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MYCOLOGICAL EXAMINATION OF POULTRY FEEDSTUFF WITH SPECIAL REFERENCE TO MYCOTOXIN PRODUCTION

(With 10 Tables)

By

H.A. TORKY; M.A. ABDEL KARIM; A.F. ABDEL MOGHNEY and AMAL A. GAMAL ALDEEN**

*: Animal Health Research Institute

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اختبار الفطـريات المفرزة للسموم في علائق الدواجن ومكوناتها

طلعت أحمد تركي ، عبد الكريم عبد التواب محمود ، عبد الرشيد فتحي عبد المغنى ، أمل عبد المنعم جمال الدين

تـم الفحـص الفطري لعدد ٧٧ عينة غذاء و مكون غذاء وقد تم عزل ٣٠ عترة من فطر الأسـبرجيلس فلافـس و١٥ عـترة من فطر الأسبيرجلس أوكراشيس و١١ عترة من فطر الأسـبيرجيلس نيجر و٢٦ عترة من فطر البنسيليم و٣٣ عترة من فطر الفيوزيريم و٢٠ من فطر الميوكور و٣ عترات من فطر الألترناريا و٤ أنواع من الخمائر وعترة واحدة من فطر الأسـبيرجلس كـنديدس. تم فحص ٥٩ عينة لسموم الأفلاتوكسين وكانت نتيجة الفحص أن ٥٧,٦٢% مـن هـذه العيـنات تحتوى على سموم الأفلاتوكسين وأن ٣٢,٢٨%من العينات تحـتوى على كميات من الأفلاتوكسين تقل عن الحدود المسموح بها بينما احتوت ٢٠,٢٣% من هذه العينات على كمية من الأفلاتوكسين تراوحت بين ٧-١٠ أضعاف الحد المسموح به. وبفحـص ٣٣ عيـنة لسموم الأوكراتوكسين كانت نتيجة الفحص أن ٦٩,٦٩% من العينات تحـتوى علـى سـموم الأوكراتوكسـين وأن ٣٣,٣% مـن العينات تحتوى على كمية من الأوكراتوكسـين أقـل مـن الحـدود المسـموح بها بينما ٣٦,٣٦% تحتوى على كمية من الأوكراتوكسين أكثر من الحد المسموح به ليصل إلى ٧ أضعاف. وقد تم فحص ٢٢ عينة من هذه العينات لسموم الأفلاتوكسين والأوكراتوكسين معا ووجد أن ٨٢,٨١% من هذه العينات تحتوى على سموم الأفلاتوكسين وبمعدل (١-٩٣ جزء في البليون) والأوكراتوكسـين بمعدل (١-٣٨ جـزء في البليون) بينما ٩,١% من هذه العينات تحتوى على سموم الأفلاتوكسين فقط بمعدل ٣-١٤ جزء في البليون و ٩,١% من العينات تحتوى سموم الأوكراتوكسين فقط بمعدل ٥-١٢ جزء في البليون. وبفحص ٧ عينات من العينات المختبرة وجد أن هذه العينات خالية من عترة الأسبيرجلس فلافس إلا أنهـا احتوت على سموم الأفلاتوكسين بمعـدل من

٢-٣٨ جـزء في البليون من ناحية أخرى تم فحص ٨ عينات من العينات المختبرة وجد أن هذه العينات خالية من فطر الأسبيرجلس أوكراشيس ولكن بها سموم الأوكراتوكسين بمعدل ٢ -١٠ جـزء في البليون. وباختبار مقدرة خمسة من العترات المعزولة من فطر الأسبيرجلس فلافـس على إفراز سموم الأفلاتوكسين وجد أن ٣ عترات منه لديه القدرة على إفراز سموم الأفلاتوكسـين بمعـدلات أكثر من الحدود المسموح بها وتراوحت بين (٣٨-١٤٠ جزء في البليون) .

