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RESEARCH ARTICLE

Distribution of Various Mycotoxins in Compound Feed, Total Mix Ration and Silage

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ABSTRACT

Present study was planned to assess the spectrum of natural occurrence of aflatoxins, zearalenone, ochratoxin A and A-B trichothecenes in dairy feed, silage and total mixed rations. One hundred and seventy one samples were analyzed by chromatographic technique. In cattle compound feed, there was a high incidence of aflatoxin B_1 (97.3%) followed by aflatoxin B_2 (50.3%), aflatoxin G_1 (10.7%), aflatoxin G_2 (1.5%), zearalenone (39.3%), ochratoxin A (37.5%) and deoxynivalenol (2.9%) with average values of 29, 8, 21, 10, 862, 64 and 813 ng/g respectively. Nine samples were found tainted with T-2 toxin (282ng/g), nivalenol (285ng/g) and fusarenon-x (1625ng/g) respectively. However, frequency distribution showed that positive seventy-seven (51.6%)samples found to be contaminated with aflatoxin B_1 levels higher than permissible level of European Commission (<20ng/g). For zearalenone, forty-four (32.5%) samples were tainted with levels ranging from ≥500 to 3750ng/gi.e. higher than recommendations by European commission (<500ng/g). In contrast to compound feed, mycotoxin analysis in silage samples demonstrated the high prevalence of ochratoxin A (77.8 %) followed by AFB_1 (25%) with mean of 53 and 8.71ng/g respectively. A scrutiny of mycotoxin for total mixed ration depicted that all samples were contaminated with aflatoxin B_1 and ochratoxin A with an average of 30 and 48.5ng/g respectively. As far as multi-mycotoxin co-existence is concerned, compound feed was concurrently contaminated with two, three and four types of mycotoxins.

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INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by certain types of fungi. They invade crops in field and may grow during storage under favorable environmental conditions. The detection of fungi does not necessarily entail the presence of mycotoxins, since mycotoxin production depends on various factors such as presence of toxigenic fungi, chemical composition of the substrates, moisture content, relative humidity and time course of fungal growth (Roige *et al*., 2009; Söyler *et al*., 2012; Saleem *et al*., 2012; Saleemi *et al*., 2012b; Hameed *et al*., 2013). Mycotoxins are ubiquitous in nature; however, geographic distribution of mycotoxin has diverse variation with respect to their occurrence (Lawlor and Lynch, 2005). Contamination of feed with mycotoxins accounts for significant losses in dairy industry as well as an undesirable trade barriers for raw materials and consumable products (Wu, 2006).

For genetic selection of high milk yielding cows and good quality beef, it is necessary to add increasing quantities of digestible energy rich components in cattle diet. In domestic farming, grazing makes up a large portion of diet and intake of concentrate is limited to few percent of total feed intake. Contrarily, dairying at commercial levels, concentrate may require upto70% of the total feed intake. This may increase the chance for the contamination of more than one mycotoxin in dairy feed (Fink-Gremmel, 2008). From past few years, silage is in practice by ensiling of green crops like corn, sorghum and alfalfa for continuous supply of feed to the animals. During ensiling process, several factors such as insufficient drying, condensation, moisture content, heat, insects, and other conditions could lead to undesirable growth of fungi that can cause aerobic spoilage. Fungal growth can reduce the nutritional value of feed and there may be a possibility for production and accumulation of mycotoxins (Velazquez*et al.,* 2008; Ahmad *et al*., 2012).

Prevalence of mycotoxins in dairy ration is of dual concern. Firstly they have detrimental effect on animal health in terms of production losses, immunosuppression, carcinogenicity and estrogenic effects (Bryden, 2012). Secondly, they may jeopardize the safety of food products of animal origin that are consumed by human beings.

Experimental data and clinical experience indicated that ruminants are less susceptible to mycotoxins but according to recent studies, it was observed that some of the rumen metabolites are more toxic than parent mycotoxins i.e. conversion of zearalenone to α-zearanol. Mycotoxins impair ruminal functions by exerting antimicrobial effects on rumen microflora. Furthermore, increased rate of passage of cattle feed through the rumen may possibly overwhelm the ability of the rumen to completely denature the toxins (Fink-Gremmel, 2008).

