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Occurrence of Aflatoxin B_1 Producing Fungi in Finished Commercial Broiler Feed in Quetta

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Authors' Contribution
NR, MAB and MR designed the experiments, acquired the data and drafted the manuscript. MMT, TA, AU, MZM and ZA statistically analyzed the data. MAA, MA, MS, MZM and AK helped in interpretation of data.

Key words
***Aspergillus flavus*, Fungal contamination, Aflatoxin B_1.**

ABSTRACT

Occurrence of *Aspergillus flavus* (*A. flavus*) in finished commercial broiler feed and among the isolated strains of *A. flavus* having capacity to produce aflatoxin was investigated in this study. Finished commercial broiler feed samples (n=96) were collected from broiler chicken farms in and around Quetta district. Physical properties *i.e.* moisture and pH were determined by oven drying and pH meter, respectively. Viable fungal count of the feed samples was estimated by using spread plate technique. Identification of fungal isolates was carried out on the basis of cultural and morphological characters by using slide culture technique. While mean moisture percentage was recorded as 5.3% with a significant difference ($P<0.05$), the pH was noted to be slightly acidic with non-significant difference ($P>0.05$) among seasons. Overall mean viable fungal count was $4.72 \times 10^3 \pm 708$ with a significant association ($P<0.05$) among seasons. Out comes from study showed that, 44.8% of the assayed samples were contaminated with *A. flavus*. From them, 48.8% of isolates were capable of producing aflatoxin B_1. The study revealed the need of regular mycological monitoring of compound commercial feed in order to make control strategies and prevent toxic syndromes to this type of contamination.

INTRODUCTION

Commercial compound feed is of great importance in the modern poultry production. Feed quality is necessary for the maintenance of physiological functions and defense system of birds against diseases. Generally, feed quality is specified based on nutritional value of every ingredient. However, it may be affected by the contamination of microorganisms like fungi (Okoli *et al*., 2006). Poultry feeds are designed in such a way to contain all the nutritional materials required for the growth and production of birds (Ige *et al*., 2012). On the other hand, they also serve as a rich habitat for fungi. Contamination of poultry feed with fungi is considered inevitable. Indeed, the fungi, which are estimated to have more than 100,000 species, are able to pollute feeds. The frequent genera mostly belong to *Aspergillus*, *Penicillium* and *Fusarium* (Trung *et al*., 2001; Osho *et al*., 2007). Abiotic factors like ambient temperature above 7°C, relative humidity above 65%, moisture and pH of substrate about 12% or more and between 2-11, respectively, pave the way for mould growth (Wheeler *et al*., 1991; Okoli *et al*., 2007). Fungal proliferation may result reduced feed quality (loss of nutrients of 5-100%), decreased palatability, caking, darkening, mildew smell and production of mycotoxins (Okoli *et al*., 2006).

Aflatoxins represent the group of the most prevalent, dangerous and studied mycotoxins (Krnjaja *et al*., 2008). These are produced as secondary metabolites principally from toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Rashid *et al*., 2013). Aflatoxins are highly toxic, carcinogenic, teratogenic and mutagenic for human and animals. In poultry aflatoxins not only impairs weight gain, feed intake, feed conversion efficiency and egg production (Ortatatli *et al*., 2005; Muhammad *et al*., 2010) but also increases the susceptibility to environmental stress (Allameh *et al*., 2005) and severity of diseases like crop mycosis, salmonellosis, coccidiosis, aspergillosis and Marek's disease (Ibrahim *et al*., 2000).

The mycoflora of poultry feed is continuously monitored worldwide. Studies on natural occurrence of fungi have been conducted every year in industrialized countries (Saleemi *et al*., 2010). However, there is very little related information is known in developing

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countries. To overcome the gap it is necessary to carry out periodic surveillance program regarding the occurrence of aflatoxigenic strains in poultry feed.

By considering the importance of mycoflora with reference to aflatoxins, the present study was conducted to monitor aflatoxin-producing *Aspergillus flavus* and determine mycological quality of finished commercial broiler feed in and around Quetta district.

MATERIALS AND METHODS

Period and location of study

The current study was conducted between June 2009 to May 2010 in and around Quetta district. Quetta district is located between 30°15 North latitude and 66°55 East longitudes, at an altitude of 1675 meters in the North-west part of Balochistan and at the West Central Pakistan. The Quetta district was selected since it is not only the capital city of Balochistan province but also the most populated district hosting, one third urban population of the province. There are approximately 150 broiler farms with a flock rearing capacity of 1000 to 6000 birds (Rashid *et al*., 2012).

