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Occurrence of toxigenic fungi and aflatoxinB1contamination on poultry feed in Anbar governorate, Iraq

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Abstract

This study was conducted to detect toxic fungi and aflatoxinB1 in poultry feed items supplied in AL Anbar province, Iraq. A total of 40 chicken feed samples were obtained from stores in four locations (Ramadi, Heet, Anha, and AL-Qaim). The dilute plate technique was employed to count molds . Twelve mold genera were recovered. The main of fungi were (formerly Deuteromycetes), comprised 9 genera (i.e., Aspergillus , Penicillium , Fusarium , Alternaria , Cladosporium , Acremonium , Trichothecium , Paecilomyces , and Aerobasidium), two genera of Zygomycetes (i.e. Rhizopus, Mucor) , one genus of Ascomycetes (i.e. Eurotium) . Fungi of the Aspergillus genus were found in 40 samples of the total examined samples (87.5%) . Using TLC technique , 19 isolates out of 27 of *A.flavus* , *A.nomus* and *A.parasiticus* obtained from collected poultry feed samples were positive for the aflatoxin B1 production .Depending on the a slightly modified immune affinity method , aflatoxinB1 were recovered from 28 out of 40 extracts analyzed samples of poultry feed. The levels of natural occurrence of AFB1in starter poultry feed tested samples were ranged between 59 – 117, 3.66 – 63 , 22.8 – 90 , 38.6 – 143 ppb, in Ramadi , Heet , Anha and AL-Qaim city respectively , While levels in finisher feed tested samples were 13 – 42.8 , 12 – 18 , 19 – 22.7 , 51 – 67.4 ppb, respectively . Aflatoxigenic fungus and aflatoxins as well as other potentially dangerous fungi were found in the chicken feeds investigated.

Key words: aflatoxin, toxigenic, fungus, feed, poultry, Anbar

Introduction

Feed safety and regulation are key concerns on a global scale. The economics of chicken farming are heavily influenced by feed. These account for 60-70 percent of the cost of producing eggs and poultry meat (David *et al.*2020) . coarse or whole grains, animal and/or plant protein source, agro-based industrial by-products, antibiotics, minerals, sorghum, wheat , maize ,broken rice, vitamins , decorticated cotton seed meal, and corn gluten meal are all common ingredients in poultry feed. Mycotoxins are dangerous micromolecules produced as secondary metabolites by

some fungal species. As they grow on varied substrates, fungi are able to infect numerous foods and feeds with these toxic metabolites compounds , which have bad effects on human and animal consumers feed (Mostafa *et al*. 2012;Ahmad *et al*. 2019). The Aspergillus, Penicillium, Fusarium, and Alternaria genera, account for the majority of the poisonous species. During plant growing , harvesting or even storage period , the fungi can produce secondary metabolites in many cereal and crops. Poultry feed can be polluted with a variety of toxins that can harm poultry health (Fumagulli *et al*.,2021 ; AL-Warshan ,*et al*.,2023) . Mycotoxins (Aflatoxin is of first importance) are the most lethal of these toxins, and they are produced by a toxic fungi or common poisonous molds, such as *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (Setlem and Ramlal 2022). Poultry are the most vulnerable to its toxicity, with even modest amounts of AflatoxinB1(AFB1) causing decreased eggs production and weight gain, feed conversion efficiency, also increased mortality and disease susceptibility . Moreover, Yu *et al*., (2023) added that they are responsible for many acute and chronic diseases in humans and animals such as; hepatocarcinogen ,kidney cancer, immunodeficiency, fetal mutations and malformation and various effects like stunted growth in children with many annual mortality cases. In addition to causing diseases, the presence of mycotoxins and mycotoxigenic fungi affects feed as they acquire an unpleasant odor, change their color, taste and not accepted by animals also their nutritional and marketing value is reduced. Monson *et al*., (2014) reported that they also affect poultry performance and health, leading to severe economic losses. Furthermore, Mycotoxin contamination of poultry diets has been proven to cause sanitary problems and mortality in the birds, in addition to indirectly exposure to toxins by a persons who eats meat or eggs of animals that have previously been fed contaminated diets (Phillis *et al*., 2021) . The goal of this study was to check for the presence of aflatoxigenic fungus and aflatoxinB1(AFB1) in popular chicken feed items supplied in the AL Anbar governorate of Iraq. To our knowledge, this is the first study to document the presence of aflatoxins and mycoflora in chicken feed products in Iraq's Ramadi state.

