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ORIGINAL ARTICLE

Genetic Modifications and Contamination with Fungi and Aflatoxins in Corn and Its Products in Iraqi Markets

Bashar A. Hadawi[1], Jasim M. Awda[1*], Aqel Mohamed Ali[2]

1. College of Agricultural Engineering Sciences , University of Baghdad, Baghdad, Iraq
2. Quality Control Department, Grain Board of Iraq, Ministry of Trade, Baghdad, Iraq

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**Corresponding author:*
Jasim M. Awda, PhD;
College of Agricultural Engineering Sciences , University of Baghdad, Baghdad, Iraq.
Tel: +96-47817229277
Email:
jasim.awda@coagri.uobaghdad.edu.iq
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ABSTRACT

Background: Corn is considered one of the most important global crops and a model plant for genetic and biotechnology studies. This study aimed to investigate genetic modifications and contamination with fungi and Aflatoxins in corn and its products in Iraqi markets.

Methods: Fifty corn product samples were analyzed for potential contaminants. DNA extraction was performed and its concentration was measured. Polymerase Chain Reaction (PCR) was employed to detect genetic modifications of the P35S promoter and the T-NOS terminator. Fungal isolates were cultured and identification was based on colony morphology on SDA medium and microscopic examination. Aflatoxins were extracted from the samples and their concentrations were determined by enzyme-linked immunosorbent assay (ELISA).

Results: DNA concentrations across all samples ranged from 50 to 100 µg/mL, with purity values ranging from 1.7 to 2.0 revealing to be suitable for downstream applications, including agarose gel electrophoresis (1% agarose). PCR analysis revealed that 62% of the samples (31 out of 50) contained genetic modifications. Furthermore, fungal contamination including both molds and yeasts was detected in 80% of the samples (40 out of 50). ELISA revealed that 20% of the samples (10 out of 50) contained varying levels of Aflatoxins.

Conclusion: These findings suggest a potential correlation between genetic modification, fungal contamination, and Aflatoxin presence that underscores the necessity for stricter quality control measures in corn products available in local markets. So it is essential to ensure consumer safety and compliance with international health standards.

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Introduction

Corn is considered as one of the most important global crops and a model plant for genetic and biotechnology studies. It is one of the most extensively researched plant species, due to their richness in genetic materials. Corn has been used in numerous biological investigations related to plant domestication, evolution, epigenetics, heterosis, diseases and insect resistance genetics that can lead to the production of doubled haploids, genome liberation, and breeding tools (1, 2). In the mid-1990s, genetically modified (GM) crops obtained through recombinant DNA technology were commercially marketed on a large scale for the

first time. Since then, the use of this technology in agriculture worldwide has increased significantly, leading to the widespread cultivation of GM crops and achieving substantial economic benefits. However, the potential risks associated with these crops remain a subject of debate. To ensure the sustainable use of GM crops, risk management practices must be adopted to prevent or minimize potential adverse effects on the environment and human health (3). Genetic modification has become commonly used in plants and bacteria (4-6).

Over 80% of the engineered genetic constructs in genetically modified (GM) plants are built using the P35S promoter derived from the Cauliflower mosaic virus (CaMV) or the TNOS terminator derived from the soil bacterium Agrobacterium tumefaciens. Specifically, as of 2015, the P35S promoter and TNOS terminator were utilized in 65.7% and 53.49% of GM constructs, respectively (7-9). Thus, most detection methods for genetically modified organisms (GMOs) are based on specific genetic sequences of the P35S promoter and the TNOS terminator, which are identified using polymerase chain reaction (PCR) or quantitative polymerase chain reaction (qPCR) (9-11).

