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Microbiology

Aspergillus flavus and Aflatoxin Contamination of Poultry Feeds in Tamil Nadu, India

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Abstract

A survey was conducted during 2013 in different regions of Tamil Nadu, India and a total of 51 poultry feed samples were collected from poultry farms and poultry feed dealers. The presence of *Aspergillus flavus* in the feeds was detected by a polymerase chain reaction (PCR) assay using specific primers based on the O-methyltransferase gene (*omt-A*) that is involved in the aflatoxin B1 biosynthesis. The contamination levels of Aflatoxin (AFB1, AFB2, AFG1 and AFG2) were determined using high-performance liquid chromatography (HPLC). The results indicated that 98% of the tested samples were contaminated with AFB1 and the levels ranged from 0 to 160.7 ppb and the levels of AFB1 in 29% of the samples exceeded 20 ppb. Aflatoxin B2 contamination was observed in more than 82 % of the samples tested and the levels of AFB2 in 17 % of the samples exceeded 20 ppb. The levels of AFB2 ranged from 0 to 95.5 ppb. AFG1 and AFG2 were not detected in any of these samples. In the PCR analysis using *omt-A* primers, *A. flavus* specific 300-bp PCR product was amplified from DNA extracted from most of the AFB1 contaminated feeds. However, some of the feed samples even though contain AFB1 failed to amplify the 300-bp product in PCR.

Highlights

- Aflatoxin B1 and B2 contents in 29% and 17 % of the poultry feed samples analyzed respectively exceeded 20 ppb
- The presence of the fungus *Aspergillus flavus* was detected in the aflatoxin contaminated feed samples

Keywords: Aflatoxin, *Aspergillus flavus*, HPLC analysis, PCR detection, poultry feed

Aflatoxin contamination is considered one of the most serious food and feed safety issues worldwide. These aflatoxins are a group of toxic secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Diener *et al.*, 1987; Kurtzman *et al.*, 1987). Maize and groundnut which are the main components of poultry feeds, are excellent substrates for the growth of *A. flavus* and aflatoxin production (Gourama and Bullerman, 1995) and aflatoxin contamination of poultry feeds can occur as a result of infection of crops by aflatoxigenic fungi. Consumption of aflatoxin contaminated feeds leads to the disease called “aflatoxicosis” in poultry birds. It has been demonstrated that intake of mycotoxin even at low level can result in reduced feed intake, poor growth rate, lower egg production, reduced fertility and hatchability of eggs and immunosuppression in poultry birds (Bryden, 2012).

The incidence of aflatoxicosis in poultry and livestocks due to the consumption of contaminated feeds has been recorded in different parts of India. The outbreak of aflatoxicosis in Murrah buffaloes in Andhra Pradesh, India was reported due to the consumption of aflatoxin contaminated groundnut cakes (Sastry *et al*., 1965). The incidence of aflatoxicosis due to the consumption of aflatoxin B1 contaminated feed has been reported in an experimental pig farm in Meghalaya (Ghosh *et al*., 1988) and in ducklings in Tripura, India (Roy *et al*., 1989). Dersjant-Li *et al.,* (2003) reported that each mg of AFB1/ kg feed would decrease the growth performance of broilers by 5%. Raju and Devegowda (2000) recorded 21% decrease in body weight at 35 days age in broilers fed with 0.3 mg AFB1/kg feed. The levels of AFB1 as low as 0.02 mg/kg feed have been reported to decrease weight gain of broilers by 5% in 3 weeks (Kana *et al*., 2010). The level and length of AFB1 exposure affect the amount of reduction in weight gain of broilers (Yunus *et al*., 2011). Furthermore, epidemiological studies indicate high correlation between outbreaks of Newcastle disease and aflatoxin contamination of broiler rations (Yunus *et al*., 2008).

Several studies reported the presence of residual aflatoxins in the meat of broiler chickens fed with aflatoxin contaminated feed (Trucksess *et al*., 1983; Chen *et al*., 1984; Wolzak *et al*., 1986; Micco *et al*., 1988; Kriukov and Krupin, 1993; Hussain *et al*., 2010). In the case of laying hens, aflatoxins and their metabolites can also be carried over to eggs (Trucksess *et al*., 1983). Oliveira *et al.,* (2000) demonstrated that the feed to eggs aflatoxin transmission ratio was approximately 5000:1. Al-Shawabkeh *et al.,* (2009) reported that the nutritional value of poultry meat in terms of Conjugated Linoleic Acid (CLA) contents was affected by the concentration of AFB1 in the feed. The authors demonstrated that as the concentration of AFB1 in the feed increased, the CLA content decreased in the breast and thigh meat. Since aflatoxin B1 is implicated in the etiology of hepatic cancer, the presence of aflatoxin B1 residues in eggs or meat becomes important as a potential human health hazard. Hence, the levels of aflatoxins in foods and feeds are strictly regulated in many countries (Rustom, 1997). For example, the United States Food and Drug Administration (FDA) has set a guideline of 20 ppb aflatoxin in food or feed as the maximum allowable limit (Van Egmond, 1989). The aim of this study was to investigate the level of aflatoxin (AFB1, AFB2, AFG1 and AFG2) contamination in poultry feed samples that were collected from different parts of Tamil Nadu, India using high performance liquid chromatography (HPLC) and to detect the occurrence of *A. flavus* in the feeds by polymerase chain reaction (PCR) assay.