Pakistan has temperate climatic conditions that is conducive for the occurrence of major mycotoxins like aflatoxin, zearalenone, trichothecenes, ochratoxins and fumonisins in cattle feed. For quality assurance, farmers usually focused on nutritional profile assessment. Fungal contamination was totally ignored and overshadowed by bacterial and viral infections in the past. Furthermore, indigenous data in cattle feed is scarce for commonly occurring mycotoxins except aflatoxins. In view of foregoing, present study was planned to assess the spectrum of natural occurrence of total aflatoxin (TAFS), zearalenone (ZON), deoxynivalenol (DON), ochratoxin A (OTA) and A & B trichothecenes in cattle compound feed, silage and total mixed ration (TMR).

MATERIALS AND METHODS

A total of 171 samples of dairy feed (n=149), silage (n=12) and TMR (n=10) were randomly collected from dairy high density areas for TAFS, ZON, DON, OTA and A-B trichothecenes analysis. A high performance thin layer chromatographic (HPTLC) technique was followed for the estimation of mycotoxins (Hanif *et al*., 2006). In brief, the extraction was performed for TAFS and OTA with a mixture of methanol and water (60:40; v/v) blended for 3 minutes and filtered through Whattman filter Paper No. 1. An 8 and 12ml of phosphate buffer was added in 4ml of sample extract for TAFS and OTA then pH was adjusted to 7 or 7.4 for TAFS and OTA respectively by using 0.1M NaOH or HCl as required. After pH adjustment the sample extract was loaded on AflaStar® and OchraStar® (Romer, Austria) IAC at a flow rate of 2ml/min. After washing of column with 20ml distilled water the bound TAFS and OTA were eluted with 3ml methanol at a flow rate of 0.5ml/m in (Tahira *et al.*, 2012). Furthermore the extraction for ZON, DON and A-B trichothecenes were carried out with combination of acetonitrile and water (84:16; v/v). Cleanup was carried out by using cleanup cartridges i.e. MycoSep® 226 for ZON, MycoSep® 227 and MultiStep® 216 for A-B trichothecenes (Khatoon *et al*., 2012). The purified eluants were then evaporated under vacuum by using Evap® System (Romer Labs Inc., USA). Toxins estimation was carried out by using TLC scanner (Dessaga, Germany). The limits of quantification of present method were 0.1ng/g; AFB_1/AFG_1, 0.5ng/g; AFB2/AFG2, 0.1ng/g; OTA, 75ng/g; ZON and 125ng/g for A-B trichothecenes respectively (Table 1).

RESULTS

Findings of present study revealed that all samples of TMR were highly contaminated with AFB_1 followed by compound feed and silage. Out of total samples, seventy five samples (50.3%) of compound feed were found positive for AFB_2 followed by AFG_1 (10.7%) and AFG_2 (1.3%). For ZON, prevalence was found in TMR samples (50%) followed by compound feed (39.3%) with exceeded levels as per recommendation of EC i.e. 500ppb (EC, 2006). Interestingly no silage sample was found positive for ZON. All TMR samples were observed contaminated with OTA followed by silage (77.8%) and feed (37.5%). As for as contamination of cattle feed with fusarium toxins is concerned, only compound feed samples were tainted with T-2 toxin whereas DON was evaluated only in four samples with levels below the agreeable limits (i.e. 2000ng/g) followed by NIV (285ng/g) and Fus-x (1625ng/g), respectively. Silage samples were only positive for NIV (Table 2). Data was further computed for frequency distribution and revealed the frequencies exceed the regulatory limits for Aflatoxins, ZON, and OTA (Table 3). In addition, multiple mycotoxins contamination was also explored and found two to three mycotoxins co-contamination incidences for AF, OTA and ZON (Fig. 1).