Corn is a valuable agricultural crop in most countries worldwide. It can be affected by fungal or bacterial diseases transmitted by the corn flea beetle (*Chaetocnema pulicaria*). Once the bacteria enter the intercellular space of the leaf, they cause water-soaked symptoms before spreading to the xylem tissue of the plant. There, they form a dense layer of biofilms, which obstructs water transport, leading to necrosis and wilting. This ultimately reduces crop yield and, in severe cases, can result in the death of the corn plant (12). Thus, corn can be susceptible to several mycotoxin-producing fungi during the growing season. Among these, *Aspergillus flavus* and *A. parasiticus*, which produce Aflatoxins (AFs, including AFB1, AFB2, AFG1, and AFG2), are a major concern in tropical and subtropical regions (13-15). In recent years, climatic changes recorded in temperate regions of Europe have led to the contamination of corn with Aflatoxins. Consequently, the primary objective of most studies has been to investigate the impact of weather conditions on the levels of Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) (16).

The allowable levels of Aflatoxins in most European countries are the permissible level of AFB1 in human food ranging between 3 and 5 ppb. The European Union also follows a limit of 0.05 ppb for AFM1 Aflatoxins in milk and 0.025 ppb in infant milk (17). According to the standards of the Iraqi Standardization and Quality Control Organization, a permissible limit of 10 µg/kg is allowed in maize and rice, 5 µg/kg in grains and their derived products, 10 µg/kg in food for milk-producing livestock and young calves, and 20 µg/kg in poultry food and other animal foods (18). High-performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry (LC-MS), and enzyme-linked immunosorbent assay (ELISA) are among the most common and widely used techniques for detecting Aflatoxins. These methods are known for their ability to provide accurate and reliable results. HPLC and LC-MS rely on separation and quantitative analysis techniques to precisely identify Aflatoxins, while ELISA depends on immunological reactions to detect the presence of Aflatoxins in samples. All of these methods are extensively used in food safety to ensure precise monitoring of contaminants, such as Aflatoxins, in food products (19-21).

To avoid the harmful effects of Aflatoxins-contaminated foods, both prevention of contamination and removal of toxic compounds during processing must be implemented (22). Fungal contamination not only leads to the accumulation of Aflatoxins; but also renders raw corn unsuitable for consumption as food or feed. It can, however, be used as biomass, which is one of the potential applications of corn biomass for bioethanol production. It is known that fermentation processes can eliminate contamination of grains, with the possibility of using the by-products generated as feed for livestock (23). So this study aimed to investigate genetic modifications and contamination with fungi and Aflatoxins in corn and its products in Iraqi markets.

Materials and Methods

This study was conducted at the Food Biology Laboratory, Department of Food Sciences, University of Baghdad, Baghdad, Iraq. A total of 50 samples of corn and some of its products, including 26 locally produced samples, representing 52% of the total samples, were randomly collected from different markets. The remaining 24 samples were imported products, representing 48% of the total samples. A total of 50 food samples of corn and some of its products were randomly collected during August and September 2024 from various markets in the Babylon, Baghdad, and Najaf provinces. After collection, the dry samples were ground and stored in plastic bags, while the wet samples were dried by exposing them to medium heating before being ground and placed in plastic bags.

DNA was extracted from samples of dry corn, canned and frozen corn, corn chips, corn flour, fresh corn cob, canned corn cob, boiled corn cob, and packaged corn varieties approved for the study

using the Plant Genomic DNA Extraction Mini Kit following the protocol provided by Favorgen Genomic DNA Extraction Kit (Taiwan). The DNA extraction was performed for all samples, and the extracted DNA was detected using agarose gel electrophoresis. Subsequently, the NanoDrop spectrophotometer was used to estimate the concentration and purity of the extracts. The results indicated that the DNA concentration ranged between 50 and 100 ng/mL, with a purity between 1.7 and 2.0. The DNA samples were then stored at -20°C until use.