Materials and methods

Samples collection and study area

A total of 40 poultry feeds samples (20 starter and 20 finisher) of poultry feed were collected from the local markets of Ramadi , Heet , Anah and AL-Qaim city (AL Anbar governorate / Iraq). samples of one kg were randomly collected from different regions and thoroughly combined to produce one kg of representative sample for submission. Each Sample was then divided into two parts , one for fungal count and classification, the other was kept in freezer for up to a week to be analyzed for AFB1 natural occurrence levels (Pereyra et al. 2010) .

Isolating , identifying and Counting, , fungus found in poultry feed.

On Potato Dextrose Agar (PDA) media, the surface-spread method was employed to count the number of fungal colony-forming units (CFU) (Bokhari 2010). 200 ml of sterilized saline solution (0.85 % sodium chloride) and o.o2 percent Tween 80 was added into 20 g of each mixed sample and shacked on horizontal shaker for around 20 minutes . the plates were subsequently kept at 25 °C for 7 days in an incubator. The identification of the various genera and species was done using appropriate keys and macroscopic and microscopic criteria (Pitt & Hocking 1998; Samson et al. 2000 ;Klich 2002).).The data obtained from experiments has been analyzed according to

CRD test . Samples were analyzed in the Phytopathology Laboratory of plant protection Depart. Colleg.of Agricul. Univ. of Anbar , Anbar governorate ,Iraq.

Ability AFB1 production from *A. flavus* , *A. nomius* and *A. parasiticus* strains that contaminated poultry feed .

The ability of AFB1 production for all (27) isolates of *A. flavus* , *A. parasiticus* and *A. nomius* have been obtained was investigated using Yeast Extract Sucrose (YES)(liquid media) according to (AL-Fahdawi and AL-Warshan,2023) (Figure:1).Using TLC technique (dimensions are 20 x 20 cm, and thickness is 0.20 mm) , coated with silica gel, the quantitative concentration of aflatoxin produced by isolates grown on Yes medium was measured with the aflatoxin standard substance and comparing the fluorescence of spots at the migration points with the method approved by International Agency for Research on Cancer(IARC) (Egan et al.1982) according to the following equation:

$$P = \frac{Ps\ vs\ v1}{V2\ v3}\ )\ \mu g / L\ )$$

whereas
Ps: the total concentration of the standard solution used.
Vs: the volume of the standard solution that gave a fluorescence intensity equal to the fluorescence intensity of the sample extract.
V1: volume of the final dilution solution of the sample.
V2: The volume of the sample extract gave a fluorescence intensity equal to the spot density in vs. for the standard solution.
V3: volume of the sample filter used for extraction.

Figure 1: 27 of Aspergillus isolates grown on Yeast Extract Sucrose (YES)

detection of naturally contaminated AFB1 in poultry feed samples.

A total of 40 feed samples (20 starter and 20 finisher) were analyzed for natural occurrence of aflatoxin B1. A slightly modified immunological affinity method based on the Association of Official Analytic Chemists (AOAC)(2005) method was used to test samples for AFB1. A 100 g subsample was taken for analysis after the entire sample was ground. Each sample received an equal amount(100mL) of methanol 80:water 20 solvent and 5 g sodium chloride, which was blended at high speed for 3 minutes. The filtrate was then diluted with water (1:4) and refiltered. Using filter

paper (Whatman 2V) the mixture was filtered .Through a glass-fiber filter paper 2 mL of last filtrate were put on AflaTest®WB SR Column, then eluted at 1-2 drops/s. The column was washed 3 times with 4 mL water ,then 1 mL of methanol solvent (HPLC – grade) was used to elute AFB1 from column. The methanol extract was added to 1mL a bromine developer and total concentration of AFB1 was read at 450nm emission and 360nm excitation.

