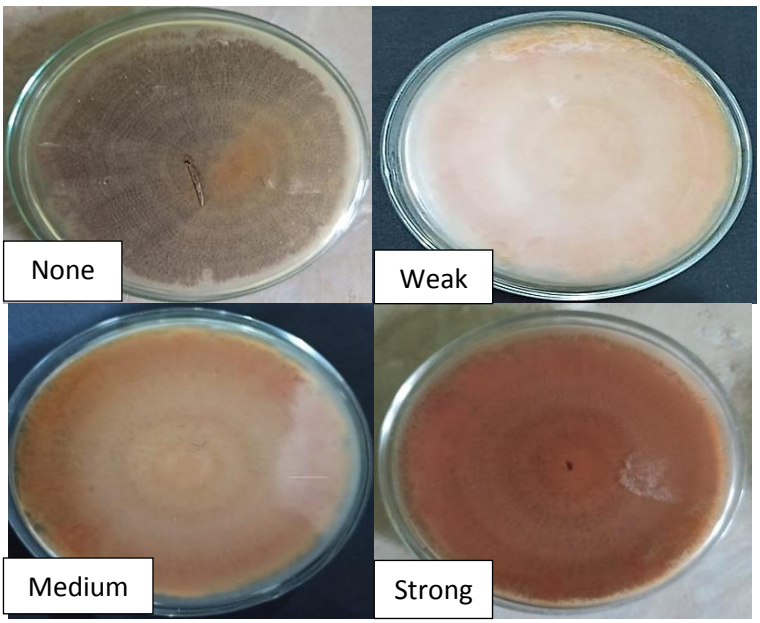

Figure (4): The color of the culture media changed according to the amounts of aflatoxins produced by the different *A. flavus* isolates.

Molecular Method of Diagnosis of *A. flavus*

Genomic DNA was extracted from *A. flavus* isolates using (TRAzol, 2012).

Many studies specializing in the use of molecular methods to determine the types of fungi have stated that the use of PCR technology is a specialized, sensitive and useful technique for early detection to identify fungal species and facilitate the implementation of procedures and measures to control microbial contamination (10).

Molecular Identification of *A. flavus AFO* gene Detection

The *AFO* gene, as a target for the specific detection of *A. flavus* by PCR. The PCR results figure (5) showed the AFO gene (679 bp) exists in 5 isolates from 62 isolation of *A. flavus* which identified the previous by morphological and microscopic methods.

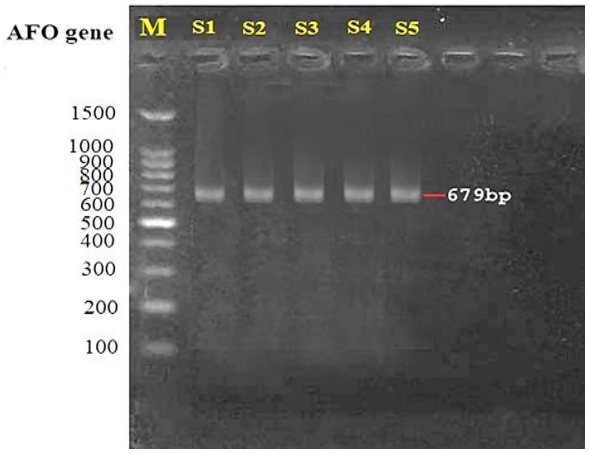

Figure (5): Gel electrophoresis of PCR product of *A. flavus* isolating using 1% agarose at 7 volt/cm for, M: marker (100-1500)bp DNA marker, lane 1-5: PCR product of *AFO*.

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