The PCR device was used to amplify DNA using the primers outlined in Table 1, along with the PreMix PCR lyophilized mixture prepared by the Korean company BioNeer. The reaction mixture, consisted of 4 µL of extracted DNA, 2 µL of forward primer, 2 µL of reverse primer, and 12 µL of nuclease-free water, was prepared to a total volume of 20 µL. This mixture was then added to the PCR tube for amplification using the thermocycler. The PCR was performed using a reaction volume of 20 µL in 0.2 mL PCR tubes, containing 2 µL of extracted DNA along with all necessary PCR components, according to the protocol provided by BioNeer Go Taq®Green master mix PCR (Korea). The PCR condition was 95ºC (heat denaturation) for 10 minutes followed by 35 cycles, 45 seconds at 95ºC, 45 seconds at 55ºC (annealing) and 72ºC for 45 seconds and followed by 72ºC for 5 minutes. After 35 cycles in the thermal cycle, the PCR products were electrophoresed in 1% agarose gel for 55 minutes at 90 V. The gel was stained with ethidium bromide stain and the results were investigated under UV light (gel documentation system).

The microbiological assessment involved isolating fungi, including yeasts and molds, present in corn samples using the Sabouraud Dextrose Agar (SDA) medium (24). To prepare the medium, 65 g of SDA powder was dissolved in 1 liter of distilled water, followed by heating to fully dissolve the medium. The solution was then sterilized in an autoclave at 121°C for 15 minutes. After placing the samples in petri dishes and pouring the medium over them, the plates were incubated at 25°C for 5-7 days. Fungal colonies were counted using a colony counter device. Fungi identification was conducted in the Biology Research Unit for Tropical Regions at the College of Science, University of Baghdad. This was done by taking a smear from each plate, placing it on a glass slide, adding a drop of lacto-phenol purple stain (25). The Aflatoxins test was conducted in the laboratories of the Quality Control Department, Animal Resources Directorate, Ministry of Agriculture, Baghdad, Iraq using the ELISA technique to examine the mycotoxin content in the samples, following the protocol steps provided by Neogen Veratox for Aflatoxins Kit (USA).

Results

Theoretically, genetically modified organisms (GMOs) can be detected using PCR technology, and the results were shown in Figure 1 and 2. Totally, 31 samples out of 50 samples were genetically modified thought detected of 35S promoter and Nos-terminator. The result showed 8 samples with 35S-promoter and 25 samples with Nos-terminator. Notably, samples No. 6 and 27 were positive for both transgenes (P35S and T-NOS). In current study, the results of fungal isolation of 50 samples showed that 10 samples were free of fungal isolates, representing 20% of the total samples. In contrast, fungal isolates were found in the remaining 40 samples, represented 80% of the total samples. They were 20 mold isolates including 14 yeast isolates, and 6 isolates containing both molds and yeasts, representing 50%, 35%, and 15%, respectively of the total isolates (Figure 3).

Also, the study revealed by ELISA technique that 10 samples were positive for Aflatoxin, representing 20% of the total studied samples as follows: 13, 19, 21, 22, 23, 29, 30, 33, 44, and 50 (Figure 4). These samples contained Aflatoxins at varying levels, most of which were within the acceptable limits according to the Central Organization for Standardization and Quality Control (COSQC), which allows for a maximum of 10 µg/kg in raw corn and rice. However, sample number 22, which was dried and bagged local corn, had Aflatoxins level of 49.7 µg/kg. This number accede the maximum acceptable limit of COSQC; while, rest of the samples were fit for human consumption despite containing some level of Aflatoxins, but with acceptable limits. In the current study, it was shown that 25 corn samples contained genetic modification along with fungal contamination. Additionally, five samples were found to contain genetic modification, fungal content, and Aflatoxins (Figure 5).

Table 1: The primers used in PCR for detection of genetically modified corn.

