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Ochratoxin-A and Mold in Some Broiler Farms of Ismailia, Egypt and Evaluation of Common Mycotoxin Binders

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Abstract: Ochratoxin A (OTA) is a serious problem affecting the poultry industry as well as a health hazard for humans consuming contaminated poultry meat. The aim of this study was to determine OTA levels in the feed used in some poultry farms in Ismailia, Egypt and in organs of birds from those farms. Commercial ELISA kits were used to detect levels of OTA in poultry feed samples and main feed ingredients as well as its residues in bird's tissues (Kidney, liver and muscles). Poultry feed samples were also tested for mycological contaminations. The most common commercial toxin binders were also evaluated *in vitro*. OTA concentration in poultry feed and ingredients ranged from 7.10-20.72 µg/kg. Soybean meal samples showed the lowest concentration, while rations formulated within the farm had the highest mean concentration. Producers using different toxin binders had a significant lower OTA concentration in their rations ($p<0.05$) as compared to those not using binders. OTA residues in different tissues were high in kidney and liver as compared to muscles. *Asprigillus flavus* was isolated from 80% of the examined feed samples. Different binders showed different abilities to bind OTA *in vitro* with highest binding capacity for yeast as compared to other commercially available binders.

Key words: OTA, ochratoxin A, mycotoxin, ELISA, poultry feed

INTRODUCTION

Poultry industry in Egypt depends mainly on imported feed ingredients, which are prone to fungal contamination either during production stages or during transportation from their production sites to port of exportation or through the long journey from their country of origin as they get exposed to variations in environmental temperatures and humidity. The last stage at which they could get contaminated is the period of storage in the Egyptian market and within poultry farms. For these reasons, there is a considerable opportunity for contamination of feed ingredients by mold and their mycotoxin metabolites which may take place throughout the food system which in turn may lead to economic losses (Bhat, 1988). Commercially formulated poultry ration reaches poultry producers in grinded or pelleted forms that make it very difficult to distinguish any mold growth.

Ochratoxin A (OTA) is a secondary toxic metabolite produced mainly by some species of *Aspergillus* and *Penicillium*. These species can grow in different climates. *Aspergilli are* found in tropical regions, whereas *Penicillia* are common in temperate regions and can grow when the temperature is as low as 5°C (WHO, 2002). *A. flavus* and *A. fumigatus* are responsible for many clinical diseases in humans. Particularly common clinical syndromes associated with *A. flavus* include chronic granulomatous sinusitis, keratitis, cutaneous aspergillosis, wound infections and osteomyelitis following trauma and inoculation (Hedayati *et al*., 2007). The presence of Aspergillus in the air is a major risk factor for both invasive and allergic aspergillosis (Denning, 1998).

In general, OTA formation occurs mainly after harvesting on insufficiently dried cereal and cereal products. Factors influencing OTA production include environmental conditions, such as temperature and humidity, but also the type and integrity of the seeds. While *A. ochraceus* grows better in oilseeds (peanuts and soybeans) than in grain crops such as wheat and corn, *P. verrucosum* may grow better in wheat and corn (Madhyastha *et al*., 1990). Fungi growth on different kinds of feed ingredients increases the final OTA concentrations in animal feed.

OTA-contaminated feed has a major economic impact on poultry industry. In general, exposure to OTA contaminated feed reduces animal growth rates and affects animal health. Signs of poultry ochratoxicosis are reduction in weight gain, poor feed conversion, reduced egg production, poor egg shell quality and nephrotoxicity,

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which may lead to confusion in diagnosis with other infectious diseases (nephropathic infection by infectious bronchitis virus) and lead to economic losses of mis-treatments. The effects depend on the level of the toxin and time of exposure. However, numerous studies showed that even exposure to low levels of OTA (0.5 mg/kg feed) adversely affected performance including decreased feed consumption and growth rate and poor feed conversion efficiency (Prior *et al*., 1980; Wang *et al*., 2009). Weight loss, diarrhea, excessive urine excretion and renal lesions have been noted in chickens fed a diet contaminated with 2 mg OTA/kg (Dwivedi and Burns, 1984). Exposure to OTA-contaminated feed was found to impair chick immune function even at concentrations as low as 0.25 mg/kg of OTA (Wang *et al*., 2009).