Materials and Methods

Survey and collection of samples

A survey was conducted during 2013 in different regions of Tamil Nadu, India and a total of 51 poultry feed samples (1-2 kg of weight) were collected from poultry farms and poultry feed dealers in order to understand the magnitude of aflatoxin contamination of poultry feeds.

DNA extraction and PCR amplification

Approximately 100 mg of each sample was ground to a powder in liquid nitrogen and the DNA was extracted by using the CTAB extraction method (Doyle and Doyle, 1987). PCR amplification was performed in 20-µl reactions containing 50 ng of template DNA, 10 µl of 2X Go Taq master mix (Promega Corporation, Madison, WI) and 10 pmol each of omt-F and omt-R primers (Shweta *et al*., 2013). PCR amplification was performed in a Eppendorf ep gradient S Master cycler (Eppendorf, Hamburg, Germany) with an initial step of 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. After the cycling reactions there was a final extension step of 72°C for 10 min. PCR products were resolved in 1.2 % agarose gel electrophoresis at 80 V for 1 h. The gel was stained with ethidium bromide (0.1 mg/l) and viewed under UV light. A 100- bp ladder (Bangalore Genei Pvt Ltd, Bangalore, India) was used as a size standard.

Extraction of aflatoxins

Powdered feed samples (50 g) were vigorously shaken with 250 ml of methanol: water (55:45 v/v), 100 ml of hexane and 2 g of sodium chloride. The extract was filtered and methanolic layer was separated and then extracted with 25 ml of chloroform thrice. The chloroform fractions were pooled and evaporated to dry *in vacuo*. The residue was dissolved in 1 ml of methanol, filtered using 0.2 µm filter (Millipore, USA) and the filtrate was used for HPLC analysis.

High Performance Liquid Chromatography (HPLC) analysis of aflatoxins

HPLC (Agilent technologies 1200 series) equipped with a fluorescence detector was used to separate and detect

aflatoxins. A C18 reverse-phase column (250 mm x 4.6 mm I.D. 5 µm) was used to separate aflatoxins and was maintained at 30 °C in a column chamber. The samples (20 µl) were injected into the system using a manual injector. The mobile phase used was water: methanol: acetonitrile (64:23:13; v/v/v) with a flow rate of 1 ml/min. To 1 l of mobile phase, 119 mg of potassium bromide and 100 µl of nitric acid were added for post-column electrochemical derivatisation using Kobra Cell (R-Biopharm Rhone Ltd, Glasgow, UK). Aflatoxins were detected at an excitation and emission wavelength of 365 and 435 nm, respectively after post-column derivatization.

Results and Discussion

In this study, 51 poultry feed samples were analyzed for AFB1, AFB2, AFG1 and AFG2. As shown in Table 1, aflatoxin B1 contamination was observed in 98% of the poultry feed samples tested and its levels ranged from 0 to 160.7 ppb and the levels of AFB1 in 29% of the samples exceeded 20 ppb. Aflatoxin B2 contamination was observed in more than 82 % of the samples tested and the levels of AFB2 in 17 % of the samples exceeded 20 ppb. The levels of AFB2 ranged from 0 to 95.5 ppb. AFG1 and AFG2 were not detected in any of these samples. A number of studies reported the occurrence of aflatoxins in feedstuffs (Vijayasamundeeswari *et al*., 2009; Dutta and Das, 2001; Njobeh *et al*., 2012; Kana *et al*., 2013). Dutta and Das (2001) reported that AFB1 content in feed samples collected from different parts of Northern India was very high with an average of 0.412–0.514 ppm. Vijayasamundeeswari *et al.,* (2009) reported that AFB1 was detected in 30 out of 53 poultry feed samples collected from Tamil Nadu, India and its levels ranged from 0.7 to 31.6 µg/kg. Njobeh *et al*., (2012) analyzed a total of 92 commercial compound feeds from South Africa for aflatoxins and reported that 30% of collected samples were contaminated with aflatoxins and the levels ranged between 0.2 and 71.8 µg/kg with a mean of 9.0 µg/kg. Kana *et al*., (2013) analyzed a total of 201 samples of maize, peanut meal, broiler and layer feeds collected from three agroecological zones of Cameroon and reported that approximately 9% of maize samples, 100% of peanut meal, 93.3% of broiler feeds and 83.0% of layer feeds were positive for aflatoxin, and the concentrations ranged from <2 to 42 µg/kg for maize, 39 to 950 µg/kg for peanut meal, 2 to 52 µg/kg for broiler feed and 2 to 23 µg/kg for layer feed. The authors concluded that peanut meal was a high risk feed. Multi-mycotoxin studies conducted in Europe reported that 75-100% of the samples contained more than one mycotoxin (Streit *et al*., 2012). Rodrigues and Naehrer (2012) carried out a worldwide mycotoxin survey and a total of 6,058 feedstuffs and finished feed samples used in the livestock production industry worldwide were collected and analyzed for the presence of the mycotoxins by HPLC and reported that 31, 44, 50, 56 and 27% of analyzed samples were positive for aflatoxins, deoxynivalenol, zearalenone, fumonisins and ochratoxin A, respectively.