No.	Target sequence	Sequence 5› to 3’	No. of bases	Reference
1	CaMV P 35S	F-GCTCCTACAAATGCCATCA	19	QL-ELE-00-001
		R-GATAGTGGGATTGTGCGTCA	20	
2	NOS Terminatar	F-GAATCCTGTTGCCGGTCTTG	20	QL-ELE-00-006
		R-TTATCCTAGTTTGCGCGCTA	20	

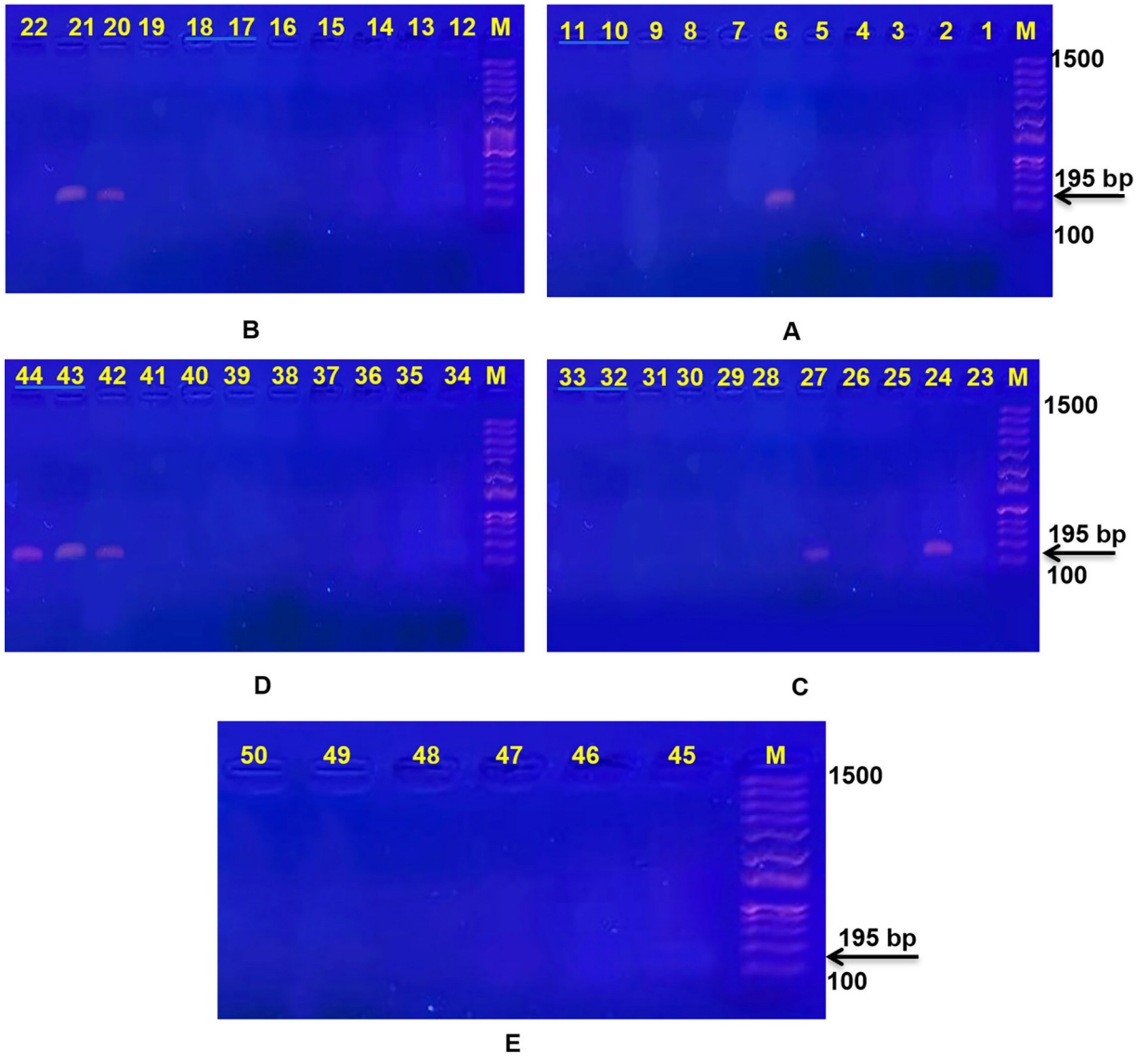

Figure 1: The electrophoresis findings of PCR using the P35S primer on 1% agarose gel.

Table 2: Types of fungal isolates.