Results

The results in table 1 shows frequency , percentage ,range and average of identified fungal genera in starter and finisher feed samples from four cities. Total fungal counts in feed samples ranged from 0.1 $\times 10^1$ to 4.7$\times 10^6$ cfu.g^{-1}, with an average of 2.3 $\times 10^5$ cfu.g^{-1}. Within the so-called mitotic fungi (previously Deuteromycetes), 9 genera (Aspergillus, Penicillium, Fusarium, Alternaria, Cladosporium, Acremonium, Trichothecium, Paecilomyces, and Aerobasidium) were found, whereas 2 genera (Mucor, Rhizopus) belonged to Zygomycetes . Fungi from the genus Aspergillus were the most common, which were found in 35 of the 40 samples ,with a mean value of 2.3$\times 10^6$ and a range of 0.1 $\times 10^6$-4.7 $\times 10^6$. Penicillium molds, which were found in 29 of the 40 samples (72.5%), were ranked second, with a mean of 2.2 $\times 10^6$ and a range of 0.2$\times 10^5$ - 4.4 $\times 10^6$. Zygomycetes , Mucor and Rhizopus molds ranked third and fourth, respectively, with a range of 1.2$\times 10^6$ -2.3$\times 10^6$ and 1.6$\times 10^5$-2.2$\times 10^5$, and a mean of each of these molds of 1.7$\times 10^6$ and 1.9$\times 10^5$. These molds came from 28 and 25 samples (70 percent and 62.5 percent). Fusarium and Alternaria molds were recovered from 22 and 19 samples, respectively, with percentages of 55 and 47.5 percent respectively and a range of 3.3 $\times$ 10 ^{5} – 4.2 $\times$ 10^5 and 8 $\times$ 10 ^{3} - 1.8 $\times$ 10^5, with a mean of 3.8 $\times$ 10^5 and 9.4 $\times$ 10^4 respectively. Cladosporium and Eurotium genera were recovered from 12 and 7 samples (30 percent and 17.5 percent), which constitute a range of 1.5 $\times 10^4$- 2.3$\times 10^4$ and 1.7$\times$ 10^3 – 1.9 $\times 10^3$ and a mean of 1.9$\times$ 10^4 and 1.8$\times 10^3$ respectively. Acremonium, Trichothecium, Paecilomyces, and Aureobasidium genera that belong to mitotic molds were identified from less than seven samples, with an average of 170, 55, 25 and 2 CFU g^{-1} respectively.

Table 1: frequency ,percentage, range and average of fungal genera obtained from 40 samples of chicken feed.

Molds	Frequency of Positive samples	Percentage of mold genera frequencies	Range	Average counts cfu g^{-1}
Aspergillus spp.	**35**	**87.5**	**0.1$\times 10^6$ – 4.7$\times 10^6$**	**2.3$\times 10^6$**
Penicillium spp.	**29**	**72.5**	**0.2 $\times 10^5$ - 4.4$\times 10^6$**	**2.2 $\times 10^6$**
Mucor spp.	**28**	**70**	**1.2$\times 10^6$ – 2.3$\times 10^6$**	**1.7$\times 10^6$**
Rhizopus spp.	**25**	**62.5**	**1.6$\times 10^5$ – 2.2$\times 10^5$**	**1.9$\times 10^5$**
Fusarium spp.	**22**	**55**	**3.3$\times 10^5$ – 4.2$\times 10^5$**	**3.8$\times 10^5$**

Alternaria spp.	**19**	**47.5**	**8×10^3 - 1.8×10^5**	**$9.4 X 10^4$**
Cladosporium spp.	**12**	**30**	**$1.5\times10^4 – 2.3\times10^4$**	**1.9×10^4**
Eurotium spp.	**7**	**17.5**	**$1.7\times10^3 – 1.9\times10^3$**	**1.8×10^3**
Acremonium spp.	**6**	**15**	**1.1×10^2 -2.3×10^2**	**1.7×10^2**
Trichothecium spp.	**3**	**7.5**	**0.4×10^1- 0.7×10^1**	**5.5×10^1**
Paecilomyces spp	**2**	**5**	**0.2×10^1 - 0.3×10^1**	**2.5×10^1**
Aerobasidium spp	**1**	**2.5**	**$0.1\times10^1 – 0.3\times10^1$**	**0.2×10^1**

Toxin-producing isolates of the Aspergillus spp .

