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CONTAMINATION OF COMMERCIAL POULTRY FEEDS AND FEED INGREDIENTS WITH *ASPERGILLUS FLAVUS* AND AFLATOXIN B1 IN PUNJAB PAKISTAN

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ABSTRACT

The occurrence of mycotoxins in feed is a major problem in poultry because it deteriorates the feed quality and thus contributes to economic losses in the form of decreased profitability. The study was carried out to assess the prevalence of A. flavus and AFB1 in poultry feed and feed ingredients during one full year. Results revealed that 34.2% feed samples were contaminated with *A. flavus* and 29.2% with AFB1, while 26.9% and 26.4% samples of feed ingredients were found contaminated with *A. flavus* and AFB1, respectively. The contamination with *A. flavus* and AFB1 was highest for corn followed by cotton seed meal and broken rice. Higher percentages of samples were found contaminated during humid summer or in spring. The AFB1 contamination in feed ingredients revealed a significant difference. More than 30% samples of corn, broken rice and cotton seed meals were found contaminated with AFB1 and more than 50% of corn and 40% of broken rice and cotton seed meal with *A. flavus*. The highest percentages of feed samples were found contaminated with *A. flavus* and AFB1 during July to September. Majority of feed samples had AFB1 levels were below 200ppb. The feed and feed ingredients were invariably contaminated with *A. flavus* and AFB1 and the prevalence varied with the season and there is a direct correlation between *A. flavus* and AFB1 contamination.

Keywords: *A. flavus*, AFB1, feed, feed ingredients, Pakistan.

INTRODUCTION

Pakistan has both tropical and subtropical zones and its climate has both strong sunshine and high rain fall, which is highly favourable for fungal growth, especially thermo-tolerant species such as Aspergillus (Raper and Fennell, 1965). These species are usually the fungal contaminants in Asia and are responsible for both crop spoilage and mycotoxins contamination of foodstuffs (Mantle, 2002; Ehrlich *et al*., 2007; Gao *et al*., 2007). *Aspergillus flavus* usually grow ideally at 37°C, however, 12 to 48°C temperatures are regarded as favourable for mould growth (Riba *et al*., 2008). Among moulds, *A. flavus* is supposed to be the predominant fungi responsible for aflatoxin synthesis in crops before harvesting and during storage (Yu *et al*., 2004). Among various fungi, *Aspergillus* species are known to be the most prevalent and commonly found in stored grains (Marquardt, 1996). Fungi normally develop from spores and found ubiquitously in the environment and can grow on the most organic matter. *A. flavus* can be identified by its spreading yellow green colonies on media with rough wall strips, and smooth to finely roughened conidia.

The occurrence of mycotoxins in feed is a problem of major concern in the poultry industry because it deteriorates the feed quality and thus results in high economic losses in the form of decreased profitability in poultry sector (Hamilton, 1984). Fungal metabolites known as mycotoxins are structurally diverse and contaminate animal / poultry feed throughout the world. About 25% of the world's crops are contaminated with mycotoxins and among mycotoxins, aflatoxins are the most notorious and perilous metabolites (FAO, 2002). Aflatoxins, a group of closely related and biologically active mycotoxins, are produced by strains of *A. flavus* and *A. parasiticus*. The AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 are the most common forms, but AFB1 is considered the most toxic (Nilipour, 2002). They commonly occur as a contaminant in poultry feeds (Edds and Bortell, 1983). The aflatoxins severely affect the poultry, animal and humans and can cause teratogenic, carcinogenic and mutagenic effects and inhibit body growth due to poor utilization of feed in the GI tract (Wild and Kleinjans, 2003; Sur and Celik, 2003). Aflatoxicosis has deleterious effects on the reproductive efficiency of both male and female, domestic animals, particularly poultry. The AFB1 severely affects the reproductive system of layers (Ortatatli, 2002). The TLC is a more precise way of determining the aflatoxinB_1 concentration in feed and feed ingredients. Many workers adopted TLC technique for the identification of mycotoxins being short, economical and accurate (Tung *et al*. 2008). As the environment of Pakistan is highly suitable for the growth of moulds capable of producing mycotoxins, it was planned to have a fresh study to find out the contamination level of commercial feeds and

various feed ingredients with Aspergillus and aflatoxin B1 during different seasons of the year.