No.	Mold type	Number of dishes
1	Pencillium ssp.	6
2	Aspergillus niger	4
3	Aspergillus flavus	4
4	Aspergillus fumigatus	2
5	Aspergillus oryzae	2
6	Aspergillus ochraceus	1
7	Cladosporium ssp.	2
8	Mucaor ssp.	3
9	Rhizopus ssp.	2
Total		26

Table 2 presents the results of fungal isolate purification from the 26 plates in which molds were observed after incubation for 7 days at a temperature of 25°C.

Discussion

Theoretically, genetically modified organisms (GMOs) can be detected using PCR technology, and the results shown in Figures 1 and 2 indicating that this technique is effective for detecting GMOs using the CaMV 35S promoter and the Nos terminator as described before (26). Several studies have demonstrated the effectiveness of conventional PCR in identifying GMOs using these two elements of CaMV 35S promoter and the Nos terminator (27-29).

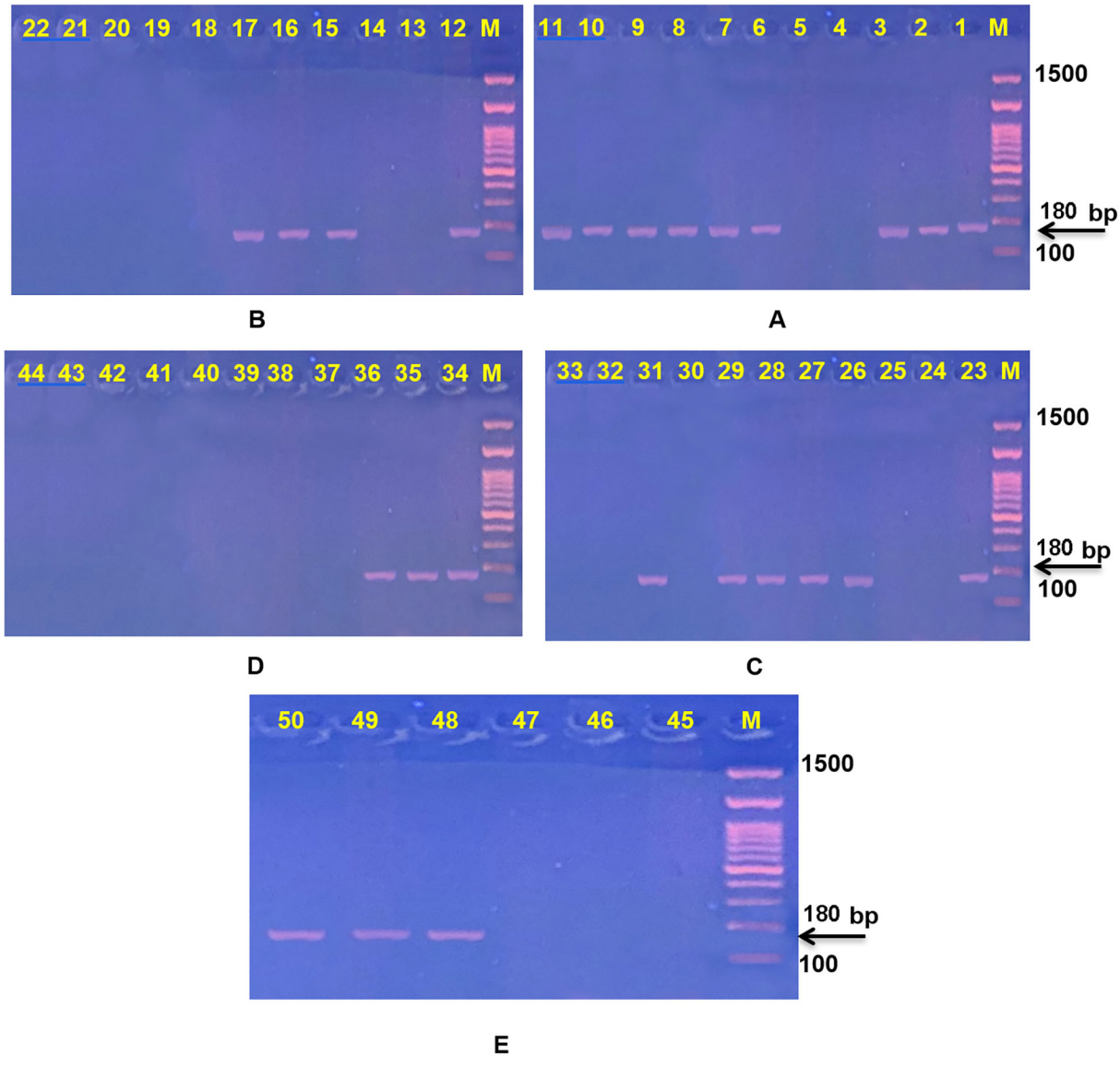

Figure 2: The electrophoresis results of PCR using the T-Nos primer on 1% agarose gel.

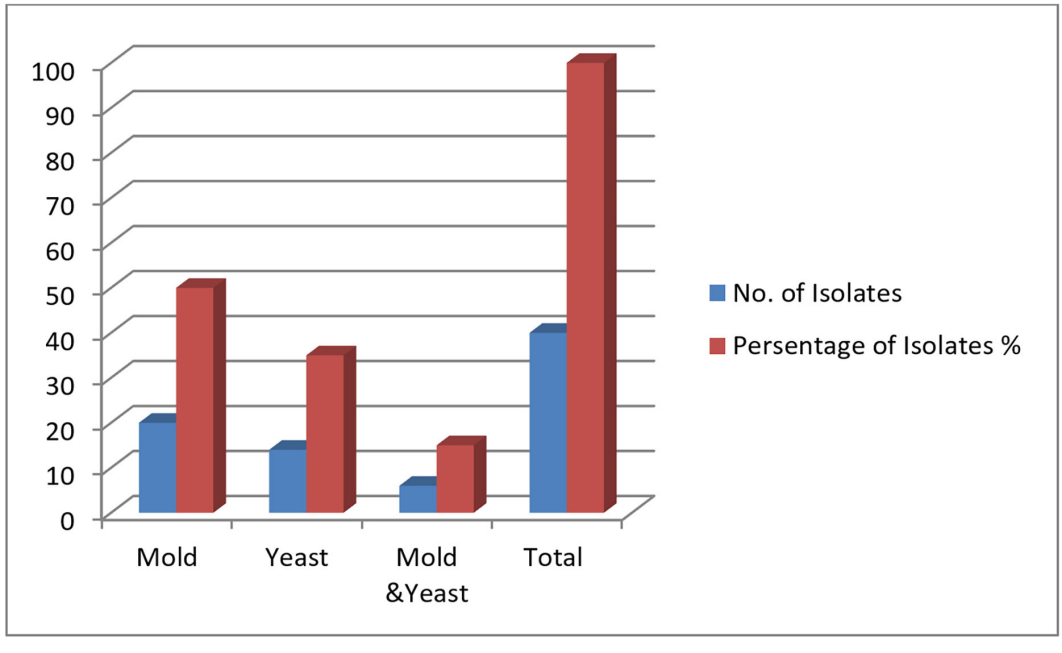

Figure 3: The percentage of fungal infections in corn samples.