Table (2) shows the prevalence of aflatoxigenic Aspergillus species isolated on PDA medium, where *A. flavus* was recovered from 15 samples, 5 from Ramadi and 3 samples from Heet city, 1 sample from Anah and 6 sample from AL-Qaim city .The *A. nomius* was recovered from 4 samples, 3 samples from Ramadi city and one sample from AL-Qaim city . Also *A . parasiticus* was recovered from eight samples , 3 samples recovered from Ramadi city and 5 samples from Anah city . Using TLC technique the ability of AFB1 production by *A. flavus* , *A. parasiticus and A. nomius* strains were investigated. Most of the isolates were AFB1 producers .As shown in table (2) (Figure:2) , Nine isolates out of fifteen isolates (60%) of *A .flavus* , three isolates out of four isolates (75%) of *A . nomius* and seven isolates out of eight isolates (87.5%) of *A . parasiticus* were able to producing AFB1. The concentration of the AFB1 was ranging between 55- 60 , 80-110 , 30-50 , 115 - 170 ppb in the isolates of Ramadi , Heet , Anah and AL-Qaim city respectively . the most production ability for AFB1 were the isolates of AL-Qaim city(115 -170)ppb , flowed by isolates of Heet city(80-110)ppb , Ramadi city(55-60)ppb and the lowest levels was of Anah city (30-50)ppb .

Table 2: The frequencies of occurrence of aflatoxigenic species *(A.flavus ,A. nomius* and *A.parasiticus)* isolated on PDA .

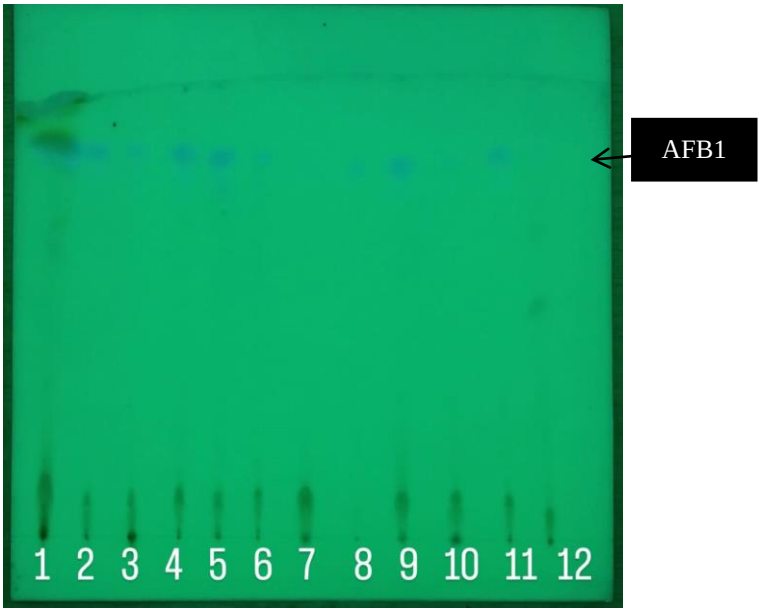
Asper

City	Aspergillus species			Total of AFB1 concentration ppb	
	A. flavus	*A. nomius*	*A.* parasiticus	Min	Max

Ramadi	5(3)	3(2)	3(3)	55	60
Heet	3(2)	-*	-	80	110
Anah	1(1)	-	5(4)	30	50
AL-Qaim	6(3)	1(1)	-	115	170
Total	15(9)	4(3)	8(7)		

Numbers in brackets are the positive isolates for AFB1 production in YES medium

*: not present .



(1 – 7 , 9 – 12) The spots of Aspergillus isolates , (8) standard substance (AFB1)

Figure 2: TLC plat showing the ability of some Aspergillus isolates to produce AFB1 on (YES) medium accompanied with the standard substance.

Natural occurrence of AFB1 in feed samples.

