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Short Communication

ANALYSIS OF AFLATOXIN IN DUCK AND POULTRY FEED IN WEST BENGAL

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ABSTRACT: Aflatoxin is a major metabolite of *Aspergillus* species which is liberated in feeds and feed stuff. The B_1 component of aflatoxin is more mutagenic, hepatogenic and nephrotoxic to poultry. A total number of eighteen samples from different parts of West Bengal have been analyzed by Lateral Flow Immuno-Chromatographic Assay (LFICA) and followed by detection in HPLC in 2475 multi fluorescence detector system. The samples were tested for presence of total aflatoxin by LFICA which detected seven samples positive with a cut off level ≥ 4 ppb (55.56%), ten sample between 1-4 ppb (38.89%) and one sample negative (5.55%). The positive samples were further confirmed by HPLC system using C_{18} column (5 µm and 4.6x 150 mm in length), methanol: water as mobile phase (45:55) and standard for B_1 prepared from 2 ppb- 1000 ppb. All seven samples showed presence of B_1 in permissible limit ranging from 6.25 ppb to 12.50 ppb. Therefore, it was concluded that the presence of aflatoxin in the tested feeds are within permissible limit and regular detection and monitoring of aflatoxin in feed samples of all the farms may be an important control measure of aflatoxicosis in the poultry farms.

Key words: Aflatoxin, Poultry feeds, LFICA, HPLC system.

Mycotoxins are group of toxic compounds detected in 1960s (Asao *et al.*1965) found in grains contaminated with *Aspergillus flavus* and *Aspergillus parasiticus*. Grain contaminated with aflatoxins recognized as a threat to human and animals through consumption of contaminated feeds (Kim *et al.* 2002). Mycotoxins are anti-nutritive factor present in feed which lead to transfer of toxin through meat and egg to human beings. There is 25% of contamination of feed throughout the world (Fink-Gremmels 1999). There are four principle types of aflatoxin *i.e.* B_1, B_2, G_1 and G_2 which are named for their respective fluorescent properties (Bennett and Klich 2003). US Food and Drug Administration (2009) framed levels of aflatoxin within 300 ppb for breeding bulls and matured poultry, 20 ppb in livestock and chicken and 3 ppb in ducklings.

Aflatoxin was first discovered around 1960 as Turkey X disease in Great Britain which was *A. flavus* toxin (Wannop 1961). First outbreak of mycotoxin occurred in poultry farms of Mysore and other parts of Karnataka with a sudden death of 2219 poultry bird (Gopal *et al.* 1969). Subsequent report on drop in egg production from 85% to 40% in Warangal in Andhra Pradesh (Sastry *et al.* 1965) and post mortem examination of dead birds revealed liver lesion with 600 ppb aflatoxin (Choudary 1986). First human outbreak of mycotoxin was reported from Banswada district of Rajasthan and Panchmahal district of Gujrat with symptom of hepatitis and death of 106 people was recorded from consumption of 2000-6000 ppb mycotoxin contaminated feed (Krishnamachari *et al.*1975). First correlation between aflatoxin contamination and hepatomegaly has been report among the children of Canara district of Karnataka (Sreenivasmurthy 1977).

Clinical signs in chicken are in appetence, reduced growth, feather picking, lameness, ataxia and convulsion. Main pathological features are enlarged liver, kidney, hydro pericardium and ascites. The half-life of aflatoxin B_1 in laying hens is about 67 hours (Jacobson and Wiseman 1974), transmission ratio is about 5000:1 (Trucksess *et al.* 1983). B_1 accumulated in reproductive organs and its subsequent transmission to eggs and hatched progeny in poultry (Foster *et al.* 1983). It causes transversion mutation to from G to T in DNA results in tumor formation (Trucksess *et al.* 1983). It causes coagulopathy due to reduced synthesis of vitamin-K (Bababunmi *et al.* 1997), oxidative damage to hepatocytes by lipid peroxidation (Shen *et al.* 1997), inhibits cyclic

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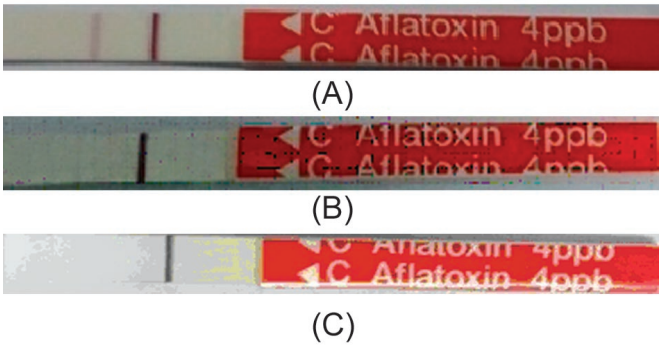

Fig. 1. Lateral Flow Immuno-Chromatographic Assay for total mycotoxin A) for negative sample having two lines, B) for 1-4 ppb range of mycotoxin samples having one bright line and another faint line and C) for positive samples having one bright line only.