MATERIALS AND METHODS

Collection of samples: Commercial feed samples were collected from 20 randomly selected poultry farms of four main districts, i.e., Faisalabad, Lahore, Multan and Rawalpindi (five farms from each location) of the Punjab Province for isolation and identification of *A. flavus* and quantification of AFB1. These districts were selected because they have a good poultry population. Feed samples were collected monthly for one year from farms within 20 kilometres radius of cities on and around 15th of each month. A total of five samples from each of the four cities for twelve months were collected. Thus, a total of 240 feed samples for one year were collected in fine UV light sterilized polythene bags. The samples were then stored at 4^0C till further analysis. The samples of five poultry feed ingredients, i.e., corn, wheat, broken rice, cottonseed meal and soybean meal were collected from storage facilities/silos of different feed mills of the Punjab Pakistan for one year. The names of the feed mills are not shown as was promised with the feed mill owners. A total of 15 feed mills/silos facilities were randomly selected for collection of feed ingredients. A total of 75 samples for five seasons from 15 feed mills/silos of each feed ingredient were collected in fine UV light sterilized polythene bags and stored at 4^0C for further analysis. The meteorological data for the year under study was also collected from the Ayub Agricultural Research Institute, Faisalabad.

Isolation of *Aspergillus flavus*: For the isolation of the *A. flavus* from different samples of commercial feeds and feed ingredients, two different culture media, i.e., Saboraud's and potato dextrose agar (PDA) were used. The growth of *A. flavus* was identified based on their growth morphology and microscopic appearance/characters by using methods as described earlier that included aspergillum-like spore-bearing structure and some cells enlarge, develop a heavy cell wall and form 'T' or 'L' shaped 'foot cells'. The size and arrangement of the conidial heads as well as the green coloured spores they bear are important identifying characteristics (Raper and Fennell, 1965).

Estimation of AFB_1 through Thin Layer Chromatography (TLC): For quantitative analysis of AFB1 the feed and feed ingredient samples were processed for estimation by thin layer chromatography (Jones, 1970). Briefly, to each sample, 90 ml chloroform, 10 ml distilled water and 2 gm sodium chloride was added. The contents of the flask were shaken on orbital shaker for 5 min at room temperature. Toxin extraction was done with chloroform and transferred into the screw capped vials (Jones, 1970). The silica gel was used to form slurry and TLC plates were coated having about 0.3 mm thickness. The coated TLC plates were spotted with 3 µl, 5 µl, and 7 µl of AFB_1 standard (10 µg/ml) on the baseline of the TLC plate with a distance of 2 cm from the bottom and 1 cm among spots and spotted 3 µl, 5µl and 7 µl of sample on TLC plates. These plates were then placed in a chromatography tank pre-filled (upto ~0.5 cm and/or was kept lower than the origin line/spots) with a solvent mixture of chloroform: acetone (95:5). The plates were then removed when the solvent front raised to about 15 cm from the starting line. The finish line of the solvent was also recorded. Plates were air dried and examined under UV lamp at 364 nm wave length. The blue fluorescent spots observed were encircled and *RF* value of each spot was determined and compared against those of standards of AFB1.The matched spots with respect to Rf and intensity of fluorescence were selected for determining the concentration of the aflatoxin in the sample.

The data were interpreted as percentage. The 95% confidence limits were also worked out and chi-square analysis was also performed.

RESULTS

The results revealed that 34.2% feed samples were contaminated with *A. flavus* and 29.2% with AFB1, while 26.9% and 26.4% samples of feed ingredients were found contaminated with *A. flavus* and AFB1, respectively.

The results of *A. flavus* and AFB1 levels in different feed ingredients during five seasons are presented in Table 1. In overall, contamination with *A. flavus* and AFB1 was highest for corn followed by cotton seed meal and broken rice. The highest percentages of samples were found contaminated during humid summer or in spring. The AFB1 contamination in feed ingredients by raw analysis and after controlling for the season revealed a significant difference. More than 30% samples of corn, broken rice and cotton seed meals were found contaminated with AFB1 and 60% of corn and 46.7% of broken rice and 40% of cotton seed meal with *A. flavus* in humid summer (Table 1).

