

BIOCHEMICAL AND HISTOPATHOLOGICAL ANALYSIS OF AFLATOXICOSIS IN GROWING HENS FED WITH COMMERCIAL POULTRY FEED.

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ABSTRACT

The poultry industry probably suffers greater economic loss than any of the livestock industries because of the greater susceptibility of their species to aflatoxin than any other species. Aflatoxicosis of animals is usually manifested by pathologic changes in liver but they have been found to be a carcinogenic and teratogenic as well as causing impaired protein formation, coagulation, weight gains and immunity. In this study, the effect of aflatoxin in hens' growth was biochemically and histopathologically analyzed. The mycotoxigenic fungus was isolated and characterized as *Aspergillus flavus* and *Aspergillus niger*. The aflatoxin was extracted from *A.flavus* and their impacts on growth patterns of hens were evaluated. The serum proteins, cholesterol, liver enzyme, were significantly altered in comparison with control hens group. The histopathological analysis reveals that lesions were found in vital organs of hens in comparison with control chick groups.

Keywords: Aflatoxin, Poultry feed, Fungal toxins, *Aspergillus* spp.

INTRODUCTION

Mycotoxins are chemical diversifies, low molecular weight compounds produced by secondary metabolism of fungal genera such as *Aspergillus* spp, *Penicillium* spp, and *Claviceps* spp. over a variety of food stuffs. These mycotoxins exhibit a wide array of biological effects and individual mycotoxins can be mutagenic, carcinogenic, embryotoxic, nephrotoxic, estrogenic and immunosuppressive. Mycotoxin contamination of various feeds continuous to be a serious quality and safety problem worldwide. Considerable global attention is being focused on mycotoxin contamination of feed because of its adverse effects on animal health¹.

The occurrence of toxin production by strains isolated from foods and animal feed does not necessarily imply the presence of mycotoxins. However, it indicates potential risk for a possible contamination with mycotoxins². Furthermore, if these feeds represent a good substratum for mycotoxin production and if the antibiotic factors especially moisture and temperature are appropriate, the contaminant hazard tends to increase³.

Aflatoxin affects all poultry species although generally takes relatively high levels to cause mortality, low levels can be detrimental if continually fed. Young poultry especially ducks and turkeys are very susceptible. As a general rule, growing poultry should not receive more than 20 parts per billion (ppb) aflatoxin in the diet⁴.

Laying hens generally can tolerate higher levels than young birds, but level should be less than 550 ppb. Aflatoxin contamination can reduce the birds' ability to withstand stress by inhibiting the immune system. This malfunction can reduce egg size and possibly lower egg production since the effects of mycotoxins on poultry are dependent on the age, physiological state, and nutritional status of the animal at the time of exposure⁵.

Aflatoxins are secondary metabolites of certain strains of *Aspergillus flavus* and *A.parasiticus*. Aflatoxin ingestion by chicken result in many different symptoms such as reduced growth, increased susceptibility to infectious agents. The growth of chicks was not affected by concentration of aflatoxin below 200mg/kg.⁶

Many mycotoxins were produced by different fungus. They are aflatoxin of *A.flavus*. Yellow rice toxin of *Penicillium* spp, Zearalenon and Zearalonal of *Fusarium* spp, Ochratoxin and Citrinin of *A.ochraceous* and *P.viridicatum* etc. Even though several number of other mycotoxin in commercial poultry production began to focus on aflatoxin in 1960, when there was an outbreak of "Turkey X disease", due to aflatoxin present in groundnut meal which was the predominant feed of turkeys.⁵

Mycotoxin affects chicks growth and cause many symptoms and affect the immune system. Gamma globulin in aflatoxin exposed groups was higher than control probably due to inflammatory response. The relative weight of Bursa of fabricus was decreased by aflatoxin.⁶

Quality of poultry feed place the most important role in poultry farming as its share is 70% good quality feed and resistant strain of hens can lead to greater production and more profit for the poultry farmer.⁷ It is suggested that use of hens, resistant to aflatoxicosis would help in minimizing the problem of poor growth rate and poor feed conversion which perhaps are the two most important factors in poultry management.⁸

The literature perusal that there is no detailed report on mycotoxigenic fungi from hens feed from poultry farms in Vellore district, Tamilnadu, India. Keeping these points the present study was carried out to isolate and identify the mycotoxigenic fungi from poultry feeds, to study the effect of aflatoxin fungi on hens' growth feed with commercial poultry feed.