Following oral consumption of OTA-contaminated feed, rapid absorption into the blood via the gastrointestinal tract is generally followed by relatively slow elimination in urine and feces (Galtier, 1991; Mantle, 2008). Based on its polar composition, OTA is widely distributed throughout the body fluids, in organs and tissues of rats, pigs, rabbits and chickens (Zepnik *et al*., 2003; Ferrufino-Guardia *et al*., 2000). Toxin binders may play an important role in reducing OTA hazards through adsorption and limiting its bioavailability in the body. The main objective of this study was to analyze concentrations of OTA in feed samples and chicken organs collected from some broiler farms in Ismailia and evaluation of binding ability of most commonly applied toxin binders in the market.

MATERIALS AND METHODS

The present study was conducted during the period from September 2011 to February 2012, where the prevailing environmental temperatures and humidity were around 20°C and 60%, respectively. Fifteen broiler farms were included in this study. Feed samples were collected from 20 feeders/farm, samples weighed around 20-25 gms/feeder, in sterile containers and transported to the laboratory. Five farms used a the mash type ration which was formulated within the farm, composed mainly of maize (60-70%), soybean meal (30-20%) and meat concentrate or corn gluten meal (10%). Some of the mash type rations were supplemented by antifungal and detoxifying premix agent containing organic acids and silicate salts (1 kg/ton). Ten farms used pelleted rations from different feed manufacturing companies that has maize, soybean meal 48% protein, corn gluten 60%, plant oil (sunflower and soya oil), with minerals, salinomycin 1.2% and amino acid supplements.

Tissue samples: Tissue samples (muscles, liver and kidney) were collected from each farm at marketing age, (38±2 days). Tissue samples were collected from a total 30 chickens in each farm. Tissues were pooled and then homogenized for OTA extraction and determination.

OTA extraction

Feed ingredients and rations: Feed ingredients and different rations were individually grinded finely and 20 grams from each sample were put in 250 ml capacity conical flask. 100 ml of 70% methanol were added to each sample and then shaken for 2-3 min and filtered. OTA concentration was measured in the filtrate.

Extraction of OTA from bird's tissues: OTA extraction from different tissues was performed according to Aoudia *et al*. (2008). Briefly thawed kidney, liver or muscle samples were pooled and homogenized. 5 g were transferred into a 100 ml round-bottom plastic tube and triturated for 3 min after the addition of 10 ml H_3PO_4 (0.5 M) and 50 ml chloroform. The chloroform phase was then isolated by centrifugation for 20 min at 830 g, and the remaining phase was extracted a second time with 50 ml chloroform. The chloroform extracts were evaporated at (35±5°C). The residue was dissolved in 50 ml of 0.5M sodium bicarbonate.

Analysis of feed and tissue samples for OTA concentration: Ochratoxin A concentrations in feed stuffs and tissue samples were determined by using ELISA kits (Helica Biosystems, Inc., Fullerton, CA, USA) as per manufacturer instructions.

Mycological examination: 10% (w/v) suspension of feed samples in sterile saline was manually shaken in 250 ml flasks for 10 min. Ten fold serial dilutions were prepared in sterile saline and then cultured on Malt Extract Agar (LAB M) and Sabouraud Dextrose Agar (BD Company) with chloramphenicol, then incubated at 25°C for 5-10 days and was considered negative after 14 days. For identification and morphological observations, fungi were cultured on Czapek-Dox agar (CDA) as per manufacturer instructions. Aspergilli were identified based on standard taxonomic systems (Christensen, 1982; Raper and Fennell, 1965; Klich and Pitt, 1988).