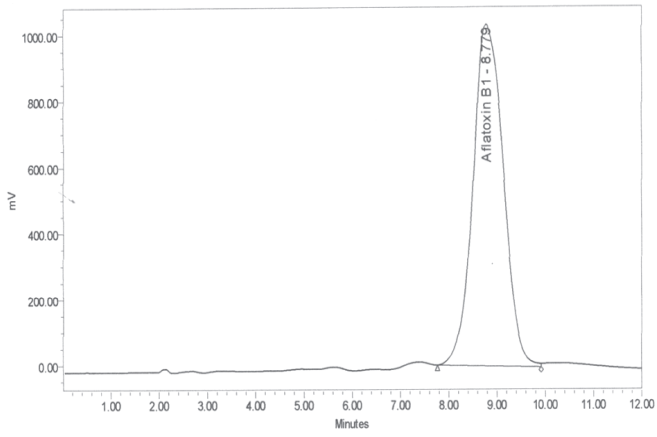

Fig. 2. Aflatoxin B_1 detection by HPLC system.

nucleotide phosphodiesterase activity in brain, liver, heart and kidney tissues (Bonsi *et al.* 1991).

Aflatoxin is analyzed by different methods like Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Enzyme Linked Immunochromatographic Assay (ELISA) and Rapid Test Kit (Betina 1985). Other emerging technologies include Evanescent wave technology, Molecular imprinted polymers and Microarray technology (Michael *et al.* 2005). Immuno-chromatographic assay is based on competitive reaction scheme that detects mycotoxin which binds to anti-mycotoxin antibody gold particle complex in a conjugate pad. This complex migrates together with second anti-antibody gold particle complex along the membrane. The positive sample shows no line in test zone whereas negative sample shows a line in test zone (Xiulan *et al.* 2005). On the other hand, aflatoxin has been detected by HPLC system using solvent mixture acetonitrile: water (60: 40, v/v) with a fluorescence detector from retail ground samples (Baydar *et al.* 2005). Therefore, present study has been envisaged to detect aflatoxin by rapid immuno-chromatographic assay and further validation by HPLC system.

Sample preparation

a) Feed samples from different sources are grinded so that 75% would pass through 20-mesh screen. 10 g of grinded samples were mixed with 20 ml of 70% methanol extraction solution (70/30 (v/v, Methanol/Water). The mixture was vigorously shaken and vortexed for 1 min. Samples were allowed to settle, filter the top layer of the extract through Whatman filter paper-1. The filter was collected subjected to lateral flow immune-diffusion assay.

b) Similarly, 10 g grinded sample were mixed with 40 ml of acetonitrile (HPLC-grade, Rankem) and homogenized properly. The content was centrifuged at 5000 rpm for 30 min. The content was allowed to precipitate and filtered through C_{18} solid phase extraction system (Sep Pak Cartridges, Waters) and filter was saved. 20 µl of filtrate was injected into HPLC system for analysis.

Lateral Flow Immuno-chromatographic Assay (LFICA)

This was conducted using rapid test kit procured from Romer Lab (Agrastrip Total Aflatoxin Test, 4 ppb). Briefly, 50 µl of assay diluents was charged to each micro well. The coating conjugates in the micro well were dissolved by pipetting the content up and down 5 times. Then 50 µl of test sample was charged on to the each well. One test strip was put in each well and allowed to develop color for 5 min. Appearance of color line in control (C) and test (T) were recorded. Two lines were visible in test line and control line clearly, considered as negative. A sharp line in the control zone and very faint line in test zone indicated weak positive sample with the detection limit from 1-4 ppb. The positive sample showed no line in test zone but a sharp line in control zone.

HPLC analysis

HPLC system, 1525 pump, 2487 fluorescence detector, rp C_{18} column, guard column (Water make) were used for HPLC analysis. HPLC graded methanol, HPLC graded-water and trifluoracetic acid, aflatoxin standard were procured from Sigma. Mobile phase was prepared with HPLC graded methanol: water (45: 55) in 0.5 % trifluoroacetic acid and passed through vacuum filter (0.2 µm, 47 mm diameter) for degassing. Standard was prepared in a final concentration of 1000 ppb in HPLC graded methanol. Then serial dilution of stock was done making concentration up to 2 ppb in methanol. Different

dilution of the standard was prepared and injected into HPLC machine (Waters 1525 Binary HPLC Pumps) for generation of standard curve in Waters 2475 multi-fluorescence detector system. 20 µl of sample was injected by Hamilton syringe and sample was passed through C_{18} column (Symmetry C_{18} 5µm, column length 4.6x150 mm length, Waters).