MATERIALS AND METHODS

Sample collection: Poultry feed samples were collected from the feed storage rooms of different poultry farms in Vellore district, Tamilnadu such as Chittoor, Ambur and Gudiyatham during July 2006.

Isolation of Mycotoxigenic fungi: The potato dextrose agar plates were prepared and the collected poultry feed samples were serially diluted in saline. The samples were inoculated into agar plates and incubated at $28\pm 2^\circ\text{C}$ for 3-5 days. After incubation the total number of fungal population per gm of feed were estimated and fungal species were identified.

Characterization of fungi: Suspected colonies were taken and emulsified in lactophenol cotton blue and observed under microscopic examination. Then each colony was inoculated into Potato Dextrose Agar separately to obtain isolated culture. Based on their morphological and cultural characteristics, the fungal isolates were identified.

Aflatoxin analysis:

Mass production of *Aspergillus flavus*: The Potato dextrose broth (PDB) was prepared to culture the fungi for aflatoxin production. The pH was adjusted to 6 and the medium was distributed in 2 liters conical flask and sterilized at 15lbs pressure for 15 mins. The flask was cooled and then inoculated with spore suspension of *A.flavus* and incubated at $28\pm 2^\circ\text{C}$ for 2-3 weeks.

Extraction of Aflatoxin: After incubation, the mycelia were removed from the medium and the liquid was filtered through Whatmann No.1 filter paper. The culture filtrate was concentrated under reduced in an evaporator on a water bath. The concentrated culture filtrate was shaken repeatedly with 100ml volumes of chloroform and the extraction was repeated 2 or 3 times. The chloroform extracts were combined and filtered through Whatmann No.1 filter paper. From the filtered chloroform extract, the toxin was extracted using sodium bicarbonate solution by shaking the chloroform extract several times with 0.5 molar sodium bicarbonate solution. All the lipid materials were removed by filtration after keeping the sodium bicarbonate extract over night in a separating funnel. Finally the pH of the solution was brought down to 2 and the toxin was extracted from the concentrate into chloroform by repeated extraction with aliquots of chloroform. The extracts was pooled and concentrated, thus the crude toxin was isolated.

Detection of aflatoxin by thin layer chromatography (TLC): Silica gel was coated on TLC plates and dried at 60°C for 1 hour. 1 ml of the concentrate of the chloroform extract was spotted in the form of a thin line on the chromatographic plates and developed with chloroform ethyl acetate formic acid toluene (50:40:10:2V/V) solvent system in a closed chamber. After drying the plate, portion of the plate was sprayed with 1% para-dimethyl amino-benzaldehyde in n-butanol dried with warm air and placed in a tank containing hydrochloric acid vapors for 15 minutes, a bright blue colour reaction to find out the presence of aflatoxin B1. The mobility of extracted aflatoxin B1 and authentic aflatoxin was compared.

Effect of aflatoxin on hens:

Preparation of aflatoxin mixed diet: Commercial poultry feed were obtained from poultry farms in Tamilnadu. They were powdered and mixed with 100ppm concentration of aflatoxin. Aflatoxin mixed feed was again palletized and dried at 37°C for 5 days so as to evaporate the chloroform.

Evaluation of aflatoxin on Hens growth: The healthy unvaccinated hens were obtained, they were separated as two groups; group A (Control) and group B (Test). Each group consists of 25 hens. Each group of hens was labeled for identification. The control hens were fed with normal diet feed obtained from a commercial poultry farm. The test hens were fed with aflatoxin mixed diet. The hens were analyzed on 25th day.

Hematological, histopathological and biochemical analysis: The study was approved by the institute ethical committee. From the test and control hens, the hens were taken and sacrificed. The blood samples were collected from control and test hens for hematological and biochemical studies. The lung, muscle, intestine, kidney and liver tissues were taken for histopathological studies.

