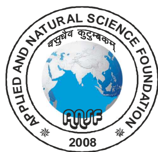

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Analysis of aflatoxin B1 and aflatoxigenic mold in commercial poultry feeds in Tamil Nadu, India

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Abstract: A total of 48 commercial poultry feed samples collected from different poultry feed manufactures in Tamil Nadu, India were examined for the contamination of aflatoxin B1 (AFB1) and *Aspergillus flavus*. AFB1 in the samples was estimated by sandwich ELISA and the presence of *A. flavus* was detected by Real-Time PCR assay. Real-Time PCR analysis using *A. flavus*- specific *omt* primers confirmed the presence of *A. flavus* in all the samples tested. ELISA results indicated that the AFB1 contents in the poultry feeds ranged from 1.0 to18.7 ppb, which were below the permissible safe limits for poultry bird consumption and health. The results suggest adoption of good manufacturing practices by the commercial poultry feed manufacturers during procurement of feed ingredients, handling, storage and processing which might have suppressed the growth of *A. flavus* and aflatoxin contamination.

Keywords: Aflatoxin, *Aspergillus flavus*, ELISA, Molecular detection, PCR

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important cereal crops worldwide and contributes to food security in most of the developing countries (Ranum *et al.*, 2014). In India, maize is emerging as the third most important crop after rice and wheat. It accounts for approximately 9 per cent of total food grain production in the country. Approximately 50% of total production goes for feed use, primarily for poultry feed. Aflatoxin contamination of maize kernels is one of the major problems worldwide. These aflatoxins are a group of structurally related polyketide-derived secondary metabolites produced by certain strains of *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare. These aflatoxins are acutely toxic, carcinogenic, mutagenic, teratogenic and immunosuppressive compounds. Common aflatoxins are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2). AFB1 is the most potent of all aflatoxins (Lee *et al.*, 2004). This toxin is the most potent carcinogen found in nature (Castegnaro and McGregor, 1998).

Although maize is infected by different species of *Aspergillus* in the field, *A. flavus* is the dominating aflatoxin-producing fungus especially in tropical regions (Calvert *et al.*, 1978, Setamou *et al.*, 1997). The fungus grows well when the temperature is between 18°C and 33°C and the relative humidity is greater than 50 per cent. The prevailing weather conditions in India favor the growth of the fungus and subsequently the production of aflatoxin in kernels. Aflatoxin contamination of maize may occur at any stage before and after harvest and during drying, storage and processing. When contaminated maize kernels are used as ingredient for poultry feed, potentially harmful amounts of aflatoxin may be ingested by poultry birds. Some of the metabolites formed during the metabolism of AFB1 are transmitted to edible animal products *viz.*, liver, muscle (Bintvihok and Davitiyananda, 2002) and eggs, which exert toxic, immunosuppressive and teratogenic effects on human beings. In order to prevent entry of aflatoxin in food chain, most developed countries have set stringent regulatory requirements on the level of aflatoxins permitted in imported and traded commodities (Van Egmond, 1989). In the United States, for example, the Food and Drug Administration (FDA) has established an action level for total aflatoxins in human food at 20 parts per billion (ppb) (Park and Liang, 1993).

Aflatoxin contamination in poultry feeds causes significant economic losses to poultry industries (Awad *et al.*, 2006). Consumption of such aflatoxin contaminated feed results in a disease called aflatoxicosis in poultry. An outbreak of aflatoxicosis in commercial poultry farms was reported in the Chittoor district of Andhra Pradesh state, India with 100% mortality (Choudary and Rao, 1982). It has been reported that each mg of AFB1/kg feed would decrease the growth performance of broilers by 5% (Dersjant-Li *et al.*,

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2003). Raju and Devegowda (2000) recorded 21% decrease in body weight at 35 days age in broilers fed with 0.3 mg AFB1/kg feed. Al-Shawabkeh *et al.* (2009) reported that feeds contaminated with aflatoxin B1 caused a significant increase in feed conversion ratios, a decrease in body gain and a significant increase in feed consumption in broiler chickens. Furthermore, the nutritional value of poultry meat (combined breast, thigh and respective skin) in terms of CLA contents was affected by the concentration of AFB1 added to the feed. The level and length of AFB1 exposure affect the amount of reduction in weight gain of broilers (Yunus *et al*., 2011). The aim of present study was to quantify AFB1 in poultry feeds from commercial poultry feed manufacturers by sandwich ELISA and detection of *A. flavus* by Real-Time PCR assay.