SUMMARY

Mycological examination of 77 samples of poultry feed and feed ingredients revealed the presence of 30 isolates of *Aspergillus flavus*, 15 isolates of *Aspergillus ochraceus*, 11 isolates of *Aspergillus niger*, 26 isolates of *Penicillium spp.*, 33 isolates of *Fusarium spp.*, 20 isolates of *Mucor*, 3 isolates of *Alternaria spp.* and 4 isolates of yeast and only one isolate of *Aspergillus candidus*. While quantitative and qualitative determination of aflatoxin for 59 samples of feed and feed ingredients using affinity column chromatography revealed that; the examined samples had aflatoxins 57.63%, and 20.33% contain high levels of aflatoxins that reached 7 to 10 times the permissible limit. In addition, 33 samples of feed were tested for ochratoxin contamination, the results showed that 36.36% of the examined samples contained high levels of ochratoxin that reached 7 times the permissible limit. Moreover, 22 of the examined samples were tested for aflatoxin and ochratoxin, the results indicated that 81.82% of the examined samples had aflatoxin and ochratoxin together at a rate of 1-93 ppb aflatoxin and 1-38 ppb ochratoxins. Seven samples were negative for *Aspergillus flavus* but carried aflatoxin at a rate of 2-38 ppb, moreover eight samples were negative for *Aspergillus ochraceus but contained ochratoxin at a rate of 2-10ppb.* Five isolates from the isolated strains of *Aspergillus flavus* were screened for their ability to produce aflatoxin, only 3 isolates produced aflatoxins at a rate above the permissible limit (38-140 ppb).

Key words: *Poultry – Feedstuff – Mycotoxin.*

INTRODUCTION

Fungi and mycotoxins in poultry feeds and feed ingredients attracted much attention during the last decades as a result of increasing awareness of health hazards presented by those fungi and their toxins produced by fungi that are grown on crops in the field after harvest, in storage or during processing of food (Clark *et al.*, 1980, and Hsieh, 1990). Almost all plants products can serve as substrates for fungal

growth and subsequent mycotoxin formation. This provides the potential for direct contamination of food, animal and poultry feeds (Shotwell *et al.*, 1966). Among many of discovered mycotoxins, aflatoxins, ochratoxin A and several others were identified (Richard *et al.*, 1993)

Aflatoxin is a group of closely related toxic and carcinogenic metabolites mainly produced by *Aspergillus flavus, Aspergillus parasiticus and Penicillium puberulum* in the field and during storage in a number of important agriculture commodities such as grains (Clark *et al.*, 1980). Feed inadequate storage conditions such as high moisture and warm temperature (25 -30C) can create conditions suitable for aflatoxin production (Cast, 1989).

Ochratoxins are nephrotoxic metabolites produced chiefly by *Penicillium viridicatum and Aspergillus ochraceus* which are commonly present on numerous grains and feed stuffs (Dwivedi and Burns, 1986).

Feeds which are contaminated with mycotoxins not only have a direct toxic effect on animal or poultry but there may be a carry over of the toxin into the product creating further exposure of human beings to this intoxication (Van Zytveld *et al.*, 1970 and Mabee and Chipley, 1973).

So the present study aimed to:

1- isolation and identification of mycotoxin producing fungi.
2- quantification of specific toxins.
3-detection of aflatoxin –producing ability of Aspergillus flavus isolates.

MATERIALS and METHODS

Materials:

Samples:

Feed samples:

A total of 77 samples of different poultry feed stuffs were collected from different farms in El Behera governorate that had a health problem in their flocks. The representative samples were obtained from the feed being fed at the time of the problem. The feed sample was thoroughly ground and mixed, then samples were taken. The samples were examined for mycotic contamination and toxic production.

Different poultry feeds ingredients examined for moulds, aflatoxin, ochratoxin, aflatoxin and/or ochratoxin.