Sampling

The finished commercial broiler feed samples (n=96) were randomly collected in sterilized polythene bags from government and private poultry farms (two samples per week). The samples (1 kg each) were collected from the feed given to the chickens in the feeders by taking randomly 12 grabs (each containing approximately 75 g of feed) and sent to Toxicology Laboratory of Center for Advanced Studies in Vaccinology and Biotechnology, University of Balochistan within 24 h for further processing and analysis (Richard, 2000).

Physical properties

The samples were thoroughly homogenized to determine the physical properties (moisture content and pH). For determination of moisture content, 2 g of feed sample (replicated five times) were dried in a hot air oven for 16 h at 80°C, weighed, and the mean moisture content was calculated on percent dry basis (Magnoli *et al*., 2002). For determination of pH 50 g of feed sample (with five replicates) was homogenized with 100 mL of deionized water for 5 min using a tissue homogenizer (Edmund Buhler 7400 Tubigen H04) and the pH was measured by using a calibrated pH meter (Jenway 3510). The sub-samples (100 g each) were further processed for mycological assessment with in four hours upon arrival or, if necessary, were stored for 2-3 days in sterilized polythene bag at 4°C.

Mycological assessment

Mycological assessment involved total viable fungal count, isolation and identification of *A. flavus*, screening and frequency distribution of aflatoxin producing strains of *A. flavus*. Standard surface spread technique was used to determine the total viable fungal count following the methodology previously adopted by Magnoli *et al*. (2002). Plates containing 10-100 colony forming units (CFU) were used for total fungal count and the results were expressed as CFU per gram. Yellow-green colonies, presumptively belonging to *Aspergillus* section *flavi* were sub-cultured on SDA plates at 28°C in dark for 4-7 days regarding their subsequent identification to species level. Taxonomic identification was based on macroscopic and microscopic characteristics. Appropriate synoptic keys were followed to identify *Aspergillus flavus* (Barnett, 1960; Raper and Fennel, 1965; Singh *et al*., 1991).

A. flavus strains having the potential to produce aflatoxin B_1 were confirmed by following the methodology proposed by Hara *et al*. (1974); fungal isolates were incubated in glass petri dishes containing 30 mL of Czapek's solution agar at 28°C in dark for 10 days. The Petri dishes were autoclaved at 121°C for 2 min and the contents were added 75 mL water + 25 mL chloroform ($CHCl_3$). The aqueous slurry was blended for 5 min using tissue homogenizer. The mixture was centrifuged and then, $CHCl_3$ layer was decanted and retained. The chloroform extraction of aqueous layer was repeated and two chloroform fractions were combined, filtered (Whatman® filter paper No. 1) and evaporated to a small volume by using rotary evaporator (Steroglass Strike 202), transferred to borosilicate vial, concentrated to dryness under gentle stream of nitrogen and re-dissolved in 4 mL methanol. This final volume was used for confirmation of aflatoxin B_1 by thin layer chromatography (AOAC, 2000). Isolation frequency (IF) of aflatoxigenic *A. flavus* (AF) isolates was calculated according to Gonzalez *et al*. (2001) as follows:

$$IF\% = N/TS \times 100$$

Where, N is number of samples with aflatoxigenic AF, TS is total number of samples.

The data regarding ambient temperature, relative humidity and rain fall were recorded from the web site (Historical Weather) on daily basis.

Data analysis

The data regarding moisture, pH and viable fungal count were analyzed by using one way analysis of variance (ANOVA). The seasonal association of *A. flavus* and aflatoxigenic strain of *A. flavus* was determined by using Chi-square test (Eyduran, 2008) in the SPSS 16 for windows program. Frequency distribution procedure

and MS Excel 2010 were used for the processing and tabulation of data.

Table I.- Moisture contents (%) and pH of finished commercial broiler feed.

Season*	Moisture Mean±SD (Range)	pH Mean±SD (Range)
Summer	5.1±0.8[b] (4.2 - 6.7)	5.5±0.2[bc] (5.2 - 6.2)
Autumn	6.5±2.8[a] (4.4 - 12.5)	5.3±0.2[c] (5.1- 6.1)
Winter	5.3±0.7[b] (3.8 -7.2)	5.7±0.5[ab] (5.0- 6.2)
Spring	4.4±0.4[b] (3.8 - 5.4)	5.7±0.3[a] (5.1- 6.1)
Overall	5.32±1.7 (3.8 - 12.5)	5.5±0.4 (5.0 - 6.2)

[abc]Values within same column followed by different superscript differ significantly (P<0.05). *, Number of samples in each season was 24.