Evaluation of common toxin binders: Available toxin binders in the market were evaluated for their binding ability according to a modified method of Manafi *et al*. (2009). Briefly, a finely grinded feed sample (3 kg) was left in humid environment for 15 days, then thoroughly mixed and grinded again before distribution into samples of 25 gram each in 250-ml conical flask. 100 ml of citric acid-sodium dihydrogen phosphate buffer were added to each sample to get a pH 6.5. Different binders were added (0.5 ml or gm) to these flasks whereas the feed in control flasks of the respective treatment was left untreated. Flasks were incubated at 37°C for 3 h. From each flask, the content was filtered and dried at 37°C for 2 h, while the respective toxin was extracted from the content and further processed. ELISA procedures were carried out and the percentage

difference in the toxin content between treatments and control were calculated as percentages of differences in absorbencies.

Statistical analysis: Results are expressed as means ± SEM for each group. Groups were tested for differences by performing the ANOVA and Fisher's least protected significance test using Stat View 4.53 software (Abacus Concepts Inc., Berkeley, CA). Differences were considered statistically significant at $p<0.05$.

RESULTS

Analysis for OTA concentration in different types of rations and feed ingredients revealed that rations formulated by poultry producers within the farm had the highest significant concentration as compared to the main ingredients. The lowest mean was found in soybean samples. There was no significant difference between different types of rations, though there was a trend toward higher concentration in mash type ration as compared to pelleted form (Table 1).

It was clear from Table 2 that poultry producers adding mycotoxin binders in the mash type rations led to significant decreased concentration as compared to those in the market or not supplemented with binders. The maximum concentration in feed supplemented with different binders was lower than the lowest concentration found in those without binders.

It was clear from Table 3 that there were significant differences between OTA concentrations in different rations, mash type ration showing the highest significant concentration as compared to all other types of pelleted form rations. Different companies producing pelleted form rations showed significant differences between them in OTA concentration reflecting either different batches of ingredients or different concentrations in the same ingredients. Within tissues, it was found that both liver and kidney had almost the similar concentrations but higher as compared to muscle samples. There were no significant differences in levels found in liver samples from different farms using different kinds of feed; however kidney levels in different farms revealed statistically significant differences regardless the concentration found in feed. Muscle samples showed significant differences between different farms in accordance with the OTA concentration in the feed.

Mycological evaluation of rations revealed that the isolated fungi were *A. flavus*, *A. fumigatus*, *A. niger* and *A. terreus*, with percentages of 80, 66.6, 73.3 and 40% of the examined samples, respectively. Results showed that the most isolated fungus as *A. flavus*, which is able to produce OTA under favorable conditions.

The results of binding activity of different binders in naturally contaminated feed revealed the highest percentage of binding (28%) was observed in yeast treated samples, followed by 23% for ochrin that had active ingredients of extracts of lactobacillus culture, mannanoligosaccharide, betaine and Treade (Molasses). Nearly similar binding percentages were observed for Synertox (organic acids, propylene glycol, calcium lactate, dried bacillus subtilin fermentation extract, sodium citrate, potassium citrate, papain and sodium potassium tartarated), D-Tox (organic acids, mono and diglycerides of fatty acids, ammonium formate and copper beta sulfate) and garlic treated samples.

Table 1: Ochratoxin A (µg/kg) in some feed ingredients and poultry rations

	Formulated ration		Feed ingredients	
Concentration	Pelleted	Mash	Corn	Soybean meal
Minimum	9.55	10.30	9.40	7.10
Maximum	17.73	20.72	12.70	10.00
Mean	13.22±1.34^{ab}	15.48±1.75^{a}	10.60±0.60^{bc}	8.16±0.59^{c}

Means with different superscripts are statistically significant at $p<0.05$

Table 2: Ochratoxin-A concentrations (µg/kg) in mash type poultry rations with or without mycotoxin binder

Concentration	With mycotoxin binders	Without mycotoxin binders
Minimum	10.30	14.7
Maximum	13.93	20.72
Mean	11.78±0.78^{b}	17.70±1.20^{a}

Means with different superscripts are statistically significant at $p<0.05$

Table 3: Ochratoxin-A (µg/kg) in different types of poultry rations and in the tissues of chickens consuming those respective rations (mean ± SE)