Table 1: Method outline of sample preparations for mycotoxins analysis (Hanif *et al*., 2006)

Mycotoxins		Rf	Re-dissolving solvents	Developing Solvents
Aflatoxin B_1(AFB_1)		0.45	Tol: AcN (95:5; v/v)	$CHCl_3$:Ace (9:1; v/v)
Aflatoxin B_2(AFB_2)		0.4		
Aflatoxin G_1(AFG_1)		0.35		
Aflatoxin G_2(AFG_2)		0.3		
Ochratoxin A (OTA)		0.6	Tol: AA (99:1; v/v)	Tol: AA:FA (6:3:1; v/v/v)
Zearalenone(ZON)		0.8	Tol: AcN (95:5; v/v)	Tol: EA: FA (6:2:1; v/v/v)
A-Trichothecenes	Neosolaniol (NEOS)	0.6	Tol: AcN (97:3; v/v)	MeOH:H_2O:AA (25:15:1; v/v/v)
	Diacetocripenol (DAS)	0.5		
	HT-2 toxin	0.4		
	T-2 toxin	0.3		
B-Trichothecenes	3 acetyl-Deoxynivalenol (3ac-DON)	0.85	MeOH: Ace (2:1; v/v)	Tol: Ace (1:2; v/v)
	15 acetyl-Deoxynivalenol (15ac-DON)	0.8		
	Fusarenone-x (Fus-x)	07		
	Deoxynivalenol (DON)	0.5		
	Nivalenol (NIV)	0.3		

Abbreviations: MeOH, methanol; H_2O, water; AcN, Acetonitrile; Tol, Toluene; AA, Acetic acid; Ace, Acetone, FA, Formic acid; EA, Ethyl acetate; $CHCl_3$, chloroform; Rf, Retention factor.

Table 2: Incidence (%) of various mycotoxins in compound feed, silage and total mixed ration collected from dairy farms

Mycotoxins (ng/gm)	Total mixed ration (n=10)			Compound feed (n=149)				Silage (n=12)		
	Positive (%)	Range (ng/g)	Mean (ng/g)	Sample No.	Positive (%)	Range (ng/g)	Mean (ng/g)	Positive (%)	Range (ng/g)	Mean (ng/g)
Aflatoxin B_1	100	0.1-57	30.18	149	97.31	≤0.1-198	29	25	≤0.1-23	8.71
Aflatoxin B_2	25	0.1-15	5.8	149	50.33	≤0.5-43	7.65	0	0	0
Aflatoxin G_1	0	-	-	149	10.74	≤0.1-89	21	0	-	-
Aflatoxin G_2	0	-	-	149	1.34	≤0.5-6	2.5	0	-	-
Zearalenone	50	100-700	379	135	39.25	≤100-3750	862	0	-	-
Ochratoxin A	100	0.1-230	48.5	135	37.5	≤0.1-169	64	77.8	≤0.1-262	53
T-2 Toxin	0	-	-	135	6.66	≤100-582	282	0	-	-
HT-2 Toxin	0	-	-	135	0	-	-	0	-	-
DAS	0	-	-	135	0	-	-	0	-	-
NEOS	0	-	-	135	0	-	-	0	-	-
NIV	0	-	-	135	2.22	≤150-585	285	33.3	150-600	200
Fus-x	0	-	-	135	2.22	≤150-1625	625	0	-	-
DON	0	-	-	135	2.96	≤150-1313	813	0	-	-
3ac-DON	0	-	-	135	0	-	-	0	-	-
15ac-DON	0	-	-	135	0	-	-	0	-	-

Total tests: 2081; DAS: Diacetoscirpenol, NEOS: Neosolaniol, NIV: Nivalenol, Fus-x; Fusarenon-x, DON: Deoxynivalenol, 3ac-DON: 3acetyl-Deoxynivalenol, 15acetyl-Deoxynialenol.

Table 3: Frequency distribution of mycotoxins contamination in compound feed, silage and total mixed ration

Aflatoxin B_1					
Frequency Distribution (ng/g)	<0.1*	>0.1≤20	>20≤50	>50≤100	>100-200
Compound Feed (%)	2.6	45.6	41.61	7.38	2.6
Silage (%)	75.0	16.66	8.33	0	0
Total Mixed Ration (%)	0	23	27	50	0
Aflatoxin B_2					
Frequency Distribution (ng/g)	<0.5*	>1≤10	>10≤20	>20≤30	>30-≤50
Compound Feed (%)	49.66	41.61	6.71	0.67	1.34
Silage (%)	100	0	0	0	0
Total Mixed Ration (%)	75	25	0	0	0
Aflatoxin G_1					
Frequency Distribution (ng/g)	<0.1*	>0.1≤25	>25≤50	>50≤75	>75≤100
Compound Feed (%)	89.26	8.72	0.67	0.67	0.67
Silage (%)	100	0	0	0	0
Total Mixed Ration (%)	100	0	0	0	0
Zearalenone					
Frequency Distribution (ng/g)	<50*	>50≤250	>250≤500	>500≤1000	>1000≤4000
Compound Feed (%)	60.74	0	6.66	26.66	5.92
Silage (%)	100	0	0	0	0
Total Mixed Ration (%)	50	0	35	15	0
Ochratoxin					
Frequency Distribution (ng/g)	<0.1*	>0.1≤20	>20≤50	>50≤100	>100-250
Compound Feed (%)	62.50	6.25	6.25	18.75	6.25
Silage (%)	22.22	0	11.11	44.44	22.22
Total Mixed Ration (%)	0	60	13.50	1.5	25.00