Table II.- Meteorological data of Quetta June 2009 to May 2010 (Historical Weather).

Season*	Temperature Mean±SD (Range)	Rail Fall Mean±SD (Range)	Relative humidity Mean±SD (Range)
Summer	28.38±2.4 (13.5 – 40.0)	0.01±0.1 (0.0 – 1.02)	35.6±6.3 (17 – 54)
Autumn	16.64±6.2 (-6.0 – 35.5)	0.01±0.1 (0.0 – 1.02)	46.7±8.3 (28 – 65)
Winter	6.70±3.4 (-7.5 – 24.0)	2.09±7.1 (0.0 – 39.9)	60.6±11.2 (36–92)
Spring	20.32±4.6 (0.5 – 38.3)	0.27±1.2 (0.0 – 7.9)	44.8±12.3 (16–70)
Overall	18.08±8.9 (-7.5 – 40.0)	0.59±3.7 (0.0 – 39.9)	46.9±13.2 (16 – 92)

RESULTS

The pH values of the feed samples (Table I) ranged from 5.0 to 6.2 (mean 5.5±0.4) with non-significant (P<0.05) difference among seasons, whereas moisture contents (Table I) ranged from 3.8 to 12.5% (mean 5.3±1.7%). The feed samples collected in autumn revealed significantly (P<0.05) higher (6.5±2.8%) mean moisture level.

During the data collection period, minimum and maximum temperature ranged from -7.5 to 40°C (mean 18.1±8.9°C) with a significant difference (P<0.05) among seasons. The average rain rainfall (2.1±7.1 mm) and relative humidity (60.6±11.2%) were recorded as significantly (P<0.05) high in winter season (Table II).

Viable fungal count (VFC) of the feed samples (Table III) ranged from 3×10^2 to 4×10^4 CFU g^{-1} (mean $4 \times 10^3 \pm 7 \times 10^2$ CFU g^{-1}). The significantly (P<0.05) high mean VFC was recorded in autumn ($1.12 \times 10^4 \pm 2.3 \times 10^3$ CFU g^{-1}). Overall, 44.8% feed samples (Table III) were contaminated with *A. flavus*. Among *A. flavus* isolates, 48.5% revealed the potential to produce aflatoxin B_1 (Table III). The prevalence of *A. flavus* as well as toxigenic strains of *A. flavus*, both were recorded high in autumn (66.7% and 56.3% respectively), with a non-significant association (P>0.05) among seasons (Table IV).

Table III.- Viable fungal count (CFU g^{-1}) and Prevalence (%) of *Aspergillus flavus* in finished commercial broiler feed.

Season*	Viable fungal count Mean±SD (Range)	Prevalence†(%) (Range)
Summer	$4.15 \times 10^3 \pm 2.1 \times 10^{3b}$ (7×10^2 - 8×10^3)	45.8
Autumn	$1.12 \times 10^4 \pm 11.3 \times 10^{3a}$ (7×10^2 - 4×10^4)	66.7
Winter	$1.65 \times 10^3 \pm 1.9 \times 10^{3b}$ (3×10^2 - 8×10^3)	29.2
Spring	$1.98 \times 10^3 \pm 7.5 \times 10^{2b}$ (6×10^2 - 4×10^3)	37.5
Overall	$4.72 \times 10^3 \pm 6.9 \times 10^3$ (3×10^2 - 4×10^4)	44.8

[ab]Values within same column for viable fungal count followed by different superscript differ significantly (P<0.05); *Number of samples in each season was 24; †Chi square value (χ^2=7.54, df= 95) for the prevalence doesn't fall in the rejection region (P>0.05).

Table IV.- Isolation frequency distribution (IF %) of aflatoxigenic strains of *Aspergillus flavus* in finished commercial broiler feed.

Season	TI	AF	Aft	IF% on the basis of AF	IF% on the basis of TI	IF% on the basis of SS	IF% on the basis of TS
Summer	37	11	5	45.5	13.5	20.8	5.2
Autumn	45	16	9	56.3	20.0	37.5	9.4
Winter	28	7	3	42.9	10.7	12.5	3.1
Spring	32	9	4	44.4	12.5	16.7	4.2
Overall	142	43	21	48.8	14.8		21.9

TI, total isolates; AF, *Aspergillus flavus;* Aft, aflatoxigenic strains of *Aspergillus flavus;* SS, seasonal sampling (n=24/Season); TS, total sampling (n=96).