Table 1:

Sample	Total No.	No. of samples examined for moulds	No. of samples examined for aflatoxin	No. of samples examined for ochratoxin	No. of samples examined for aflatoxin and/or ochratoxin
Poultry ration	48	48	30	23	22
Ration concentrates	7	7	7	--	--
Yellow corn	10	10	10	5	--
Soybean	5	5	5	--	--
Fish meal	7	7	7	5	--
Cumulative totals	77	77	59	33	22

Media for isolation:

The following media were used for isolation of fungi:

1- Sabouraud's dextrose agar medium with chloramphenicol (0.05g/l) prepared according to Cruickshank *et al.* (1975).

2- Potato dextrose agar medium prepared according to Shotwell *et al.* (1966).

Stains:

- Lactophenol cotton stain prepared according to Raper and Fennel (1965)

Apparatus:

Flurometer (VICAM, V series 4).

Mycological examination:

Isolation of fungi:

The dilution of the examined samples till the 4^{th} dilution (10^{-4}) was carried out according to Dalcero *et al.* (1998), from each dilution 0.1 ml is removed into a sterile Petri dish containing Sabouraud's dextrose agar with chloramphenicol 90.05g/l) using surface spread method and incubated at 25-28C for 7-10 days. After incubation, the plates were examined visually and microscopically by making films, individual suspected colonies were selected depending upon their morphological characters. Stock cultures were made from each isolate and maintained in Sabouraud's dextrose agar slopes in refrigerator for further identification.

Identification of moulds:

Identification of moulds was carried out according to the method of Raper and Fennel (1965) and Frey *et al.* (1979) in which all the positive mould cultures were purified by sub culturing on Sabouraud's dextrose agar plates incubated at 25-28C for 3-5 days and examined for gross and microscopical characteristics (Collins and Lyne, 1984)

For direct microscopical examination, a small portion of the periphery of a fresh colony was picked up using the sticking surface of a piece of a solutip and placed with its sticking surface down on a clean slide with a drop of lactophenol cotton blue stain and examined microscopically to detect separation of hyphae, roughness or smoothness of conidiophores, the shape of the vesicles, arrangement and number of rows of the sterigmata.

Extraction of aflatoxin, and ochratoxin from feed samples:

Itq was carried out according the instructions of VICAM manufacturer Kit.

Aflatoxins and ochratoxins were determined quantitatively by using affinity column chromatography (Aflatest PTM column and ochratest column) and fluorometer (VICAM series 4) with 360 nm excitation filter and 450 nm emission filter.

Detection of aflatoxin producing ability of *Aspergillus flavus* isolates: Production of aflatoxins:

Aflatoxins were produced by growing *Aspergillus flavus* on sterile polished rice by the method of Shotwell *et al.*, (1966) and the detection was carried out according VICAM kit instructions.

RESULTS

As shown in Tables (2, 3, 4, 5, 6, 7, 8, 9 & 10)

DISCUSSION

Mycotoxins are considered as unavoidable contaminants in foods and feed ingredients because agronomical technology has not yet advanced to the stage at which pre-harvest infection of susceptible crops by fungi can be eliminated (Clark *et al.*, 1980).

Mycological examination of 77 poultry feeds for *A.flavus* revealed the presence of other fungi isolated from the cultural media of examined samples (table 2). The most prevalent species were *Fusarium* which appeared in 33 samples out of 77 examined samples (42.86%), followed by *Penicillium spp.* (33.77%), *Mucor spp.* (25.97), *Yeast* (5.19%) and *Alternaria spp.* (3.90%). These results agreed with those of Abd El-Hamid *et al.* (1989) who isolated *Fusarium spp.* at a rate of 31% out of 73 poultry diets, and Dalcero *et al.* (1997) who reported that *Fusarium spp.* occurred in a high level in poultry feeds reaching 87%. Christensen, and Sauer (1982), considered *Fusarium spp.* A field -borne

fungi, which exist on the grain on arrival, once *Fusarium* invaded a corn crop, it continue to propagate during storage if the moisture content remains high.

