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Detection of Fungi Producing Aflatoxin B1 and Ochratoxin A In Poultry Feed in Anbar

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Abstract

The study was conducted at the Department of Plant Protection, College of Agriculture, Anbar University to detect toxigenic fungi contaminating poultry feed and the concentrations of aflatoxin B1 (AFB1) and ochratoxin A (OTA) in them. The results showed the feed contaminated with various fungi, the most prevalent being Aspergillus spp at 57%, followed by Penicillium spp, Fusarium spp, and Mucor spp at 26, 9 and 3%, respectively. Colorimetric analysis on thin layer chromatography (TLC) plates revealed that isolates producing AFB1 formed 66.66% of the total number of isolates while it was 100% for OTA. AFB1 detection in poultry samples showed that the highest concentration of the toxin was in samples from the city of Hit at 50-120 ppb, 30-75 ppb in those from Saqlawiyah, and 40-60 ppb in samples from Fallujah and Al-Qa`im city. As for OTA, the results showed lower contamination levels than for AFB1 at 45-50 ppb in samples from the city of Al-Karmah, and 30-40 ppb in Ramadi and Al-Qa`im. Samples from Fallujah city recorded the lowest OTA concentration at 30-40 ppb while none was detected in feed samples from the city of Saqlawiyah, Hit, and Ana.

Keywords: Fungi, Aflatoxin B1, Ochratoxin A, Feed, Poultry.

الكشف عن الفطريات المنتجة للافلاتوكسين B1 والاوكراتوكسين A في علائق الدواجن في محافظة الانبار

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الخلاصة

اجريت الدراسة في قسم وقاية النبات/ كلية الزراعة/ جامعة الانبار للكشف عن الفطريات السامة الملوثة لعلائق الدواجن وتراكيز الافلاتوكسين والاوكراتوكسين فيها. اظهرت النتائج تلوث العلائق بالعديد من الفطريات، وكان الفطر Aspergillus spp هو الاكثر تواجدا بنسبة 57% يليه الفطر Penicillium spp ثم Fusarium spp و Mucor spp بنسب 26، 9 و3% على الترتيب. كشفت نتائج التحليل اللونية على الواح Thin Layer Chromatography (TLC) ان نسبة العزلات المنتجة للافلاتوكسين كانت 66.66% من مجموع العزلات الكلي، في حين كانت العزلات المنتجة للاوكراتوكسين 100%. بينت نتائج الكشف عن الافلاتوكسين في عينات الدواجن ان اعلى تركيز للسم وجد في عينات مدينة هيت اذ تراوحت بين 50 –120 ppb، في حين تراوحت بين 30 – 75 ppb في عينات الصقلاوية و40 – 60 ppb في عينات الفلوجة والقائم. اما نتائج الكشف عن الاوكراتوكسين، فبينت النتائج تلوثها بتراكيز اوطأ مما هو في تراكيز الافلاتوكسين، اذ بلغ اعلى تركيز للاوكراتوكسين بين 45 – 50 ppb في عينات الكرمة. وكان تركيز الاوكراتوكسين بين 30 – 40 ppb في عينات الرمادي والقائم. وسجلت عينات الفلوجة اقل تركيز للاوكراتوكسين (30 – 40 ppb). ولم يتم الكشف عن اي تلوث بالاوكراتوكسين في عينات اعلاف مدينة الصقلاوية، هيت وعنه.

كلمات مفتاحية: فطريات، الافلاتوكسن B1، الاوكراتوكسن A، اعلاف، دواجن.

Introduction

The poultry industry is an major economic resource of many countries, especially some developing countries, as it is an important source of national income for them. In addition, it meets the growing demand for poultry meat and eggs (4). Poultry meat is distinguished by its high nutritional value in terms of protein and other important nutritional components. The industry faces many challenges, including the quality of materials used in production, such as feed, medicines, food additives, and others (10). Ensuring that feed does not contain any dangerous pathogens represents the most important challenge facing those concerned with the safety of poultry meat for

consumption. Poultry diets consist almost entirely of agricultural products, especially grains, such as yellow corn, which represents approximately 50% of its components, in addition to wheat, barley, soybeans, peanut, and others.