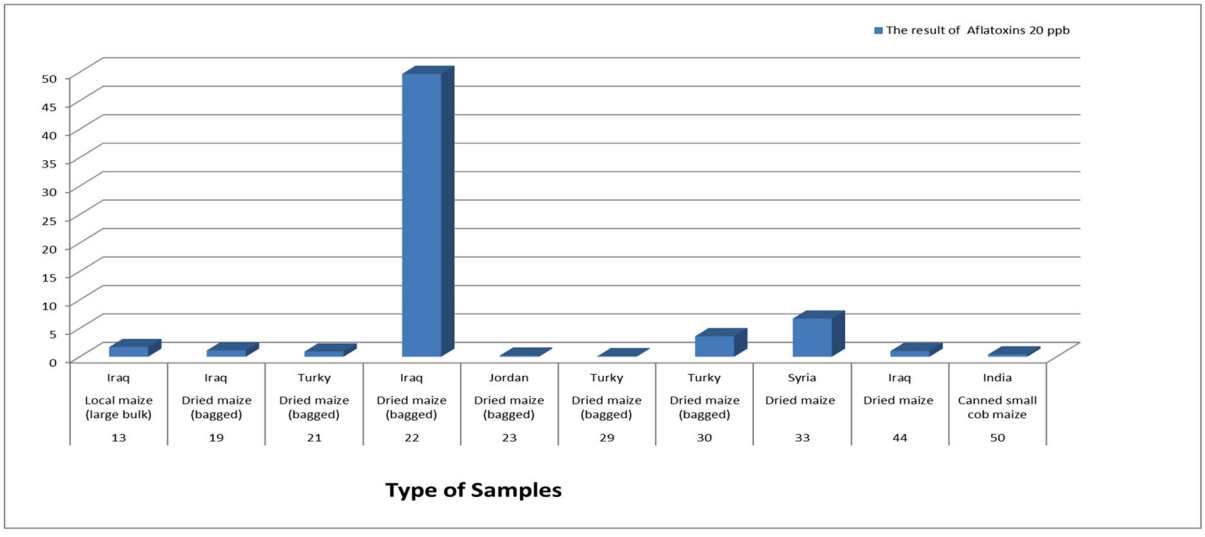

Figure 4: The origin of corn samples and its products, as well as their Aflatoxin contamination (20 ppb).

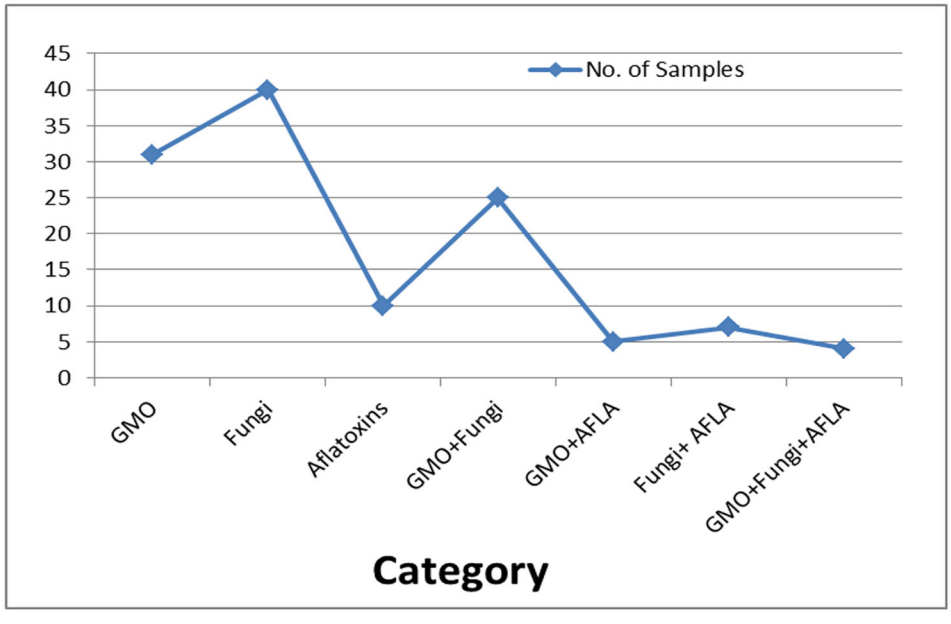

Figure 5: The number of samples and their contaminations.

Additionally, Safaei *et al.* in Iran found that 2 out of 81 samples (i.e., 2.46%) were positive for the CaMV 35S promoter, while they were negative for the Nos terminator in the remaining samples (30).