*Denotes the detection limit of analytical procedure.

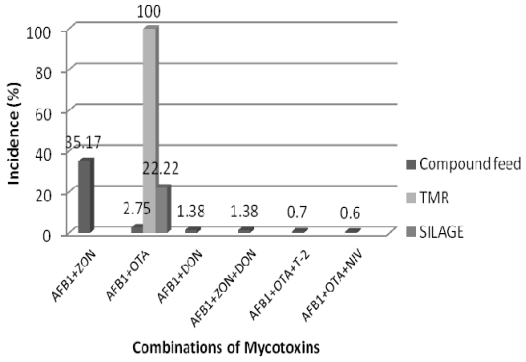

Fig. 1: Co-contamination of different mycotoxins in compound feed, TMR and silage.

DISCUSSION

Findings of present study revealed the high incidence of different mycotoxins in cattle feed. AFB_1 is most prevalent and frequently (97.3%) present mycotoxin in compound feed and average value was slightly high (i.e.29ng/g) as per recommendation of EC (2005) i.e. 20ng/g. Similar studies with respect to contamination of feed for AFB_1 have been conducted (Charoenpornsook and Kavisarasai, 2006; Akosy *et al.,* 2009). They found that 92 and 95% samples of concentrate were contaminated with average values of 7.56 and 6.81ng/g, respectively. These comparisons showed similar incidence of mycotoxin contamination but mean levels of AFB_1 was found higher (29ng/g) than their findings. Aflatoxin, the most comprehensively reviewed group of all mycotoxins is of serious concern under warm and humid climatic conditions. In Pakistan, environmental conditions are conducive for growth of mycotoxin producing fungi like *Aspergillus flavus* and *Aspergillus parasiticus*, the two *Aspergillus* species primarily responsible for aflatoxin production. Furthermore, temperature, moisture, insect damage, storage conditions and process of transportation further increase the risk for fungal growth and AFS production (Saleemi *et al*., 2010). Cereal grains and their by-products have a unique importance in animal feed from a nutritional point of view as they are primary source of carbohydrate for farmed

animal. Cereals and its related by-products are easily colonized by fungal growth; as they provide an excellent substrate in the field, after harvest and during storage. Among cereals corn is most frequently contaminated by aflatoxins, whereas among byproducts like rice polish, corn gluten feed, cotton seed cake and sunflower are highly susceptible commodities and selection of ingredients may be an additional factor for aflatoxin contamination (Saleemi *et al*., 2012a). AflatoxinB_1 is most potent mycotoxin and 100 times more toxic than rest of group members and classified as class 1A carcinogen for humans (Anonymous, 1993). It may cause liver damage, reduce feed consumption and milk production that leads to overall retarded growth and development (Akande *et al*., 2006). Aflatoxin B_1 is only major feed associated mycotoxin of concern with respect to safety of milk and milk products. Aflatoxin B_1 also has a carryover effect as it is excreted into milk in the form of AFM_1 within 12 hours with residue approximately equal to 1.7% of the dietary aflatoxin level (Diaz *et al*., 2004).