The problem is that the metabolites produced (T-2toxin, diacetoxyscirpenol, deoxynivalenol, and zearalenone) by these *Fusarium spp.* are strongly suspected to be responsible for mycotoxicoses for human and animals, which cause hemorrhage, with dermatitis, immunosuppression and cancer (Sato *et al.*, 1975 and Marasas *et al.*, 1983). Regarding the risk of the high incidence of penicillium spp is that the most important toxin produced by it is Ochratoxin A, A complicated factor is that some penicillin isolates (P.viridicatum) were found not to produce Ochratoxin A, but rather produced a group of other toxic metabolites, unrelated to Ochratoxin A (Stack *et al.*, 1977).

Table (3) shows that 30 isolates of *Aspergillus flavus* (38.96%) were recovered from the examined samples followed by *Aspergillus ochraceus* (16.88%). *A.niger* (14.29%) and *A.candidus* (1.30%). This indicates that *A.flavus* is the most prevalent isolated species. The results agreed with those of Magnolli *et al.* (1998) who recorded that *A.flavus* is the most prevalent isolated species recovered from poultry feed samples and Connole *et al.* (1981) who reported that 200 (56.33%) *A.flavus* isolates were recovered from 355 feed samples. This result disagrees with Le Bars, (1982) and Bragulat *et al.* (1995) who reported that *Aspergillus flavus* present in feed samples at low level.

The presence of *Aspergillus flavus*, *Aspergillus ochraceus A.niger* and *A.candidus* with their respective percentages reflect how much the problem will be by their metabolites. Frisvad (1986) reported that (aflatoxins, aflatrem, aflavinin, aspergillic acids, neoaspergillic acids, B-nitropropionic acid) are the metabolites of *Aspergillus flavus*,, while (kojic acid, ochratoxins,penicillic acids) are related to *Aspergillus ochraceus*, moreover, *A.niger* produce (Emodin, ochratoxins, malformins, naphthopyrones, nigragillin)), *A.candidus* produce (candidulin, terphenylin, xanthoascin).

Table (4) shows that 23.37% of poultry ration, 6.49% of ration concentrates, 3.89% of yellow corn, 2.59% of soybean and 2,59% of fish meal are contaminated with *Aspergillus flavus*. The high level of *A.flavus* in poultry ration may be due to unsuitable storage conditions or may be due to the ration ingredients contamination with fungi pre-or post-formulation. These results agree with Payne (1987) who mentioned that corn is an important crop in the Americans, Europe, and many southest Asian countries. *A.flavus* is the dominant aflatoxin-producing

spp. on this crop, and aflatoxin contamination, either pre-or post harvest has been demonstrated in all the producing area of the world. In the U.S pre-harvest contamination is a serious problem with *A.flavus* colonizing undamaged external corn silks and then invading the developing kernels while insects infestation and kernel damage contribute to fungal penetration and toxin production, Environmental temp, mechanical damage, drought stress are also important.

Results in Table (5) represent the distribution of aflatoxin in different feed ingredients. 22 samples (37.28%) have low levels of aflatoxin which do not reach the permissible limit (20 ppb for all foods and feed ingredients (Schuller *et al.*, 1983), twelve samples (20.33%) of the examined samples contained high levels that may reach up to 7 times of the permissible limit.

Concerning the detected levels of aflatoxin, there do not appear to be truly safe levels of mycotoxins, the prudent person should assume that any level carries with it a risk (Schaeffer and Hamilton 1991)

Results in Table (6) represent the distribution of ochratoxin in different examined feeds. Eleven samples (33.33%) have low levels of ochratoxin (1-5ppb). Twelve samples (36.36%) of the examined samples contained high levels that may reach 7 times of the permissible limit (5 ppb for all feeds) (FAO, 1995). The effects of ochratoxicoses in broilers are longer lasting than those typically associated with aflatoxin, Huff and Doerr (1980). Ochratoxin A has immunossuppressive , embryonic and carcinogenic effects in animals. Because Ochratoxin A is fat soluble and not readily excreted, it accumulates in the fatty tissue of affected animals. It has been suggested that Ochratoxin A is a causal agent of human endemic nephropathy (Krogh *et al.* 1974)