One of the most common risks in poultry feed manufacturing operations is contamination by toxigenic fungi or mycotoxins which are secondary metabolic products of some fungi that have a genetic ability to secrete these chemical compounds (23). These secondary metabolic products have significant negative impacts on poultry health even at minor contamination levels. Scientific studies in recent decades have shown the danger of these fungi in stored infected grains prepared for human and animal consumption. The effect of these compounds may extend to consumers, exposing them to poisoning indirectly through the food chain when they consume the meat of poultry previously fed mycotoxin contaminated diets (10 and 11). Many toxic fungi have the ability to grow and secrete toxins on various types of grains that make up animal feed, including poultry feed, because they provide a nutritional medium that is very suitable for their growth. Mycotoxins are usually found in grains that are not processed well, such as appropriate levels of drying, to prevent fungi growth on them, or when damaged during handling or storage.

A particular mycotoxin may be secreted from a specific type or group of fungi that share the production of the same toxin, and one fungus variety may produce a specific or multiple types of toxins depending on the environmental conditions and growth medium (12). The most harmful and widespread mycotoxins in poultry feed are aflatoxins, ochratoxins, fumonisins, and zearalenone. AFB1 is the most toxic fungal compound due to its biological effects on humans and animals in very low concentrations, and its wide prevalence in the environment and temperature ranges. This research investigates the types of toxigenic fungi contaminating poultry feed and examines the levels and risks of AFB1 and OTA in some areas and districts of Anbar governorate.

Materials and Methods

Sample collection: Random samples of poultry feed were collected from feed factories and stores in different districts and locations in Anbar Governorate, including Al-Karmah, Fallujah, Saqlawiyah, Ramadi, Hit, Ana, and Al-Qa`im. Three samples of 1 kg each were taken from different locations to detect toxigenic fungi contamination and to estimate the concentrations of AFB1 and OTA in them (Table 1). The samples were placed in polyethylene bags and labeled with the dates and locations.

Table 1: Poultry feed samples from feed factories and stores in Anbar governorate.

Location	Sample Type	Sample No	Collection Date
Al-Karmah	Poultry feed	3	**20 / 4 / 2024**
Fallujah	Poultry feed	3	**20 / 4 / 2024**
Saqlawiyah	Poultry feed	3	**22 / 4 / 2024**
Ramadi	Poultry feed	3	**22 / 4 / 2024**
Hit	Poultry feed	3	**26 / 4 / 2024**
Ana	Poultry feed	3	**26 / 4 / 2024**
Al-Qa`im	Poultry feed	3	**30 / 4 / 2024**

Detection of fungi in poultry feed: Potato dextrose agar (PDA) was used to detect fungi in the poultry feed, according to the manufacturer's recommendation (BIOMARK) by dissolving 49 grams of the substance in 1 liter of distilled water. After mixing and dissolving the powder, the flask was placed in the autoclave at 121 °C and a pressure of 1.5 bar cm^{-3}. The culture medium was removed from the incubator after sterilization, and before it solidified tetracycline at 200 $mg.L^{-1}$ was added to it and then poured into sterile plastic Petri dishes and left to solidify. Fungi were detected in samples of poultry feed by the dilution method where 1 gram of each feed sample was placed in a test tube containing 10 mL of sterile distilled water.

A series of dilutions were conducted up to a dilution of 10^{-4}, after which 0.5 mL of the last dilution was taken and spread in dishes containing the PDA culture medium. The dishes were placed in an incubator at 25 °C. After 4 days, the fungi growing on the medium were detected and classified based on the phenotypic characteristics of the mycelium, conidia, shape, size, type of spores, and color of the colonies, using the taxonomic keys developed by (5 and 15). The percentage of occurrence of fungal species was calculated based on the equation:

IF% = number of samples of occurrence of fungi species/ total number of samples × 100

Detection of toxigenic fungi producing AFB1 and OTA: Isolates of *A. flavus* or *A. parasiticus*, which produce AFB1, and isolates of *A. ochraceus* that are most likely to produce OTA were grown based on the method by (3), and their ability to produce the toxin was tested. The isolates were grown on a yeast extract and sucrose (YES) medium which was prepared by adding 200 grams of sucrose and 20 grams of yeast extract to 1 liter of sterile distilled water (21). After dissolving the ingredients in the water, the solution was poured into 100-ml flasks which were then sterilized in the autoclave at 121 °C and a pressure of 1.5 bar cm^{-3} for 20 minutes. The flasks were cooled and inoculated with a half-cm disc taken from the edge of 1-week-old fungal isolates from the diagnosed isolates, then placed in an incubator at 27± 2 °C for 21 days. Meanwhile, the OTA-producing fungi isolates were grown according to the method by (17 and 18). AFB1 and OTA were extracted from the nutrient media of the isolates according to the methods by (3 and 18), respectively.