The results of percentages of feed samples found contaminated with *A. flavus* and AFB1 during different months of the year are presented in Table 2. The highest percentages of samples were found contaminated with *A. flavus* and AFB1 during July to September, however, with non-significant difference statistically. The results of AFB1 levels in samples of different feed ingredients are presented in Table 3. Most of the samples had AFB1 levels below 200ppb. The results of AFB1 levels during different months of the year are presented in Table 4. Results revealed <200 ppb levels from January to June and October to December, while in other months higher than 200ppb of AFB1 levels were also detected.

Table 1. Prevalence of *A. flavus* and AFB1 in different samples of feed ingredients collected from different stores/silos of feed mills of Punjab, Pakistan.

Ingredients	Autumn + ve (%)	Winter + ve (%)	Spring + ve (%)	Dry Summer + ve (%)	Humid Summer + ve (%)	Overall	
						+ve (%)	95% CI
A. flavus							
Corn	5 (33.3)	4 (26.7)	6 (40.0)	4 (26.7)	8 (53.3)	27 (36.0)	25.76 - 47.31
Wheat	3 (20.0)	2 (13.3)	3 (20.0)	2 (13.3)	4 (26.7)	14 (18.7)	11.04 - 28.68
Broken rice	4 (26.7)	3 (20.0)	4 (26.7)	4 (26.7)	6 (40.0)	21 (28.0)	18.73 - 38.94
C.S.M	5 (33.3)	3 (20.0)	5 (33.3)	4 (26.7)	7 (46.7)	24 (32.0)	22.20 - 43.16
Soybean meal	3 (20.0)	2 (13.3)	4 (26.7)	2 (13.3)	4 (26.7)	15 (20.0)	12.10 - 30.18
	Mantel-Haenszel test P < 0.069					*chi-sq. P < 0.107*	
AFB1							
Corn	5 (33.3)	4 (26.7)	6 (40.0)	4 (26.7)	9 (60.0)	28 (37.3)	26.96 - 48.67
Wheat	3 (20.0)	1 (6.7)	3 (20.0)	2 (13.3)	3 (20.0)	12 (16.0)	8.97 - 25.61
Broken rice	5 (33.3)	3 (20.0)	5 (33.3)	4 (26.7)	7 (46.7)	24 (32.0)	22.20 - 43.16
Cotton seed meal	5 (33.3)	3 (20.0)	5 (33.3)	4 (26.7)	6 (40.0)	23 (30.7)	21.04 - 41.76
Soybean meal	2 (13.3)	2 (13.3)	3 (20.0)	2 (13.3)	3 (20.0)	12 (16.0)	8.97 - 25.61
	Mantel-Haenszel test P < 0.005					*chi-sq. P < 0.005*	

Table 2. Prevalence of *A. flavus* and AFB_1 in different feed samples during different months.

Months	Average temperature and relative humidity		No. of samples	A. flavus		AFB1	
	Temp (°C)	R. Humidity		+ve (%)	95% CI	+ve (%)	95% CI
Jan	13	75.1	20	5 (25)	9.79 - 47.02	4 (20)	6.70 - 41.49
Feb	16.55	66.4	20	5 (25)	9.79 - 47.02	4 (20)	6.70 - 41.49
March	23.85	42.7	20	6 (30)	13.16 - 52.28	4 (20)	6.70 - 41.49
April	29	31.5	20	6 (30)	13.16 - 52.28	5 (25)	9.79 - 47.02
May	32	36.9	20	6 (30)	13.16 - 52.28	5 (25)	9.79 - 47.02
June	35.6	42.6	20	5 (25)	9.79 - 47.02	5 (25)	9.79 - 47.02
July	33.9	69.5	20	11 (55)	33.28 - 75.36	10 (50)	33.28 - 75.36
Aug	34.3	76.4	20	12 (60)	37.89 - 79.39	11 (55)	33.28 - 75.36
Sep	30	66.3	20	10 (55)	33.28 - 75.36	9 (45)	24.64 - 66.72
Oct	23.6	55.2	20	7 (35)	16.77 - 57.30	6 (30)	13.16 - 52.28
Nov	19.6	66	20	4 (20)	6.70 - 41.49	3 (15)	3.96 - 35.61
Dec	14.35	67.2	20	5 (25)	9.79 - 47.02	4 (20)	6.70 - 41.49
Total			**240**	**82 (34.2)**	**28.37 - 40.34**	**70 (29.17)**	**23.68 - 35.16**

Table 3. Occurrence of AFB1 levels in number of samples of different feed ingredients during different seasons.