Poultry ration type	Ration	Liver	Kidney	Muscle
A	21.43±0.56^{a}	57.49±2.47	53.87±5.87^{b}	34.57±2.37^{ab}
B	9.12±0.45^{c}	63.77±4.66	60.83±1.53^{ab}	30.62±2.22^{b}
C	14.57±0.58^{b}	62.02±2.02	68.32±3.23^{a}	36.32±1.34^{a}
D	6.50±0.55^{d}	57.94±2.47	62.00±5.87^{ab}	21.60±2.37^{c}

A = mash type; B, C and D = different companies producing pelleted form ration. Means within column with different superscripts were statistically significant at $p<0.05$

Table 4: Number of feed samples positive for *Asprigillus* mold

Isolated mold	Number of positive samples	% of positive samples
flavus	12/15	80.0
fumigatus	10/15	66.6
niger	11/15	73.3%
terreus	6/15	40.0%

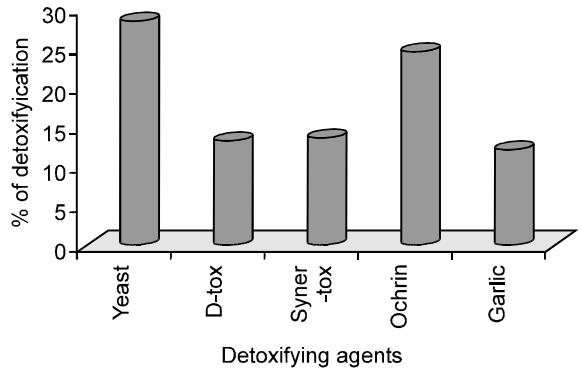

Fig. 1: Percent of ochratoxin-A bound by different common binders

DISCUSSION

The widespread contamination of food and animal feeds by fungi and their metabolite OTA may present a serious hazard to human and animal health. Production of ochratoxins is influenced by various factors including medium composition, temperature and water activity. Levels observed in different poultry feed and feed ingredients in this study were comparable to levels reported from many parts of the world, whereas in Venezuela, 98% of poultry feed samples were contaminated with OTA in range from 2.56 to 31.98 µg/kg feed (Figueroa *et al*., 2009). Schiavone *et al*. (2008) found in 10 poultry farms that all collected feed samples were contaminated by OTA with values ranging from 0.04 to 6.50 µg/kg. Levels recorded in our study are almost identical to those recorded by Agouz and Anwer (2011) in their OTA detection in fish feed in Sharkia, Egypt (15 µg/kg) and less than ranges recorded by Azza Zohair and Salim (2006), who found levels of OTA in cereals ranging from 18-421 µg/kg in samples collected from Egyptian countryside. Higher OTA concentration in formulated rations in farms (mash type) as compared to company manufactured rations may reflect the differences in hygienic measures. The lowest concentration detected in soybean samples might be due to the fact that soybean may be a poor substrate for growth of ochratoxigenic fungi and production of OTA (Blesa *et al*., 2004). Ochratoxin in poultry feed has many deleterious effects; it affects performance even at low level of 0.5-1.5 ppm (Nedeljkovi *et al*., 2004). Verma *et al*. (2004) found that cell mediated immunity was significantly reduced in the chicks fed Aflatoxin B1 (AFB1) at the dose of 2 mg/kg feed and OTA at the dose of 4 mg/kg feed in combination. Antibody titer against SRBCs was also significantly lower in the group fed higher doses of OTA alone or in combination with AFB1.

Some of the greatest difficulties in detecting mycotoxins in animal feed include their heterogeneous distribution in the raw and finished products and the need to be able to detect low levels of these contaminants. In contrast, their direct detection and quantification in the birds themselves (in the plasma, liver, kidneys and muscles) confirms the existence of the problem and minimizes the errors involved in feed sampling and analysis (Furlan *et al*., 2001). Blood and tissue levels of toxins are influenced by various factors including the form of the toxin ingested, health status of the animals, age and sex of the animal. In chickens, the tissue distribution follows the order of kidney > liver > muscle > fat (Harwig *et al*., 1983). Schwerdt *et al*. (1999) reported the potential of OTA to bind in a highly specific way onto different organelle and cytosolic proteins. Among them the most important one was 62 kDa organelle proteins present in the cells originating from the proximal tubules of the kidneys. Although this protein is low in abundance, due to this specific binding, it seems that kidney is the primary target organ affected by OTA. Our results are in agreement with previous observation as it showed almost the same trend with higher levels in kidney followed by liver and then muscles. OTA (5 ppm) in chicks was found to provoke strong degenerative changes in liver and kidneys, degenerative changes and depletion of cells in lymphoid organs, edematous and degenerative changes in the brain, muscular haemorrhages and fatty changes in the bone marrow. The target organs for carcinogenic effect of OTA in chicks were found to be kidneys and liver (Stoev, 2010).