Detection of AFB1 and OTA in fungal isolates grown on the YES medium: The proportions and number of isolates producing AFB1 for *A. flavus* and OTA for *A. ochraceus* were detected using thin layer chromatography (TLC) plates coated with 0.2-cm-thick silica gel. The dripping process for the isolates extract was carried out

on TLC plates using a micro syringe accompanied by the standard materials for AFB1 and OTA. Plates for detecting AFB1 were placed in a tank containing chloroform and methanol (97:3), while those for detecting OTA were placed in a tank containing benzene, methanol, and acetic acid (18:1:1). The plates were removed from the tank when the solution reached 2 cm below their upper edges, dried vertically in laboratory air, and placed under ultraviolet rays to identify the fluorescent (toxin-producing) isolates. A five-degree scale indicated the fluorescence intensity of the AFB1 and OTA: - = no fluorescence; + = weak fluorescence intensity; ++ = medium fluorescence intensity; +++ = high fluorescence intensity; ++++ = very high fluorescence intensity.

Measuring AFB1 and OTA concentrations in the poultry feed samples: Samples of poultry diets from animal feed stores and factories in different areas of Anbar governorate were analyzed based on the method approved by the European Council Communities for extracting AFB1 and OTA (9). Fifty grams of each sample were placed in a 500 ml flask to which was added 250 ml chloroform and 25 ml distilled water. The flasks were placed on a shaker for an hour and filtered, and 50 ml of the filtrate was taken and placed in a 250 ml separation flask to which 100 ml of hexane was added. After simple shaking, the hexane layer was removed and the chloroform layer added sequentially to the chromatographic column.

Before the chloroform level reached the surface of the anhydrous sodium sulfate in the chromatographic column, 100 ml of diethyl ether were added to remove dyes. When the level of the last compound reached the surface of the anhydrous sodium sulfate, 150 ml of chloroform methanol (97:3) was added gradually and the mixture from the bottom of the chromatographic column with a clean beaker was received. Then, the chloroform and methanol were evaporated with hot air until dry. The extract was re-dissolved with 5 ml of chloroform and integrated into a small vial. The extract was completely dried to preserve it until the detecting process was performed, and this method was adopted for the other samples. The AFB1 concentration was measured using TLC plates according to the equation (9):

$$W = \frac{PS\ VS\ V2\ V3}{m\ V1\ V4}\ ppb$$

where Ps = concentration of standard aflatoxin B1solution (ppm)

Vs = volume of standard B1 solution giving fluorescence intensity equal to that sample spot (µl)

V1 = volume of sample extract spot (µl)

V2 = volume of final extract (2 ml of chloroform)

V3 = volume of chloroform employed in extraction (250 ml)

V4 = volume of filtrate used in clean-up of the extract (50 ml)

Mass = mass of ground sample (50 g)

The same method was followed for extracting OTA using the chromatographic column with different extraction solutions where 25 ml of phosphoric acid (0.1 molar) was used with 250 ml of chloroform. The sample was then filtered after placing it on the shaker for 30 minutes and the filtrate added to the column. The

chromatographic column was washed with 100 ml of hexane and the filtrate from it discarded. Then, the OTA was washed from the chromatographic column by adding a mixture of acetic acid and benzene (5:95). The liquid was collected in a 125 ml flask, evaporated to near dryness using a stream of air, and the remaining quantity transferred to an 8 ml vial after adding 5 ml of an acetic acid and benzene mixture (1:99). The mixture was then dried in the vial and the dripping process conducted on TLC plates by adding 500 micro liters of the acetic acid and benzene mixture. OTA concentrations were measured according to the equation (9):

$$W = \frac{PS\ VS\ V2\ V4}{V1\ V3\ m}\ ppb$$

where Ps = mass concentration of working standard solution (ppm)

Vs = volume of working standard solution giving spot with fluorescence intensity equal to that of a given sample extract spot (ppb)

V1 = volume of sample extract giving spot with fluorescence intensity equal to Vs ppb of working standard solution (ppb)

V2 = volume of final dilution of sample extract (500 µl acetic acid – benzene)

V3 = volume of chloroform extract from the column chromatography (50ml)

V4 = volume of chloroform employed for extraction (250 ml).

m = mass of sample extract (50 g).