The serum was separated from test and control hens plasma by centrifugation, and the following biochemical analyses were performed by the standard methods: urea test (DAM method), creatinine test (Alkaline Picrate method), total protein test (Bi-uret method), albumin test (BCG method), SGOT test (Raitman and Frankels method), SGPT test (Raitman and Frankels method), Alkaline phosphatase test (modified Kind and King's method), triglycerides test (GPO/PAP method).

Histopathological analysis: The lung, muscle, intestine, kidney and liver tissues were taken for histopathological studies. After sacrifice, each animal was necrotized and organ lesions were described, with special attention focused on gizzards. Samples of lung, liver, intestinal tissue and kidney tissue were taken. They were fixed in 10% buffered formalin, embedded in paraffin and cut on a microtome in slices 4-5mm thick and stained with hematoxylin-eosin staining

RESULTS AND DISCUSSION

In the present study, totally three samples were collected from poultry farm in Vellore district, Tamilnadu. From the feed, the fungal populations were studied. Totally, 40 fungal isolates were observed in all three samples. The maximum fungal population of sample I was 15 colony forming units (cfu) from Chittoor, sample II was 0 cfu from Ambur and sample III was 25cfu from Gudiyattam. Among the total 40 fungal isolates, the dominant two fungal isolates were selected for characterization. The isolate I showed velvety, yellow to green or brown colonies, their conidiophores were of variable length, rough pitted and spiny characteristics sporting head, conidia were globous and echinulate. The hyphae were hyaline, septate, branched dichotomously; the sterigmata only cover half of the conidia and were single.

The characteristic of isolate II recorded that, initially white to yellow, then turning dark brown to black colonies and their conidiophores is of variable length, sterigmata were

double, cover entire vesicle from radiate head. Hyaline, septate hyphae were present. Conidial head was large in size with black to brownish black in colour. The sterigmata are double, the primary sterigmata are long, and the secondary sterigmata were short.

Based on cultural and morphological features, the fungal isolates were identified as *Aspergillus niger* and *Aspergillus flavus*. The *A. flavus* was cultured in a production medium. The mass cultures were extracted for aflatoxin. The extracted material was separated by thin layer chromatography, and confirmed as the isolate I are producers of aflatoxin.

Aflatoxins are natural contaminants of feed stuff. Hens are most sensitive to these toxins. Although chicks are claimed to be the most sensitive poultry animals. Sensitivity tests carried on quails revealed that these animals may be easily affected by aflatoxins present in feed. The severity of poisoning aflatoxins depends upon the age, sex, species of the animal, the amount of being exposed and the duration of exposure. The vitamins, minerals and antibiotics present in feed are among the factors that change the severity of poisoning. In addition, the amount of the protein in the feed composition is closely related to poisoning. The present study reveals

about the biochemical and histopathological analysis of hens fed with aflatoxins in commercial poultry feed.

The aflatoxin producing fungi was isolated from three different poultry feed samples. Among them, sample III recorded maximum fungal population (25cfu/g), and absence of fungal population was observed in sample II. This result reveals that storage condition of poultry feed under hygienic condition of feed sample III and I respectively.

Totally 40 mycotoxigenic fungus were observed in all three different poultry feed samples. Among the 40 isolates, 2 dominant fungal isolates were selected, characterized and identified as *A.flavus* and *A.niger*. This finding is similar to the previous reports.⁹

Biochemical analysis: The in vivo effects of aflatoxin fed hens were biochemically and hematologically analyzed. The biochemical characteristics of test and control chicks are shown in table 1.

Hematological analysis: In hematological analysis, the hemoglobin level of test hens (6%) were decreased when compared to control hens (7.2%). The test hens showed the increased lymphocyte count 42, when compared to the total count of control hens as 31, shown in Table 2.

Table 1: Biochemical characteristics of serum from test and control hens.