MATERIALS AND METHODS

Sample collection: Forty eight poultry feed samples were collected from commercial feed manufacturers in Tamil Nadu, India. The presence of AFB1 in naturally contaminated poultry feed samples was detected by sandwich ELISA as described by Kannan and Velazhahan (2015).

Extraction of aflatoxins from poultry feeds: Powdered feed samples (50 g) were macerated with 250 ml of methanol: water (55:45 v/v), 100 ml of hexane and 2 g of sodium chloride. The extract was filtered; 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 0.05M carbonate buffer (pH 9.6) and used as antigen.

Sandwich ELISA: The wells of microtiter plates (Genaxy Scientific PVT LTD, Solan, India) were coated with 150 µl/well of antiserum raised against AFB1-KLH in carbonate coating buffer (0.05M carbonate buffer, pH 9.6) (1: 10,000 dilution) and incubated at 4°C overnight. The plates were washed thrice with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST). AFB1 standard/sample extract (150 µl) was added into the coated wells and incubated for 2 h at 37°C. The plates were washed thrice with PBST and 150 µl/well of antiserum raised against AFB1-KLH in 0.05M carbonate coating buffer was added into each well. After incubation for 2 h at 37°C, 150 µl of alkaline phosphatase -labelled goat anti-rabbit IgG (Genei, Merck Specialities P. Ltd. Mumbai, India) at 1:4,000 dilution in carbonate buffer was applied. The plates were incubated at 37°C for 2 h and then washed with PBST for 5 times and 150 µl of freshly prepared *p*-nitrophenyl phosphate (0.5 mg/ml) in 10% diethanolamine (pH 9.8) was added to each well. The microtiter plates were incubated for approximately 30 min and read at 405 nm on a microtiter plate reader (Sunrise™, Tecan groups Ltd, Switzerland).

Detection of *A. flavus* by real-time PCR assay: In order to detect the presence of *A. flavus* in poultry feeds, Real-time PCR assay was conducted as described by Shweta *et al*. (2013). Approximately 100 mg of feed samples were ground to a powder in liquid nitrogen and the DNA was extracted by using the CTAB extraction method (Doyle and Doyle, 1987). Real-time PCR amplification was performed using Light Cycler 480 SYBR Green Master mix (Roche

Table 1. Detection of aflatoxin B1 in poultry feed samples by ELISA.

S.No.	Poultry feed samples (Sample code)	AFB1 Concentration (ppb)
1	1786	7.7
2	3055	6.7
3	3033-3034	9.2
4	176-177	5.6
5	2132	6.4
6	704-705	5.2
7	3089	7.5
8	97 A	5.4
9	2831	6.0
10	2830	6.5
11	1778 (629 A)	5.8
12	G 1	6.9
13	2817-2818	4.1
14	753-755	3.2
15	261-262	5.3
16	627 A	2.5
17	717-720	3.1
18	741-743	5.2
19	2129	18.7
20	341	6.8
21	2704-2706	8.2
22	2130 G	6.2
23	652-654	8.5
24	655-657	6.3
25	747-749	8.4
26	795 (2724)	5.1
27	2891-2892(1779)	8.4
28	758-760	3.4
29	2703-2706	6.1
30	644-645(1860)	4.8
31	869-870	4.6
32	1898	5.6
33	3091 A	6.7
34	1941 G	5.7
35	1879 (782-783)	4.6
36	744-746	2.1
37	750-752	6.6
38	649-651	2.0
39	1788 (9-100)	1.4
40	2133 G	2.9
41	658-660	1.0
42	1938 G	9.9
43	796-797(1891)	1.3
44	1868	9.9
45	2707-2708	1.9
46	646-648	1.6
47	761-763	1.2
48	C1	1.2

Fig. 1. *Amplification curves on the detection of A. flavus through Real-Time PCR using SYBR Green, indicating the increase of the fluorescence signal in DNA obtained from naturally infected poultry feed samples.*