Table 1: Aflatoxin B1 and B2 content in poultry feeds in Tamil Nadu, India

Sample ID	Place	District	AFB1 (ppb)	AFB2 (ppb)
S1	Karamadai	Coimbatore	20.2	3.5
S2	Pallipalayam	Erode	2.1	0
S3	Athani	Erode	135.9	61.3
S4	Attur	Salem	11.4	10.9
S5	Nelpet	Madurai	14.6	0
S6	Gudimangalam	Udumalpet	70.5	25.1
S7	Perundurai	Erode	99.8	59.1
S8	Nathapalayam	Erode	76.4	43.9
S9	Vellakoil	Erode	37.7	14.8
S10	Sulur	Coimbatore	2.4	0
S11	Ukkadam	Coimbatore	4.9	1.4
S12	Palladam	Tripur	7.4	3.5
S13	Poosaripalayam	Coimbatore	5.7	4.8
S14	Linganur	Coimbatore	14.0	9.1
S15	Gettupatty	Dharmapuri	4.9	3.8
S16	Vadavalli	Coimbatore	21.6	7.1
S17	Veerakeralam	Coimbatore	19.4	8.1
S18	Navavoor pirivu	Coimbatore	52.0	95.5
S19	Dharmapuri	Dharmapuri	27.7	20.6
S20	Karur	Karur	2.1	2.1
S21	Paramathi-Velur	Namakkal	5.6	2.7
S22	Marurpatty	Namakkal	19.7	3.7
S23	Pappinayakkanpatti	Namakkal	13.6	2.3
S24	Paramathi-Velur	Namakkal	15.5	6.4
S25	Peelamedu	Coimbatore	1.2	0
S26	Kallapalayam	Coimbatore	1.5	0.5
S27	Kodangipalayam	Coimbatore	21.8	8.8
S28	Mathampatti	Coimbatore	0.9	0.9
S29	Devarayapuram	Coimbatore	8.1	0
S30	Irugur	Coimbatore	1.5	0
S31	Tiruchengodu	Namakkal	0	0
S32	Rasipalayam	Coimbatore	1.7	0
S33	Cuddalore	Cuddalore	2.6	3.6

Contd.

Sample ID	Place	District	AFB1 (ppb)	AFB2 (ppb)
S34	Ambasamudram	Tirunelveli	3.5	1.1
S35	Mohanur	Namakkal	160.7	90.2
S36	Rasipuram	Namakkal	12.3	3.6
S37	Srirangam	Tiruchirappalli	12.7	6.1
S38	Bhavani	Erode	11.7	3.7
S39	Gobichettipalayam	Erode	41.4	1.7
S40	Palayur	Coimbatore	2.5	0
S41	Sathyamangalam	Erode	41.6	23.9
S42	Seeranayakenpalayam	Coimbatore	4.7	3.9
S43	Thuraiyur	Tiruchirappalli	9.2	6.1
S44	Puduchatram	Namakkal	31.3	37.2
S45	Valayapatti	Namakkal	7.3	5.7
S46	Namakkal	Namakkal	18.6	5.6
S47	Palayamkottai	Tirunelveli	24.3	12.7
S48	Erode	Erode	5.0	1.4
S49	Thethipalayam	Coimbatore	9.5	4.2
S50	Sendamangalam	Namakkal	0.97	0.5
S51	Vallanad	Tirunelveli	18.9	8.0