Followed by aflatoxins, the detected level of ZON (862ng/g) was found to be higher for compound feed than as reported by (Driehius *et al.,* 2008; Akosy *et al.*,2009) in which they detects very low levels of ZON. Their findings were not in accordance with the result of present study that might be due to geographical and climatic variation. zearalenone is a field mycotoxin produced by *Fusarium graminearum* and *Fusarium tricintum*. These species are common on cereals and tend to develop during cool, humid and harvest season (Amadi and Adeniyi, 2009). Ingredients like cotton seed cake and rice polish are the most susceptible commodities for contamination of ZON. Zearalenone is known due to its estrogenic effects and associated with abortion in cattle, vaginitis, vaginal secretion, poor reproductive performance and mammary gland enlargement (Driehius *et al.,* 2008)

Ochratoxin A is a member of important mycotoxins like aflatoxins, zearalenone and deoxynivalenol etc. In recent study, OTA was detected in 37.5% samples of compound feed in range of 0.1-169ng/g with a mean of 64ppb which was far greater than permissible level (10ppb) as per recommendation (EC, 2005) . Similar study was conducted by (Rosa *et al*., 2008) in which they reported that twenty-five percent samples tainted with OTA in a range of 12-324ng/g. Percentage prevalence of present finding was slightly high as compare to their study but their detected levels were not in accordance with present finding. OTA is produced by *Penicillium verrucosum* in temperate or cold climates, whereas, *Aspergillus ochraceous*, *Aspergillus carbonarious and Aspergillus niger* in warmer and tropical parts of the world. Ochratoxin A has been classified as a possible class 2B carcinogen (Anonymous, 1993) and exhibits a nephrotoxic, immunosuppressive and teratogenic effects (Hassan *et al*., 2010; 2011; 2012). It poses a health risk not only to livestock but also to human and cause economic loss due to its adverse effects on dairy cattle

Mycotoxins produced by *Fusarium* fungi on cereals are matter of great concern. The most important and largest family of fusariotoxins are trichothecenes, which are comprised of several compounds divided into four subgroups, with type A (T-2 toxin, HT-2 , DAS and NEOS) and B (NIV, FUS, 3- acetyl DON and 15- acetyl DON) being the most significant. Temperate climates are associated with the development of *Fusarium* in cereals and its by-products. Lower temperatures are required by these fungi to grow and produce mycotoxins in comparison with *Aspergillus*. These are associated with, production losses, gastroenteritis, intestinal hemorrhages and necrosis. They are also known to suppress immunity, interfere with protein synthesis and nephrotoxic (Placinta *et al.,* 1999). In view of this background, current study illustrate the natural occurrence of DON (2.96%) which was followed by T-2, NIV and FUS among trichothecenes groups (A & B). Whereas, (Charoenpornsook and Kavisarasai, 2006) reported that 86% samples were contaminated with DON with an average of 34ng/g.

As far as silage is concerned, in present study the most dominant mycotoxin was OTA (77.8%) and AFB_1 (25%) whereas (Velazquez *et al*., 2008) reported the 100% prevalence of AFB_1, OTA, ZON and DON in silage samples. During ensiling process, several factors such as insufficient drying, condensation, moisture content, heat, insects and other conditions could lead to undesirable growth of fungi which subsequently lead to mycotoxin production. Cattle's feeding is basically practiced in the form of total mixed ration (mixture of feed and silage) depending upon the availability of raw ingredients. Findings of present study revealed that incidence of mycotoxin contamination are higher in feed as compare to silage.

The severity of response to mycotoxin depends to a large extent on the presence of specific mycotoxin and the level of contamination. Co-occurrence of mycotoxins is likely to arise for at least three different reasons firstly most fungi are able to simultaneously produce a number of mycotoxins secondly commodities can be contaminated by several fungi, and feed is made from various commodities. Studies have shown that multiple mycotoxins contaminations have more severe effect than individual mycotoxin and may exert synergistic additive and antagonistic effects in animals (Grenier and Oswald, 2011).

Finding of present study illustrate that problem of mycotoxin contamination exist in dairy ration and compound feed is highly contaminated with more than one type of mycotoxin. Mycotoxins contamination of crops and ensuing consumption of contaminated feed ingredients by animal is an inevitable part of animal production system. Mycotoxins produce wide range of injurious effects in animal in addition to food borne hazards to humans. The concentration levels of AFB_1 might be injurious for animal health as well as for human due to secretion of AFM_1 in milk. The high levels of mycotoxins in the buffalo/cattle feed samples and limited data on factual contamination of feed with mycotoxin imply that more emphasis should be given to the routine inspection of dairy feed and milk for mycotoxins. There is a need to improve storage practices and adopt effective strategies for mycotoxin decontamination and detoxification.

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