In this study, 22 samples were exposed to aflatest and ochratest together (Table 7). Eighteen samples (81.82%) produced aflatoxin at a rate of 1-93 ppb and also ochratoxin at a rate of 1-38ppb. Two samples (9.1%) produced aflatoxin at a rate of 3-14 ppb and 2 samples (9.1%) produced ochratoxin at a rate of 5-12 ppb (Table 7) consequently it could be emphasized that there is a relation between ochratoxin and aflatoxin (Chute and Barden, 1964) and Stock (1961). The toxicoses resulting from multiple mycotoxin contamination may result in clinically unique symptomatology. The infinite number of possible combinations of mycotoxin contamination in feedstuffs makes meaningful diagnosis of such problems extremely difficult (Smith and Henderson 1991). Elevated occurrence of aflatoxin was linked to hepatocellular carcinomaa in humans. Epidemiologiccal studies have clearly

eastablished infection of humans by hepatitis B virus in areas of the world where hepatocellular carcinoma is a primary disease (Hsieh, 1986).

From Table (8), 7 samples produced aflatoxin at a rate of 2-38 ppb but we could not isolate *A.flavus* from the samples. Also in Table (9) 8 samples contained ochratoxins at a rate of 2-10 ppb but *A.ochraceus* could not be isolated from the examined samples. These results may be due to that mycotoxins were synthesized by organisms when metabolically active in moist stored feed in sealed and unsealed silos and the species which synthesize these toxins may have been killed by heat treatment during the preparation of the feeds or may be due to the treatment of feed ingredients with antifungal additives (Smith and Henderson, 1991).

In Table (10), the results of screening of isolated random strains of *Aspergillus flavus* for aflatoxin production revealed that 3 isolates out of 5 tested isolates of *A.flavus* were found to be aflatoxin producers at a rate above the permissible limit. This was in agreement with those reported by Al-Lakany (1991) who reported that aflatoxins were produced by 69.2% of the *A.flavus* isolates. Also agree with Diener and Davis (1986) who reported that 60% of isolates of *A.flavus* group produced some aflatoxins and 40% of the isolates produced aflatoxins at a rate below the permissible limit but all the isolates are toxin producers.

In conclusion the present study revealed that: 1- High incidence of *Aspergillus flavus* strains as it was the predominant isolated fungus and it is an important aflatoxin producer which throw a strong light how much the problem is from economic point of view as an effect on the birds in addition to its health hazard for human consuming this birds that might be contain a residues of the aflatoxin. 2- The results showed that the presence of poultry feed ingredients contaminated with both aflatoxin and ochratoxin. A matter hich maximize the mycotoxin problem for birds and who consume their by-products. 3- A prophylactic addition of antimycotic and antimycotoxins as feed additives is of great importance. 4- As any level of mycotoxins carries with it a risk for animal and human, the routine mycological examination and mycotoxin detection for feed ingredients before manufacturing is essential.

Table 2: Type of fungi present in examined samples.

Examined samples	No.of examined sample	Fungi present in each of examined sample									
		Penicillium spp.		Fusarium		Mucor		Alternaria		Yeast	
		No.	%	No.	%	No.	%	No.	%	No.	%
Poultry ration	48	18	37.5	23	47.92	15	31.25	2	4.17	4	8.33
Concentrates	7	4	57.11	6	85.71	--	0.00	1	14.29	-	-
Yellow corn	10	2	20.00	2	20.00	3	30.00	-	0.00	-	-
Soybean	5	1	20.00	1	20.00	2	40.00	-	0.00	-	-
Fish meal	7	1	14.29	1	14.29	--	0.00	-	0.00	-	-
Cumulative total	77	26	33.77	33	42.86	20	25.97	3	3.90	4	5.19

Table 3: Incidence of Asperigillus species in the examined samples.

Fungal species	Samples[a]	Frequency[b] (%)
Aspergillus flavus	30	38.96
Aspergillus ochraceus	15	19.48
Aspergillus niger	11	14.29
Aspergillus candidus	1	1.30
Cumulative totals	**55**	**71.43**

a: Number of contaminated samples from total number=77.
b: Percentage of samples where each fungal species.