Results and Discussion

The isolation and diagnosis of the poultry feed from the different collection sites showed the presence of various fungal species (Table 2). They varied according to the collection sites, but were dominated by the Aspergillus spp. genus which registered a 57% frequency of the diagnosed genera in most of the sites. *A. niger* and *A. flavus* at 26 and 24%, respectively also showed convergence in appearance rates in the examined samples for the species level, while *A. ochraceus* was the least frequent (6%). The results also showed that Penicillium spp. was second in frequency (26%) followed by the genus Fusarium spp. (9%). The Mucor spp. and Alternaria spp. genus rated 5% and 2%, respectively while the lowest frequency was for the Trichoderma spp. (1%). No phenotypic detection of fungal isolates of the *P. nordicum* or *A. carbonarius* species which are likely to produce OTA was found in the tested feed samples, with the exception of *A. ochraceus*.

These results are consistent with many other studies that indicated the presence of the genus Aspergillus spp. in most grains used in the food or feed industry (1, 22, 23 and 24) due to its ability to grow and develop in wide environmental conditions and on different types of nutritional sources. The frequency sequences of the fungi in the samples show convergence in nutritional and environmental requirements between hazardous and mycotoxin-secreting fungi such as Aspergillus spp. Penicillium spp. and Fusarium spp. (16).

Table 2: Occurrence of fungi in poultry feed in the study areas.

Type of Fungi	Species Appearance Rate %	Genus Appearance Rate %
A. niger	26	57
A. flavus	24	
A. ochraceus	6	
Aspergillus spp	1	
Fusarium sp.		26
Penicillium sp.		9
Mucor sp.		5
Alternaria sp.		2
Trichoderma sp.		1

Detection of AFB1 and OTA fungal isolates contaminating poultry feed: The results of the TLC technique show that of the 27 *A. flavus* isolates, 18 produced AFB1 at different levels as revealed by their fluorescence intensity on the TLC plates under ultraviolet rays (Table 3). The percentages of AFB1-producing isolates capable of producing the toxin based on collection sites were 100%, 75%, 25%, 75%, 60%, 66%, and 66% for Al-Karmah, Fallujah, Saqlawiyah, Ramadi, Hit, Ana, and Al-Qa`im, respectively. The overall rate of AFB1-producing isolates was 66.66% of the total number of isolates. As for fluorescence intensity, the higher producing isolates were obtained from Hit (AFL13), Saqlawiyah (AFL19), and Ramadi (AFL23), as shown in Figure 3. Meanwhile, isolates AFL 11 (Al-Qa`im), AFL 20 (Ramadi), and AFL 22 (Het) gave high fluorescence intensity, while AFL 10, AFL 24 (Fallujah), AFL 12, AFL 26 (Al-Karmah), and AFL 18 (Ana) had medium fluorescence intensity. As for the rest of the isolates, their fluorescence intensity was weak, indicating poor production of AFB1 or non-toxicity. All sites included in the study revealed the presence of toxin-producing isolates at different concentrations, which makes them harmful to animal and human health. These results are similar to previous findings that most *A. flavus* isolates are able to produce AFB1 (2, 6, 8, 21 and 24).

Table 3: AFB1 and OTA production rates of the *A. flavus* and *A. ochraceus* isolates.

A. flavus	*A. ochraceus*	Isolate Sites	Fluorescence Intensity*
AB1	**OTA**		
AFL1		Fallujah	+
AFL2		Hit	-
AFL3		Saqlawiyah	-
AFL4		Ramadi	-
AFL5		Al-Qa`im	+
AFL6		Saqlawiyah	-
AFL7		Hit	+
AFL8		Ana	-
AFL9		Al-Karmah	+
AFL10		Fallugah	++
AFL11		Al-Qa`im	+++
AFL12		Al-Karmah	++
AFL13		Hit	++++
AFL14		Al-Qa`im	-
AFL15		Ana	+
AFL16		Fallujah	_
AFL17		Ramadi	+
AFL18		Ana	++
AFL19		Saqlawiyah	++++
AFL20		Ramadi	+++
AFL21		Al-Karmah	+
AFL22		Hit	+++
AFL23		Ramadi	++++
AFL24		Fallujah	++
AFL25		Saqlawiyah	-
AFL26		Al-Karmah	++
AFL27		Hit	-
	OTA1	Al-Karmah	+
	OTA2	Fallujah	++
	OTA3	Ramadi	++
	OTA4	Al-Qa`im	++
	OTA5	Al-Qa`im	+++

*Fluorescence intensity: - none; + weak; ++ medium; +++ high; ++++ very high.