S. No	Name of the test	Test Hens			Mean value	Control Hens			Mean value
		T1	T2	T3		C1	C2	C3	
1	Urea (mg/dl)	12	16	13	13.6	14	10	12	12
2	Creatinine (mg/dl)	0.8	1.6	0.8	1.06	0.2	0.2	0.2	0.2
3	Total protein (gm/dl)	5.3	2.8	5.8	4.63	0.2	0.2	0.2	0.2
4	Albumin (gm/dl)	1.1	1.01	1.1	1.06	0.1	0.1	0.1	0.1
5	SGOT (U/L)	326	311	300	321.3	200	201	201	201
6	SGPT (U/L)	13	74	20	35.6	5.3	5	5	5
7	Alkaline phosphatase (U/L)	19	32	16	22.3	1	1	1	1
8	Cholestrol (mg/dl)	271	154	202	209	124	110	112	115.3
9	Triglyceride (mg/dl)	158	155	151	155	34	40	38	37.3
10	High density lipoprotein (mg/dl)	96	62	88	82	72	60	70	67.3
11	Low density lipoprotein (mg/dl)	31	1	25	24	15	10	14	13

Table 2: Hematological analysis of hens

S. No	Name of the test	Control Hens			Test Hens		
		C1	C2	C3	T1	T2	T3
1	Haemoglobin level (%)	7.2	7.1	7.2	6	6.8	7
2	TC count (cu.mm)	3000	3500	3500	6000	7000	7500
3	DC count:						
	Neutrophil	65	159	62	47	42	48
	Basophil	3	2	3	2	1	0
	Eosinophil	7	6	4	9	11	30
	Lymphocytes	25	37	31	42	46	39

Histopathological analysis: No hence perished during the experiment. Gross lesions, except for the gizzards, were minimal and mainly involved mild degenerative changes and congestion of the liver and kidneys. More prominent lesion was noted in gizzard of aflatoxin treated animals. (Discolouration, erosion, and ulceration) compared to control groups.

In liver degenerative reversible lesion were present, from mildest of severest degree with various distributions in test groups mild parenchymatous degeneration characterized by granular appearance of hepatocytes cytoplasm was observed, severe hydropic and vacuolar degeneration. The vast majority of hepatocytes had significant cytoplasmic visualization with disseminated necrotic cells were observed in the experimental groups.

In kidney, moderate parenchymatous tubular degeneration, predominantly of the distal tubules, manifested by epithelial swelling and fine granular appearance of cytoplasm was most prominent in aflatoxin treated hens. In the experimental animals, hydropic and vacuolar degeneration was also noted, but the severe degree characterized by desquamation of epithelial tubular cells was present in almost all cells when compared to control groups.

In lungs, congestion and mild prevascular edema was noted in all animals except for the control groups. However, thickening and hyalinization of the blood vessel walls, except for the control groups. Peribronchial and perivascular lymphocytic infiltration was noted in the individual animals in each group.

In intestine, the only untreated animals (control group) there were no changes at all, but the mildest form of inflammation, catarrhal inflammation was observed. An important finding was vacuolization of mesenchymal cells of the lamina propria, which was found exclusively in test animals.

Histopathological analysis reveals that lesions were observed in tissues of liver, kidney, lungs, and intestine. This result indicates the significant damage of vital organs in hens. These findings are coinciding with the previous findings. The biochemical and histopathological analysis of hens showed that decrease of hen's weight, liver, enzyme alteration and histological changes of important organs in hens.

CONCLUSION

The present study concludes that the importance of toxin production by strains isolated from animal feed does not necessarily imply the presence of aflatoxin. However, it indicates a potential risk for a possible contamination with

aflatoxins. Furthermore, if these feed represent a good substratum for aflatoxins production and if the abiotic factors (especially moisture and temperature) are appropriate, the contamination hazards tends to increase.

Quality of poultry food plays the most important role in the poultry farming as its share is 70%. Good quality food and resistant strain of chicks can lead to larger production and more profit for the poultry farmer. Poultry industry in Tamilnadu has expanded tremendously during the last few years. However, the acute shortage of chicken meat has pushed its prices steeply up. It is suggested that use of chicks, resistant to aflatoxicosis, would help in minimizing problem of poor growth rate and poor feed conversion which perhaps are the two most important factors in poultry management.

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