Mycological examination revealed that the main ochratoxin producing molds were not detected in the examined samples. This may be because OTA producing penicillium spps grow in cool and temperate regions as *Penicillium verrucosum* (Castella *et al*., 2002; Pitt, 1987) or *P. nordicum* (Larsen *et al*., 2002), however, OTA niger strains isolated from examined samples produce a series of secondary metabolites of which only OTA can be regarded as mycotoxin. Only 3-10% of the strains examined for OTA production have tested positive under favorable conditions (Schuster *et al*., 2002). Also, *A. niger* var. *niger* is thought to be the OTA producing fungi in the mixed feeds, oilseeds and cereals in the warm zones (Accensi *et al*., 2004).

OTA binding activity of different commercial and natural binding products revealed the order of binding activity to be yeast> Ochrin>D-tox, Synertox> garlic> plant extract. Most of these products are shown to have different ingredients in their composition like yeast, lactobacillus and the chemical adsorbents. Piotrowska and Zakowska (2005) reported the adsorption of OTA onto some *Lactobacillus* and *Lactococcus* strains of bacteria.

All strains caused the reduction of OTA amount. The highest decrease, exceeding 50% of the original amount, was caused by *L. acidophilus* CH-5, *L. rhamnosus* GG, *L. plantarum* BS, *L. brevis* and *L. sanfranciscensis*. According to Turbic *et al.* (2002), viable and nonviable cells of *L. rhamnosus* reduced OTA amount. Also, *Saccharomyces* strains were able to adsorb OTA. The percentages of OTA removal were between 11% and 45% in yeast peptone medium, depending on the strain used. Ringot *et al.* (2007) reported the adsorption of OTA onto yeast byproducts: a vinasse containing yeast cell walls, a purified yeast beta glucan and a yeast cell wall fraction. The cell wall fraction was able to bind 95-100% of OTA. The adsorption of OTA onto yeast cells and yeast cell walls was also reported by Huwing *et al.* (2001) and Nunez *et al.* (2008). According to Ringot *et al.* (2007), yeast biomass may be regarded as a good source of adsorbent material, due to the presence in the cell wall some specific macromolecules, such as mannoproteins and beta glucans. Though, garlic enjoys a worldwide reputation as an antifungal folk remedy as aqueous garlic extract and concentrated garlic oil showed similar or better inhibitory effects than pharmaceutical preparations and demonstrated similar minimum inhibitory concentrations against *Aspergillus* (Pai and Platt, 1995). Also, allicin demonstrated fungicidal activity against numerous yeast and fungi (Ghannoum, 1988) and the growth of both *Aspergillus niger* and *Candida albicans* was inhibited by ajoene (garlic derivative) at concentrations less than 20 μg/mL (Yoshida *et al.*, 1987), the detoxification effect was the lowest as compared to other adsorbing agents used in this study. In general, differences in the binding ability of different binders could be attributed to their compositions and percentages of each compound with superiority of yeast as a binder.

Conclusion: OTA levels observed in feed samples from the surveyed poultry farms were clearly at levels that represent a health hazard for both chickens consuming such feed and humans consuming such chickens. Accumulation of toxin in different tissues represents a high health hazard effect of tissues in the order of kidney, liver and then muscles. Some practices applied by poultry producers such as adding of toxin binders to the rations appear to have some beneficial effects. Yeast products as toxin binders showed the best binding efficiency as compared to other commercially available binders.

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