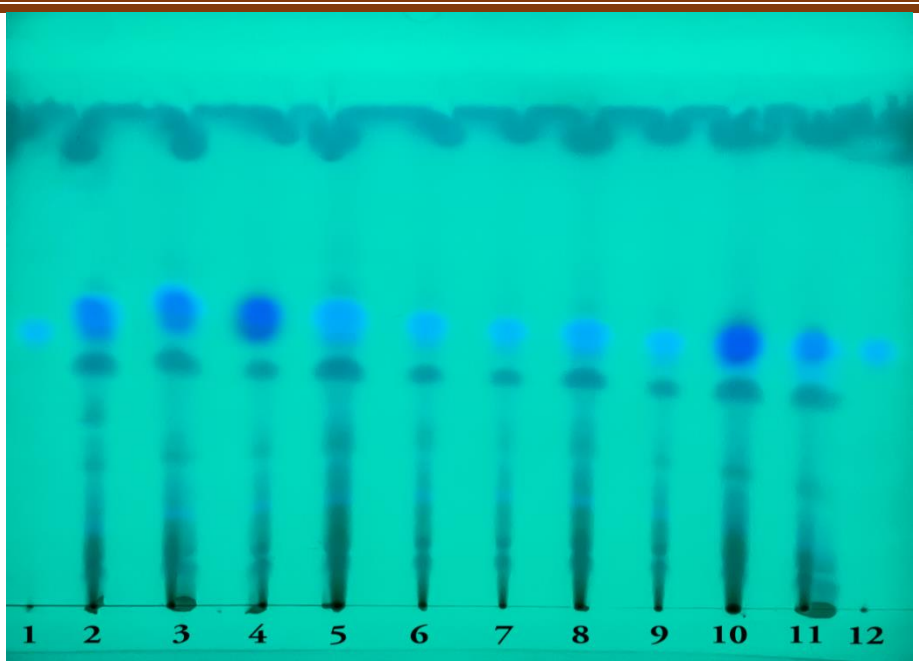

Fig. 1: Fluorescent isolates of *A. flavus* on TLC plates 1,12: standard AFB1; 4: AFL13 Het; 10: AFL19 Saqlawiyah.

As seen in Figure 2, the five isolates of the *A. ochraceus* from Al- Karmah (OTA 1), Fallujah (OTA 2), Ramadi (OTA 3), and Al-Qa`im (OTA 4), and (OTA 5) all produced ochratoxin at different levels. Isolate OTA5 had high fluorescence intensity, OTA 2, OTA 3, and OTA 4 had moderate intensity, and OTA1 (Al-Karmah) had weak fluorescence. The results show all isolates of the fungus *A. ochraceus* producing OTA in proportions that cannot be ignored, representing an additional threat to the presence of more than one fungal species capable of secreting another type of toxin. This may create a state of stimulating synergy between the toxins, leading to additional issues of feed contamination and deteriorating animal health (5).

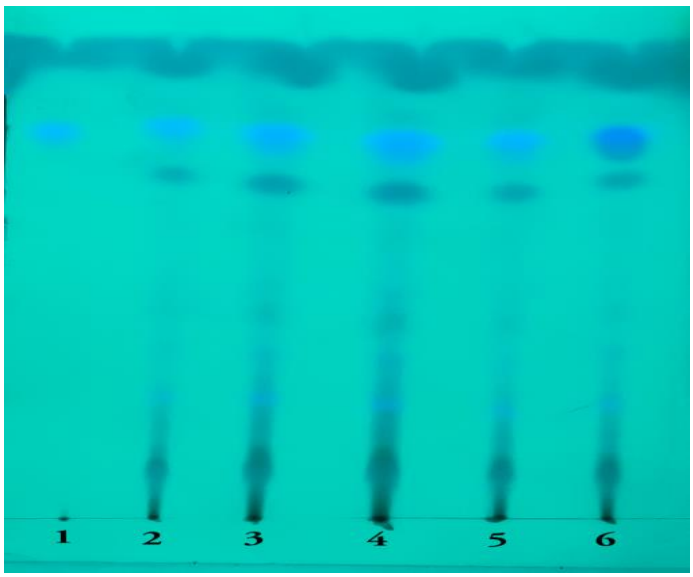

Fig. 2: Fluorescent isolates of *A. ochraceus* on TLC plates 1: Standard OTA; 6: OTA5 Al-Qa`im.