Diagnostics GmbH, Manheim, Germany) and Roche Light Cycler 96 (Roche Diagnostics GmbH, Manheim, Germany). The PCR reaction contained 5 µl of 2X Light Cycler 480 SYBR Green Master mix, 5 pmol each of *omt* forward (5' GACCAATACGCCCACA-CAG-3') and reverse (5' CTTTGGTAGCTGTTTCTCGC -3') primers and 5 ng of DNA template in a final volume of 10 µl. Thermal cycling conditions consisted of initial denaturation for 7 min at 95 °C, followed by 35 cycles of 95°C for 30 s, annealing temperature of 59°C for 30 s and extension at 72°C for 30 s. After the PCR cycle, a melting curve of the product was generated by ramping the temperature to 95°C for 10 s, back to 65° C for 60 s, and then incremental increases of 4.4°C/s up to 95°C with continuous measurement of fluorescence. The threshold cycle (Ct) value for each reaction was assessed using the Light Cycler 96 S W 1.0 software (Roche Diagnostics GmbH, Manheim, Germany).

Table 2. Real-time PCR assays in poultry feed samples.

S.No.	Sample ID	Ct value
1	1786	32.46
2	176-177	34.55
3	704-705	31.54
4	3089	34.79
5	97 A	34.05
6	2831	36.42
7	2830	34.98
8	1778 (629 A)	33.47
9	G 1	33.12
10	2817-2818	33.59
11	753-755	34.78
12	261-262	32.34
13	627 A	32.63
14	717-720	29.07
15	741-743	33.12
16	2129	34.82
17	341	34.10
18	2130 G	33.93
19	652-654	27.49
20	655-657	28.31
21	747-749	35.80
22	2891-2892(1779)	34.83
23	2703-2706	32.77
24	3091 A	28.95
25	1941 G	34.11
26	1879 (782-783)	25.97
27	744-746	33.15
28	750-752	35.09
29	649-651	27.54
30	1788 (9-100)	35.45
31	658-660	36.63
32	796-797(1891)	33.37
33	2707-2708	36.35
34	C1	28.02

RESULTS AND DISCUSSION

As shown in Table 1, AFB1 was detected in all the feed samples analyzed and the AFB1 content in the naturally contaminated poultry feeds ranged from 1.0 to18.7 ppb. However, the quantities were below the permissible limit for poultry bird consumption and health. When these naturally contaminated poultry feed samples were analyzed for the presence of *A. flavus* by conventional PCR using *omt* primers, the expected 300-bp product was amplified in most of the AFB1 contaminated feed samples (Data not shown). However, some of the feed samples even though contain AFB1 failed to amplify the 300-bp product in conventional PCR. The same samples showed positive results when analyzed by Real-time PCR (Fig. 1). The intercalating dye, SYBR Green showed satisfactory results in the detection of *A. flavus* in poultry feeds. The Ct values ranged from 19.61 to 26.11 for $7.3x10^{11}$ to $7.3x10^{7}$ copies, respectively, of the target DNA se-

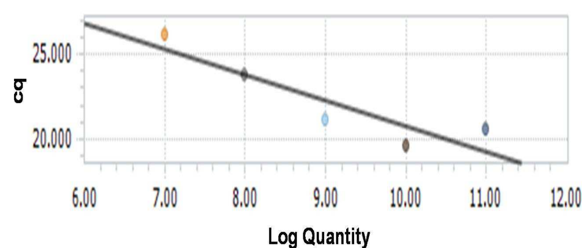

Fig. 2. *Real-time PCR standard curve of the log of amount of A. flavus DNA, versus the corresponding cycle quantitative (Cq) values.*