PCR-based methods are widely used for detection of aflatoxin-producing fungi in foods or feeds (Shapira *et al.*, 1996; Sweeney *et al.*, 2000; Criseo *et al*., 2001; Degola *et al*., 2007; Cruz and Buttner, 2008; Passone *et al*., 2010; Luque *et al*., 2012; Rodriguez *et al*., 2012; Shweta *et al*., 2013). In the present study, DNA extracted from the poultry feed samples was subjected to the PCR using *omt-A* primers in order to detect the fungus. As expected, *A. flavus* specific 300-bp PCR product was found in most of the AFB1 contaminated feeds (Fig. 1). However, some of the feed samples even though contain AFB1 failed to amplify the 300-bp product in PCR. Hence, it can be concluded that the absence of *A. flavus* in the poultry feeds as analyzed by PCR does not guarantee freedom from aflatoxins as the fungus could have already died or removed during processing of feeds while leaving the toxin intact.

Poultry industry in India is transforming gradually from small-scale backyard farming into large-scale, Hi-tech industry. In India the value of output from poultry sector is nearly ` 15,000 crores and this sector provides direct or

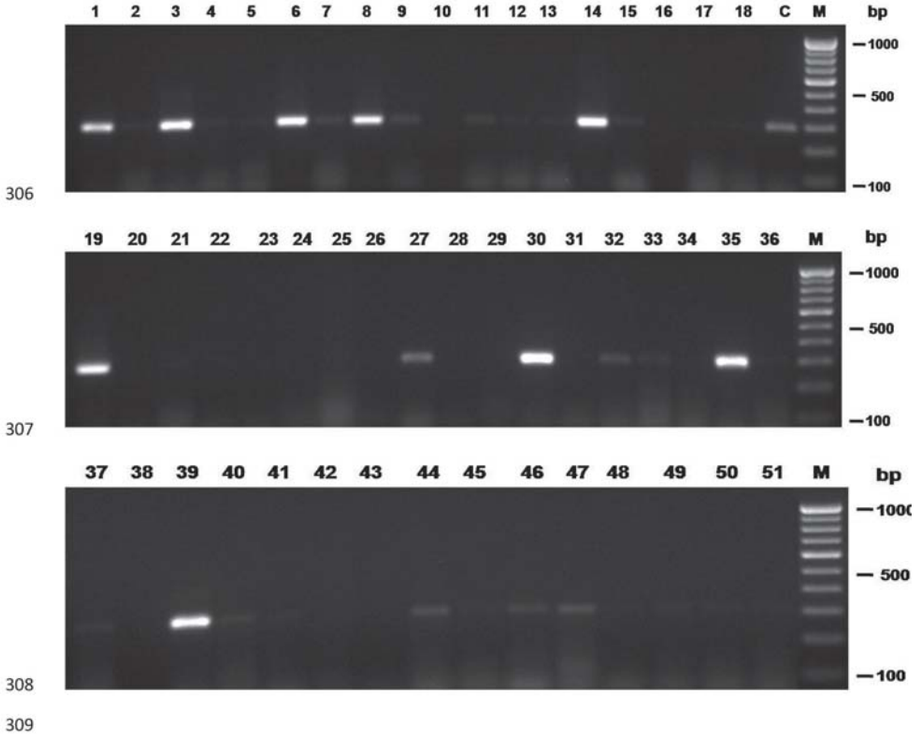

Fig. 1: Detection of *A. flavus* in poultry feeds by PCR using omt-A primers Lane M, 100-bp DNA ladder; Lane numbers indicate poultry feed sample ID; Lane C, positive control (Purified DNA of *Aspergillus flavus*).

indirect employment to over three million people. In addition to reducing the value of feed (Nichols, 1983), aflatoxin contamination has been linked to increased mortality in farm animals (Smith and Moss, 1985), and increased incidence of liver cancer in humans (Magnussen and Parsi, 2013). Li *et al.*, (2001) reported that the levels of Aflatoxins B_1, B_2 and G_1 were significantly higher in corn from the high incidence areas for human hepatocellular carcinoma. Hence, regular monitoring of the levels of aflatoxins in poultry feeds is very important to ensure the safety and quality of poultry products. In the present study, aflatoxin concentrations in general are well below the guidance values although some samples exceeded the admissible limits. Inappropriate storage conditions and field contamination of grains with *A. flavus* could be implicated in the *A. flavus* growth and aflatoxin production. Since the absence of *A. flavus* as determined by PCR is of no assurance that the feeds will be free from aflatoxins, the feed samples have to be analyzed for the presence of aflatoxins by employing sensitive methods like HPLC or ultra-performance liquid chromatography/tandem mass spectrometry.

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