Table 4: Incidence of aspergillus flavus in the examined samples.

Fungal species	Number of examined samples	Aspergillus flavus from examined smples	
		No.	%
Poultry ration	48	18	23.37
Ration concentrates	7	5	6.49
Yellow corn	10	3	3.89
Soya corn	5	2	2.59
Fish meal	7	2	2.59
Cumulative totals	77	30	38.96

Percentages were calculated in relation to the total number of examined samples.

Table 5: Incidence of aflatoxin in the examined samples.

Examined samples	No.of examined sample	Aflatoxin producing samples expressed in ppb									
		-ve rate.		1-20ppb		21-50ppb		51-100ppb		>100ppb	
		No.	%	No.	%	No.	%	No.	%	No.	%
Poultry ration	30	6	20.00	19	63.33	4	13.33	1	3.33	- -	0.00
Concentrate	7	3	42.86	1	14.38	1	14.28	1	14.28	1	14.28
Yellow corn	10	7	70.00	1	10.00	1	10.00	1	10.00	- -	0.00
Soybean	5	4	80.00	1	20.00	- -	0.00	- -	0.00	- -	0.00
Fish meal	7	5	71.43	--	0.00	- -	0.00	- -	0.00	2	28.57
Cumulative total	59	25	42.37	22	37.28	6	10.16	3	5.08	3	5.08

Permissible limit is 20ppb (Schuller *et al.*, 1983)

Table 6: Incidence of ochratoxin in the examined samples

Type of examined samples	No.of examined sample	Aflatoxin producing samples expressed in ppb									
		-ve rate.		1-5ppb		6-10ppb		11-20ppb		>20ppb	
		No.	%	No.	%	No.	%	No.	%	No.	%
Poultry ration	23	2	8.7	11	47.83	8	34.78	2	8.70	-	0.00
Yellow corn	5	4	80	--	0.00	-	0.00	-	0.00	1	20.00
Fish meal	5	4	80	--	0.00	1	80.00	-	0.00	-	0.00
Cumulative total	33	10	30.3	11	33.33	9	27.27	2	6.06	1	3.03

Permissible limit is 5ppb (Schuller *et al.*, 1983)

Table 7: Feed samples contaminated with aflatoxin and/or ochratoxin.

Number	Rate	Rate of ochratoxin producing samples (ppb)	Rate of aflatoxin producing samples (ppb)
2	9.10	5-12	0
2	9.10	0	3-14
18	81.82	1-38	1-93

Total number of examined samples =22

Table 8: Samples positive for aflatoxin but negative for aspergillus flavus strains.

Serial number of samples	Type of sample	Aflatoxin (ppb)	Isolated fungi
1	Poultry ration	38	Fusarium
2	Ditto	36	-ve
3	Ditto	27	-ve
4	Ditto	18	Fusarium, penicillium
5	Ditto	14	Fusarium, A.niger
6	Ditto	10	a.niger, mucor
7	Ditto	2	-ve

Permissible limit = 20ppb (Schuller *et al.*, 1983).

Table 9: Samples positive for ochratoxin but negative for aspergillus ochraceus strains.

Serial number of samples	Type of sample	ochratoxin (ppb)	Isolated fungi
1	Poultry ration	2	Fusarium
2	ditto	2	Fusarium+penicillium
3	ditto	2	Fusarium+penicillium
4	ditto	5.4	A.niger+mucor
5	ditto	5.6	-ve
6	ditto	6	Mucor+penicillium
7	ditto	7	A.niger+penicillium
8	ditto	10	-ve

Permissible limit = 5ppb (Schuller *et al.*, 1983).

Table 10: Toxigenicity of aspergillus flavus isolates.

Isolate serial no.	Source	Amount of afs (ppb)
1	Poultry ration	19
2	Ditto	14
3	Ditto	59
4	Ditto	140
5	Ditto	38

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