Ingredients	20-25	51-80	81-110	111-140	141-170	171-200	201-230	231-260	261-290	291-320	321-350	351-380	381-410	411-440
Autumn														
Corn	-	-	-	1	2	2	-	-	-	-	-	-	-	-
Wheat	2	1	-	-	-	-	-	-	-	-	-	-	-	-
Broken rice	-	-	2	1	1	1	-	-	-	-	-	-	-	-
CSM	-	-	1	2	2	-	-	-	-	-	-	-	-	-
Soybean meal	-	1	1	-	-	-	-	-	-	-	-	-	-	-
Winter														
Corn	-	2	2	-	-	-	-	-	-	-	-	-	-	-
Wheat	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Broken rice	-	2	1	-	-	-	-	-	-	-	-	-	-	-
CSM	-	-	1	2	-	-	-	-	-	-	-	-	-	-

Soybean meal	-	2	-	-	-	-	-	-	-	-	-	-	-	-
Spring														
Corn	-	-	-	-	2	-	2	1	1	-	-	-	-	-
Wheat	2	1	-	-	-	-	-	-	-	-	-	-	-	-
Broken rice	-	-	-	-	-	2	2	1	-	-	-	-	-	-
CSM	-	-	-	-	1	2	-	2	-	-	-	-	-	-
Soybean meal	1	1	1	-	-	-	-	-	-	-	-	-	-	-
Summer (dry)														
Corn	-	-	2	2	-	-	-	-	-	-	-	-	-	-
Wheat	1	1	-	-	-	-	-	-	-	-	-	-	-	-
Broken rice	-	-	2	1	1	-	-	-	-	-	-	-	-	-
CSM	-	-	1	2	1	-	-	-	-	-	-	-	-	-
Soybean meal	-	2	-	-	-	-	-	-	-	-	-	-	-	-
Summer (humid)														
Corn	-	-	1	2	1	-	3	-	-	1	-	-	1	-
Wheat	-	2	1	-	-	-	-	-	-	-	-	-	-	-
Broken rice	-	-	1	1	-	2	1	1	-	1	-	-	-	-
CSM	-	-	1	-	1	1	-	-	1	-	2	-	-	-
Soybean meal	-	1	2	-	-	-	-	-	-	-	-	-	-	-
Total	**6**	**17**	**20**	**14**	**12**	**10**	**8**	**5**	**2**	**2**	**2**	**-**	**1**	**-**
%	**1.6**	**4.53**	**5.33**	**3.73**	**3.2**	**2.67**	**2.13**	**1.33**	**0.53**	**0.53**	**0.53**	**-**	**0.27**	**-**

(Number of samples and percentages in each season: 1 = 6.7%; 2 = 13.3%; 3 = 20%)

Table 4. Levels of AFB1 recorded in different poultry feed samples collected during one year.

	20 - 50	**51 – 100**	**101 - 150**	**151 - 200**	**201 - 250**	**251 - 300**	**301 - 350**	**351 - 400**	**400 – 450**	**451 - 500**	**501 - 550**	**551 - 600**	**601 - 650**	**651 - 700**	**701 - 750**	**751 – 800**
January	2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
February	1	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-
March	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-
April	-	2	1	1	1	-	-	-	-	-	-	-	-	-	-	-
May	-	1	3	1	-	-	-	-	-	-	-	-	-	-	-	-
June	1	2	1	1	-	-	-	-	-	-	-	-	-	-	-	-
July	-	-	-	1	-	2	-	1	-	-	2	-	-	2	-	2
August	-	-	-	1	-	2	1	-	2	-	-	1	-	1	2	1
September	-	-	1	1	1	1	1	-	1	2	2	-	-	-	-	-
October	-	-	2	3	-	-	-	-	-	-	-	-	-	-	-	-
November	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-
December	-	1	2	1	-	-	-	-	-	-	-	-	-	-	-	-
Total	4	10	18	10	2	5	2	1	3	2	4	1	-	3	2	3
(%)	(1.67)	(4.16)	(7.50)	(4.16)	(0.83)	(2.08)	(0.83)	(0.42)	(1.25)	(0.83)	(1.67)	(0.42)		(1.25)	(0.83)	(1.25)