Result

In this study, total 18 numbers of feed samples were analyzed by LFICA method for presence of total aflatoxin and followed by HPLC methods. Out of this, 07 numbers of samples were positive by LFICA which detected at ≥4 ppb around cut off value. 10 number of samples were detected with 1- 4 ppb. Rest 01 number of feed sample was negative for total aflatoxin (Fig. 1). Then seven positive samples were further analyzed in HPLC system to detect the actual concentration of B_1 fraction as described in materials and methods. The system detected all samples in different concentration of mycotoxin B_1 from 6.25-12.50 ppb Fig. 2. and Table 1. Concentrations of mycotoxin B_1 standard chromatogram is having retention time 8.779 (Fig. 3).

Aflatoxin detection in poultry feed samples is of great importance to ameliorate the negative effects that could be achieved through physical examination of the contaminated feed components used in poultry feeding (Beg *et al.* 2006). The emergent sophisticated and easier techniques for testing residues led to increased safety awareness in food and feed production. The monitoring of mycotoxin requires precise and reliable analytical methods. Different separation and detection methods *i.e.* ELISA, LFICA HPLC and TLC require suitable samples extraction steps (Stroka *et al.* 1999). The immunological

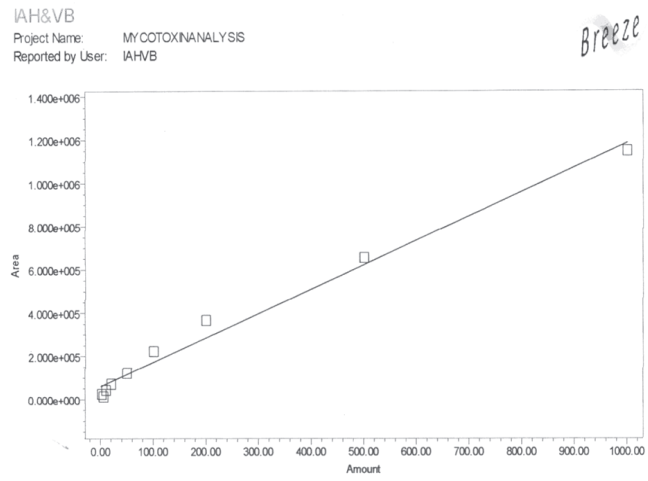

Fig. 3. Standard curve for Aflatoxin B_1 in HPLC system.

Table 1. Results of different feed by LFICA and HPLC system.

Sl. No. of Farm	Types of samples	No. of sample received	Level of aflatoxin by LFICA			Concentration detected in HPLC (ppb)
			Negative	1-4 ppb	≥ 4 ppb	
1.	Duck grower	01	-	-	01	6.25
	Duck layer feed	01	-	-	01	7.50
2.	Layer mash	01	-	-	01	8.25
3.	Layer feed	01	-	01	-	
4.	Layer feed	04	01	02	01	9.25
	Layer mash	01	-	-	01	6.25
5.	Duck mash	01	-	01	-	-
6.	Maize (duck feed)	01	-	01	-	-
	Soya bean (duck)	01	-	01	-	-
7.	Chick & Layer feed	02	-	02	-	
	Grower chick mash	01	-	-	01	8.75
8.	Wheat bran	01	-	01	-	-
	Wheat crush	01	-	01	-	-
9.	Grower chick mash	01	-	-	01	12.50
Total		18	01	10	07	
Percentage			5.55%	55.56%	38.89%	

based methods are widely used due to less time and reduced expenditure required for analysis (Richard *et al.* 1993).

In this study, firstly total aflatoxin was determined by immune-chromatographic assay and followed by detection of B_1 in HPLC assay. LFICA accurately detected total aflatoxin in feed samples which showed 38.89 % positivity for ≥4 ppb, 55.56 % for 1-4 ppb and 5.55% were total negative. Further the positive samples were validated in HPLC system that detected all the samples from 6.25-12.50 ppb (Table 1). This result revealed that low level of aflatoxin concentration in feed within the permissible level. In Iraq, there was 15% positive and 85% negative in residual aflatoxin from broiler pellet feed sample by rapid immune-chromatographic assay (Khalaf *et al*. 2015). In another study it has been found that 71% and 23% feed samples were found positive to aflatoxin by LFICA in North and South America with an average 92 µg/kg and 7 µg/kg respectively (Naehre *et al.* 2012). Presence of aflatoxin from 0.03-3.16 ppb in retailed ground samples has been detected by HPLC method in Turkey (Baydar *et al*. 2005). Presence of residual aflatoxin has been recorded in the highest concentration in liver (2.12 ppb), lowest concentration in the breast muscle (0.63 ppb) from feed contaminated with 965.12 ppb mycotoxin and in eggs (0.66 ppb) fed from 894.12 ppb contaminated feed by HPLC method (Saqer *et al.* 2013).

The present study clearly has indicated the presence of mycotoxin within the permissible limit in the poultry feed samples. Further intensive investigation with more number of samples from more sources is required to know the actual effect of aflatoxin on poultry health and production as well as possible residual amount present in different poultry tissues and eggs used for human consumption.

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