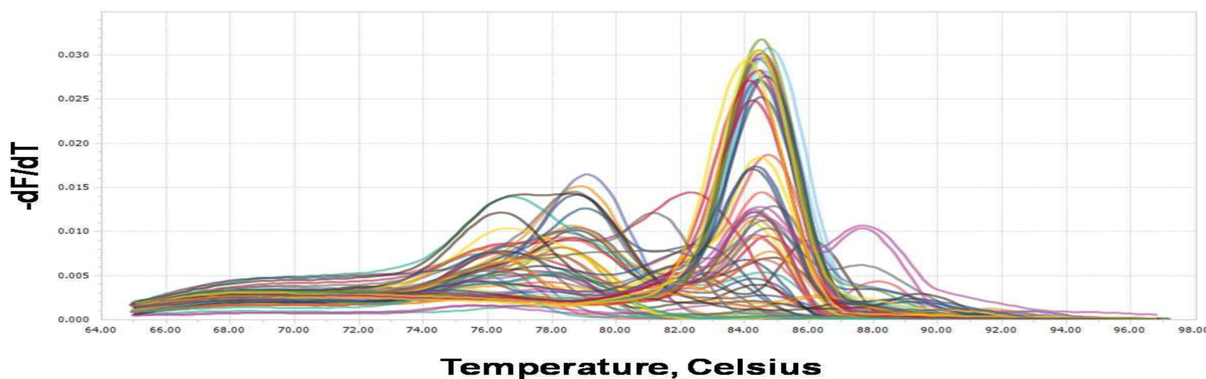

Fig. 3. *Melting curve for the target amplicon of A. flavus in naturally infected poultry feed samples as analyzed by Real-Time PCR.*

quence (Fig. 2). The Ct values were between 25.97 and 36.63 (Table 2) for the naturally infected poultry feed samples and confirmed the presence of the fungus in the samples. The R^2 value of 0.93 in standard curve strongly supports the reliability of the results. The melting curve analysis showed a major peak with a melting point of 84°C (Fig.3). The RT-PCR assay is reproducible between replicate samples. The evaluation of the Real-time PCR products, obtained from the amplification of DNA fragments of naturally contaminated poultry feed samples with *omt* primers designed for *A. flavus*, through electrophoretic analysis in 1.0% agarose gel generated bands of 300 bp (Data not shown), indicating specificity of the primers.

Bhat *et al.* (1997) reported that 26 % of maize kernels collected from different parts of India were contaminated with AFB1 beyond the level of Indian standard for consumption (30 µg/ kg). Waliyar *et al.* (2003) reported that 43 % of maize samples collected from retail shops or super markets in Hyderabad, Andhra Pradesh, India were contaminated with toxin with the highest AFB1 level of 806 µg/ kg. Using RT-PCR technique, Shweta *et al.* (2013) proved the presence of *A. flavus* in maize kernel samples. Vijayasamundeeswari *et al.* (2009) reported that 58 % of the maize kernel samples collected from different agro-ecological zones of Tamil Nadu, India was contaminated with AFB1 and the levels of AFB1 in 26 % of the pre- and post-harvest maize kernels exceeded 20 µg kg^{-1}. Kannan *et al.* (2014) reported that 98% of the poultry feed samples collected from poultry farms and poultry feed dealers of Tamil Nadu, India 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. Kannan and Velazhahan (2015) reported that AFB1 contamination was found in more than 88 % of the poultry feeds samples collected from Tamil Nadu, India and its level ranged from 5.4 to 125.4 µg/kg.

The presence of aflatoxins in agricultural commodities poses a serious health threat to both humans and domestic animals. Several studies reported the presence of residual aflatoxins in liver and meat of broilers when fed with aflatoxin contaminated feeds (Oliveira *et al*., 2000; Hussain *et al*., 2010; Herzallah, 2013). In the case of laying hens, aflatoxins and their metabolites were detected in the eggs (Trucksess *et al*., 1983). The results of this study indicate that the AFB1 content in the poultry feeds were below the tolerance level fixed by the FAO for poultry bird consumption suggesting adoption of good manufacturing practices by the feed manufacturers during the procurement of feed ingredients, handling, storage and processing of feeds to ensure the safety of feeds for poultry consumption. The amplification of target DNA fragment might be due to the indiscriminate detection by PCR independent of the viability status of the fungus. Novel PCR-based strategy, propidium monoazide - Real-Time quantitative PCR, that differentiate nucleic acids associated with viable cells from those associated with inactivated cells has to be tested (Nocker and Camper, 2009; Crespo-Sempere *et al*., 2013; Cangelosi and Meschke, 2014).

Conclusion

It could be concluded that *A. flavus* was detected in all poultry feed samples tested by Real-time PCR. However, the poultry feeds were contaminated with low levels of aflatoxin B1 (1.0 -18.7 ppb), suggesting adoption of good manufacturing practices by the feed manufacturers during the procurement of feed ingredients, handling, storage and processing of feeds to ensure the safety of feeds for poultry consumption. The amplification of target DNA fragment in PCR might be due to the indiscriminate amplification by PCR independent of the viability status of the fungus. It is necessary to increase the awareness among farmers and traders about the importance of aflatoxins and to adopt improved management practices to minimize aflatoxin contamination in feed ingredients.

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