The obtained results showed that, more than half of poultry feed tested samples were contaminated with natural occurrence of AFB1 (Table 3). AFB1 were recovered from 28 out of 40 extracts analyzed samples of poultry feed (starter and finisher)from four cities in AL Anbar governorate (Ramadi , Heet , Anha and AL-Qaim).The percentage of positive tested samples for starter and finisher were (80,80) ,(60,40) ,(80,60) and (100,60) in the city of Ramadi, Heet ,Anha and AL-Qaim respectively .The highest percentage of contaminated samples were starter feed of AL-Qaim city (100%) and the lowest samples were finisher feed of Anha city (40%). The levels of natural occurrence of AFB1in starter poultry feed tested samples were ranged between 59 – 117, 3.66 – 63 , 22.8 – 90 , 38.6 – 143 ppb, in Ramadi , Heet , Anha and AL-Qaim city respectively , While levels in finisher feed tested samples were 13 – 42.8 , 12 – 18 , 19 – 22.7 , 51 – 67.4 ppb, respectively . Starter feed analyzed samples of AL-Qaim city appeared highest level of AFB1 (143)ppb . On the other hand, the lowest level were the samples of Anha city (3.66 – 63) ppb . Also the finisher tested samples of AL-Qaim city recorded highest levels (51 – 67.4)ppb , and the lowest levels were samples of Heet city (12 – 18)ppb . The average amount of AFB1 recovered in this investigation was 80%, with starting feed samples averaging 73.33ppb, and finisher feed samples averaging 39.7ppb

Table 3: Tested and positive samples with the percentage of detected levels for AFB1in Poultry feed .

city	Poultry feed samples							
	starter				Finisher			
	Tested samples	Positive samples	% of positive samples.	Levels of AFB1(ppb)	Tested samples	Positive samples	% of positive samples.	Levels of AFB1(ppb)
Ramadi	5	4	80	59 - 117	5	4	80	13 – 42.8
Heet	5	3	60	3.66 – 63	5	2	40	12 – 18
Anha	5	4	80	22.8 - 90	5	3	60	19 – 22.7
AL-Qaim	5	5	100	38.6 - 143	5	3	60	51 – 67.4

Discussion

Our investigation found that the overall fungal load in the studied feed samples was roughly 104 cfu.g^{-1}, which is greater than the 103 cfu.g^{-1} reported in Slovakia (Labuda *et al*, 2005). Close to outcomes were seen in Turkey (Heperkan and Alpeeden, 1988) and Argentina (Dalcero , 1998). The prevalence of mold genera was similar to Saudi Arabia's (Abdul Wahab, 1996), which could be explained by some similarity in the ecological and climatological factors. In terms of mold contamination, the genus Aspergillus appeared to be the most common (87.5%). Because of the possibility of mycotoxin production especially AFB1, these molds are extremely important (Raghda et al,.2022). For toxin-producing fungal isolates Aflatoxigenic of *A. flavus, A. nomius* and *A. parasiticus* have been documented in the literature by several authors (Vishwambar *et al*,2021). As a result, the existence of these species received more attention , Being a source of secretion of the most toxic fungal compounds(AFB1). And about differences in the production capacity of AFB1 between isolates is basically due to genetic capabilities if other factors of growth are same (Caceres *et al*.2020) . In current study all the positive isolates, produced high levels of AFB1 on liquid media (YES) more than the regulation levels of mycotoxins(20 ppb) in Iraq and many countries . The results showed the gravity of poultry feed contamination with such molds , and the potential for AFB1 production on these feed . Observing the results of the analysis of poultry feed samples from the areas included in the study ,shows the level of risk of exposure to AFB1 and the negative effects that it may have on birds consuming these contaminated feeds. In comparison to other studies , the current results were higher than those reported inTaiwan, Korea and China (Lu *et al*.,2023) Saudi Arabia (Alisraa *et al* ., 2021) . The concentrations of AFB1 in the feed samples were much higher than the permissible limits in animal feed, which are supposed to not exceed 20 ppb. This result give us a warning that the samples contained with high concentration of a toxic substance that could pose a threat to the human health and chicken industry.

Conclusion:

There were aflatoxigenic fungus and aflatoxins in AL Anbar governorate's chicken feeds together with other harmful fungus, particularly those from the Penicillium,

Fusarium, and Aspergillus species. In order to lower feed contamination levels, more severe prevention and control procedures must be used to prevent the death of poultry as well as the transfer of carcinogenic mycotoxins through the food chain.

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