DISCUSSION

Fungal contamination of poultry feed especially *A. flavus* is undesirable as the inhaling of spores may cause aspergillosis to the exposed community whereas, high level of fungi can affect palatability, reduce nutrient adsorption and may result in caking of feed. Furthermore, the mold has potential to produce aflatoxins. In the present study, moisture contents of the analyzed samples were below 12% which was in agreement with Addass *et al*. (2010) and Ali *et al*. (2010) reported 4 and 8%, respectively. Moisture content of finished feed is significantly affected by moisture level of the stuff used to formulate feed, manufacturing process, warehouse conditions and storage time. Maintaining low water activity (α_w) is a useful practice to reduce fungal growth on feed stuff during storage (Rosa *et al*., 2009). Water activity less than 0.65 (α_w = 0.65 equivalent to an equilibrium moisture content of 13% in cereal grain) is considered safe to limit fungal growth (Atanda *et al*., 2011). The mean pH value was recorded as slightly acidic and similar result (5.67) was reported by Gerbaldo *et al*. (2011).

Viable fungi count of poultry feed not only emphasizes the risk of mycotoxins but also it is one of the criterion to evaluate feed hygiene. In the present study total viable fungal count showed moderate values (between 3 x 10^2 and 4 x 10^4 CFU g^{-1}, mean 4.72 x 10^3 CFU g^{-1}). All of the samples were contaminated with fungi; however, most (> 90%) of the samples did not exceed the mycological hygienic feed quality limits of 1 × 10^4 CFU g^{-1} as proposed by Good Manufacturing Practices Plus (GMP+, 2010). Results concerning the fungal counts in poultry feeds from Slovak Republic have been reported by Labuda and Tancinova (2006) and from Brazil by Oliveira *et al*. (2006) with mean viable fungal count below the maximal allowable limit. On the other hand, Dalcero *et al*. (1997) from Argentina reported mean fungal count exceeding the hygienic limit. The variation in viable count might be associated with abiotic factors.

The present study revealed the occurrence of *A. flavus* in finished commercial broiler feed. Contamination of poultry feed with *A. flavus* is not an unusual phenomenon and has been reported by various researchers from different parts of the world including Argentina (Magnoli *et al*., 2002; Astoreca *et al*., 2011), Egypt (Azab *et al*., 2005), Slovakia (Labuda and Tancinova, 2006), Nigeria (Osho *et al*., 2007); Serbia (Krnjaja *et al*., 2008) and Iran (Azarakhsh *et al*., 2011). In the present study, prevalence of *A. flavus* in poultry feed was observed to be 44.79%. Azab *et al*. (2005), Saleemi *et al*. (2010) and Astoreca *et al*. (2011) reported its prevalence as 36, 10 and 49%, respectively. Abiotic factors like geographical area (Dersjant-Li *et al*., 2003), moisture level and pH value of substrate, oxygen to carbon di oxide ratio (O_2:CO_2), ambient temperature, relative humidity of the atmosphere, incubation time, light, type and quality of raw material used to formulate feed (Magnoli *et al*., 1998; Thompson and Henke, 2000; Tabuc and Stefan, 2005) and storage conditions (Azarkakhsh *et al*., 2011) considerably affect proliferation and prevalence of fungi. This not only increases tremendous fungal growth but also highly raises the risk of mycosis and mycotoxin formation.

Isolation of aflatoxigenic *A. flavus* in broiler feed is of particular interest. It is both an important indicator of mycological quality and alarm of aflatoxicosis. Among total isolated strains of *A. flavus,* almost half (49%) reflected the ability to produce AfB_1, which was in agreement with Magnoli *et al*. (1998) and Azab *et al*. (2005) who reported 47 and 45%, respectively. However, studies of Saleemi *et al*. (2010) and Gerbaldo *et al*. (2011) revealed that more than 70% isolates of *A. flavus* had the potential to produce AfB_1. The variation in the nature of *Aspergillus flavus* strains (toxigenic and non-toxigenic) might be associated with genetic differences among strains.

The prevalence of *A. flavus* and aflatoxigenic *A. flavus* in commercial feed for broiler chicken consumption emphasizes the importance of the study on fungal contamination. This is the first report from the study area describing the existing status of mycological contamination of finished commercial broiler feed. Subsequel risks and losses of fungal contamination and propagation in poultry feed or feed stuff might be limited by controlling both abiotic and biotic factors either in the field, during storage, processing and transportation. Thus, improved pre- and post-harvest practices, strict hygiene during storage processing and transportation, periodic surveillance of poultry feed and feed stuff regarding fungi and fungal toxins should be adopted in order to lessen the risk of aflatoxicosis, enhance poultry production, and to build future strategies.

Statement of conflict of interest

Authors have declared no conflict of interest.

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