Based on the cultural and morphological characteristics defined by researchers (31, 32), fungal isolates were identified using a light microscope. Our findings are in consistent with Mohamed and Al Shamary, where Aflatoxin-producing fungi were isolated and identified from stored wheat. Several fungal species were obtained, including *Aspergillus flavus*, *A. niger*, and *Alternaria spp.* (33). The results of this study are also in consistent with the findings of Khalifa *et al.* regarding contamination of pistachio and walnut seeds collected from local markets in Baghdad, Anbar, Erbil, Sulaymaniyah, and Diyala with *A. niger*, which has the ability to produce ochratoxin A (34). Hassan demonstrated in his research the use of the ELISA technique to determine the quantity of Aflatoxins produced by *A. flavus* strain contaminating pistachio samples collected from local markets in Sulaymaniyah Governorate. The results showed contamination in 20 out of 32 pistachio samples (35).

Ahmed and Al Shamary tested 26 samples from various sources, including soil, damaged fruits, wheat, barley, corn, and spoiled bread and exhbited the potential to produce Aflatoxins at varying levels (36). James *et al.* also demonstrated that corn sold in West Africa had high levels of Aflatoxin contamination, ranging from 0.4 to 490 µg/g in Ghana, 0.2 to 120 µg/g in Benin, and 0.7 to 110 µg/g in Togo (37). The corn samples collected from southern Guinea were also contaminated with Aflatoxins, containing

77 µg/kg (38). Meanwhile, corn samples collected in Croatia between 1996 and 1997 showed Aflatoxin levels ranging from 224 to 614 µg/kg (39). Similarly, it was shown that 89% of corn samples in Albania contained aflatoxins, with concentrations ranging from 0.38 to 109 µg/kg (40). All these previous studies, whether in consistent with or differred from the current study, indicate the presence of fungal contamination in addition to genetic modification, with varying levels of Aflatoxins in our region. These factors may pose significant risks to human health, potentially leading to various diseases. Therefore, it is essential for all relevant authorities responsible for grain inspection to ensure the safety of grains from these contaminants.

Our PCR results demonstrated high accuracy and reliability in detecting genetic modifications in the studied samples using specific primers (P35S promoter and T-NOS terminator) targeting genetically modified models. Corn crops and their products in Iraq were not free from genetic modification. It was shown that 31 out of 50 corn samples and related products available in local markets were genetically modified (62%). Of these, 25 genetically modified samples contained fungi, while 6 were free from fungi. The remaining 19 samples were negative for the primers used, accounting for 38% of the total samples. Among these, 15 samples contained fungi, and 4 were free from fungal contamination.

The absence of genetically modified genes in the 19 samples does not necessarily indicate the absence of genetic modification. The results also revealed that 17 of the genetically modified samples were local varieties, while 14 were imported, representing 54.8% and 45.2%, respectively. Fungal isolation results illustrated that 40 samples (80% of the total) contained fungi, while 20% were free from fungi. Among the fungal isolates, molds represented 50%, yeasts 35%, and samples containing both molds and yeasts represented 15%. Aflatoxin testing results indicated that 80% of the samples were free from toxins, while 20% contained aflatoxins at varying levels. Most of these levels were within the acceptable limits set by the Iraqi Standardization and Quality Control Authority (10 µg/kg). However, one sample had an extremely high level of Aflatoxins, exceeding the allowable limit, making it unsuitable for human consumption. Notably, half of the aflatoxin-containing samples were genetically modified, while the other half was non-genetically modified.

Conclusion

Based on the results, it can be concluded that most of the available corn and its products in local markets were genetically modified, with a significant proportion containing molds that produced Aflatoxins. It is possible that some samples were free from fungi due to manufacturing processes, but they may still contain toxins produced earlier.

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Authors' Contribution

BA drafted and wrote the first version of the manuscript. JM and AM contributed to the conception of the research, supervised the work, conducted the investigation, collected and validated the data, and participated in the study design and implementation of the methodology. BA, JM, and AM were all involved in the analysis and interpretation of the results, reviewed the final manuscript, and approved its submission.

Conflict of Interest

The authors declare no conflict of interest.

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