(Number of samples and percentage in each month: 1 = 5%; 2 = 10%; 3 = 15%)

DISCUSSION

The contamination of feed and feed ingredients collected from various sources with *A. flavus* was not unusual, rather it has been reported in various countries of the world (Kumari *et al*., 1995; Jaimez *et al*., 2003). The contamination by *A. flavus* of 20% feed samples were recorded with maximum contamination of 60% samples. The contamination of poultry feed by *A. flavus* has been reported previously (Benkerroum and Tantaoui, 2001; Dutta and Das, 2001). The growth of *A. flavus* was favoured by environmental conditions like high humidity, temperature and the availability of the substrate (Riba *et al*., 2008; Tung *et al*., 2008). In tropical countries like Pakistan the natural humid environment during the months of July to September enhanced the contamination levels and it has been reported that improper storage and management of feed have greater impact on the occurrence of *A. flavus* (Mantle, 2002). The present results showed that there was a direct correlation with the increase in the occurrence of *A. flavus* and the levels of AFB1 recovered from poultry feeds. It has been reported that during the metabolism, *A. flavus* not only produce aflatoxin but also induce deteriorating effects on the nutritive value of the feed (Cawood *et al*., 1991). The

present study showed that 34.2% of feed samples were contaminated with *A. flavus*, 29.2% with AFB1 and the levels varied from 22 to 800 ppb. Another study reported levels between 20 to 200 ppb, but in four samples higher levels of 2000 to 5625 ppb in commercial poultry feed has also been reported (Kichou and Walser, 1994). Most of the workers had attributed such a high prevalence of AFB1 contamination of feed to storage, temperature, moisture content of feed and environmental precipitation that varies at different months of the year (Gao *et al*., 2007; Riba *et al*., 2008).

Among feed ingredients, the contamination by *A. flavus* was observed in 13.3% of soybean meal and wheat samples during the dry summer and winter, respectively. While higher percentages of samples of 53.35, 26.7, 40.0, 46.7 and 26.7% of corn, wheat, broken rice, cotton seed meal and soybean/wheat were contaminated with *A. flavus* during the humid summer. Autumn was the other season where higher percentages of *A. flavus* contaminated samples were observed. The highest prevalence of *A. flavus* was obvious due to high ambient temperature of storage and elevated humidity during the humid summer season that may be helpful in promoting the growth of *A. flavus* in the corn ingredient of the feed. Moreover, the corn ingredients contain proportionately high levels of starch and carbohydrates, which are thought to be helpful in promoting the growth and multiplication of *A. flavus* (Gao *et al*., 2007; Pacin *et al*., 2001). In most of the feed formulations, corn has been included as a major feed ingredient, therefore the chances of occurrence regarding *A. flavus* were higher in poultry feed. In the present study it was recorded that maize was the major ingredient contaminated with *A. flavus* during hot and wet season as reported earlier as well (Asia, South America and Equatorial Africa) (Pacin *et al*., 2001). The highest prevalence of *A. flavus* in the corn ingredients was further supported by the persistently high prevalence of AFB1 in corn samples, followed by broken rice and cotton seed meal samples. The results of the present study showed a direct correlation of occurrence in *A. flavus* with the prevalence of AFB1 from respective feed ingredients. The contamination of AFB1 in the broken rice may be correlated with the specific fatty acids and starch contents which are abundantly present in the rice and are responsible for the growth of *A. flavus* and production of aflatoxins (Sales and Yoshizawa, 2005). The results of the present study showed lower prevalence of *A. flavus* and AFB1 contamination in wheat and soybean meal samples which are said to be as less efficient substrate for the synthesis of AFB1(Riba *et al*., 2008).

Based on the results, it is suggested to the feed millers and poultry farmers to improve the feed ingredient and feed storage facilities.

Conclusions: The feed and feed ingredients are invariably contaminated with *A. flavus* and AFB1 and the prevalence varies with the season and there is a direct correlation between *A. flavus* and AFB1 contamination. The corn, broken rice and cotton seed meals are relatively more contaminated with both compared with other ingredients.

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