Detection of AFB1 and OTA in the poultry feed samples: Analysis of the three poultry feed samples from the study sites in Anbar governorate indicated the presence

of AFB1 in different concentrations in most of the samples (Table 4). The highest AFB1 concentrations were in the samples from the city of Hit at 50 to 120 ppb, followed by Fallujah and Al-Qa`im (40 – 60 ppb each), and Saqlawiyah (30 - 75 ppb). AFB1 contamination rates reached 100% in samples from Fallujah, Hit, and Al-Qa`im, 66.66% from those in Al-Karmah and Saqlawiyah, while the lowest were in Ramadi (33.33%). No contamination was detected for samples from Ana city. The results show that for all samples in which AFB1 was detected, its concentration exceeded the health standards set for diets (20 ppb) (2 and 15). These results give an indication of the level of risk from poultry consuming such contaminated feeds, which affects production performance and the quality of meat or eggs produced. It also indicates the lack of care in the quality of raw materials used in the manufacture of feed or their poor storage, and its subsequent impact on consumers.

Table 4: AFB1 concentrations in the poultry samples.

Sample Site	Sample Numbers	Contaminated Samples	AFB1 Concentrations (ppb)	AFB1 in Samples (%)
Al-Karmah	3	2	110 - 60	66.66
Fallujah	3	3	60 - 40	100
Saqlawiyah	3	2	75 - 30	66.66
Ramadi	3	1	100 - 70	33.33
Hit	3	3	120 - 50	100
Ana	3	0	0	0
Al-Qa`im	3	3	60 - 40	100

Analysis of the feed samples indicated lower levels of OTA contamination concentrations compared to AFB1, at between 30 to 50 ppb (Table 5). The highest concentration was in the samples from Al- Karmah (45-50 ppb), and the lowest for Ramadi and Al-Qa`im at 30-40 ppb for both, followed by 40 ppb for the Fallujah samples. The percentage of contaminated samples was 66.66% in the Al-Karmah, Ramadi, and Al-Qa`im samples, and 33.33% for Fallujah. No OTA contamination feed samples were detected from Saqlawiyah, Hit, and Ana.

Contamination of feed with more than one type of mycotoxin presents the most complex problem for those concerned with the quality of feed or its contents. Feeds may be examined to determine a specific mycotoxin expected to be present without examining for other types of mycotoxins. Studies indicate that the presence of more than one mycotoxin in poultry feed causes greater losses in product quantity and quality (13 and 19). The failure to detect AFB1 and OTA in some samples does not necessarily indicate no toxin contamination, as the concentrations may be below the TLC testing threshold. Also, testing only once is not a permanent guarantee that these feeds are free of mycotoxins (20).

Table 5: OTA concentrations in the poultry samples.

Sample Site	Sample Numbers	Contaminated Samples	OTA Concentrations (ppb)	OTA in Samples (%)
Al-Karmah	3	2	50 - 45	66.66
Fallujah	3	1	40	33.33
Saqlawiyah	3	0	0	0
Ramadi	3	2	40 - 30	66.66
Hit	3	0	0	0
Ana	3	0	0	0
Al-Qa`im	3	2	40 -30	66.66

Conclusions

The study indicates that contamination of feed by toxigenic fungi cannot be easily avoided, if not impossible, because the feed components offer a suitable medium for their growth. The most harmful types of toxin-producing fungi, such as A. flavus, are more often found in feed samples than other types. Also, the detection of multiple mycotoxins, such as AFB1and OTA, in the samples under study should alert authorities concerned with animal health to strengthen control over the quality of feeds and their raw materials, and the necessity for conducting regular checks on animal feed factories.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Salim H. S. Al-Warshan: methodology, writing—original draft preparation; Farah Kareem Mizil and Sara Thamer Hadi writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Data available upon request.

Conflicts of Interest:

The authors declare no conflict of interest.

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