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Estimation of aflatoxins in cattle feed used in and around Lahore District

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ABSTRACT

There are more than 100 types of fungi that can yield mycotoxins. They are secondary metabolites and their main producing agents are *Aspergillus flavus, Aspergillus nomius* and *Aspergillus parasiticus* that are famous for Aflatoxin B1, B2, G1, and G2. The present study was conducted to quantify the aflatoxins in bovine feed provided to cattle found in and around Lahore. Total 50 samples were collected from two different localities of Lahore and categorized in two groups: Group A (Raiwind, 30 samples), Group B (Bedian Road, 20 samples). All samples were analyzed for estimation of aflatoxins level using thin layer chromatography (TLC) method. The results showed that out of 50 samples, 20 (40 %) were fit as no level of aflatoxins detected in these samples. However, 30 (60 %) were positive for aflatoxins. 15(50 %) samples were highly contaminated and unfit. However, the other 15 (50 %) samples were within the tolerance range of aflatoxins. Aflatoxin B1 was detected in all 30 samples and aflatoxin B2 was noticed in only 10 samples. However, Aflatoxin G1 and Aflatoxin G2 were not detected in any sample. It was concluded that there is a need to regulate managerial practices to improve the quality of bovine feed.

Keywords: Aflatoxins, Fungi, Mycotoxins, Raiwind market, Bedian Road.

INTRODUCTION

Livestock such as cows, buffalos, goats, sheep, donkeys, and horses plays an important role in the national economy. Most of the animals are providing high-quality food to human beings. In Gross Domestic Production (GDP) of Pakistan, the dairy products contribute up to 46.8%. The agriculture sector is significantly contributing in the form of milk, milk products, meat, hides, skin, hair, wool and bone meal (Alao *et al.,* 2017; Rehman *et al.,* 2017; Chikwanha *et al.,* 2018; Joshi *et al.,* 2020). Agricultural productivity also plays a major role to assuage poverty in rural areas (Christiaensen *et al.,* 2006; Abedullah *et al*., 2009; Cervantes-Godoy and Dewbre, 2010). Even though agriculture is improving due to various practices such as the use of medicine, nutrition, and managerial practices. But still, the feed which is provided to the cattle is at risk of contamination (Ayofemi, 2020). Natural pasture, crop residue, improved pasture and forage, and other by-products like food and vegetable refusal are different animal feed resources, of which the first two contribute the largest feed types (Alemayehu, 2003). The cattle feed mixed with kitchen and bakery waste products may get affected by fungus and contribute to the production of mycotoxins in cattle feed (Ilyas, 2007).

Mycotoxins are chemically diverse secondary metabolites which are toxic in character and produced by different kinds of fungi (Lanyasunya *et al.,* 2005). Mainly they are produced by Aspergillus, Alternaria, Penicillum, Claviceps, and Fusarium genera (Akande *et al.,* 2006; Haque *et al.,* 2020). There are different kinds of mycotoxins which are: aflatoxins, trichothecenes, zearolenone, ocratoxin,and fumono sins. Aflatoxins are produced by some strainsof *Aspergillus flavus* and *Aspergillus parasitic*

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us (Lanyasunya *et al.,* 2005; Chilaka *et al.,* 2012). According to Peles *et al.* (2019) toxins produced by Aspergillus species are possible source for the feed and food adulterations. They affect farm animals deleteriously and can cause acute or chronic manifestations of mycotoxicoses.

Aflatoxin is produced at a temperature of 12-40°C and requires 3-18% moisture (Duncan and Hagler, 2008). They were first discovered in 1960 when more than 100.000 young turkeys on poultry farms in England died in the course of a few months from an apparently new disease that was termed "Turkey x disease" (Cordova - Izquierdo *et al.,* 2007). Aflatoxins are of different kinds such as B1, B2, G1, and G2; metabolized to aflatoxins M1, and M2 (Boudra *et al.,* 2007). Out of all types, the Aflatoxin B1 is more potent and carcinogenic which is found in dairy products and affects humans via milk (Maridgal-Santillan *et al*., 2007; Awuchi *et al.,* 2020; Tumukunde *et al.,* 2020).

Aflatoxins are of great importance because of their detrimental effects. They can cause infections and damage to organs (kidney, liver and intestine), organ systems, hepatitis, reduce production and reproduction, and increase diseases by reducing immunity in swine, poultry, and cattle (Sultana and Hanif, 2009; Zaki *et al.,* 2012; Magnoli *et al.,* 2019; Megerssa *et al.,* 2020; Kannojia *et al.,* 2020; Kemboi *et al.,* 2020; Yang *et al.,* 2020). They are also carcinogenic, mutagenic, teratogenic and immunosuppressive in nature. That's why they gain more attention than any other type of mycotoxins (Lanyasunya *et al*., 2005; Ozay *et al*., 2008; Turkoglu & Keyvan, 2019; Awuchi *et al.,* 2020; Kannojia *et al.,* 2020). They can cause vomiting, diarrhea, hepatitis, cirrhosis, miscarriages in both animals and humans (Morales-Moo *et al.,* 2020).

The physical properties of Aflatoxins show they do not have any kind of flavor and scent. Aflatoxins can be seen under the ultraviolet light because they are fluorescent in nature (Cordova-Izquierdo *et al*., 2007). Aflatoxins are soluble in different chemicals like methanol, chloroform, acetone, acetonitrile. *Aspergillus flavus* typically produces AFB1 and AFB2, whereas *Aspergillus parasiticus* produce AFG1 and AFG2 as well as AFB1 and AFB2. Tajkarimi *et al*. (2007) also report that the change of season affects the production level of aflatoxins.

In Pakistan due to hot and humid type of environment, the chances of occurrence of major mycotoxins like aflatoxin, zearalenone, trichothecenes, ochratoxin, and fumonisins are more likely in the livestock feed. So, being an agricultural country, there is also need to update the knowledge about aflatoxins on regular basis especially considering spatio-temporal variations. There was also need to report data on levels of aflatoxins in animal feed from Lahore and its vicinity as no literature was reported yet from this region. The aflatoxins has various adverse effects for animals and also deteriorating human health via food chain. This study endorsed on need of synergistic approach to support farmers and highlight the contamination levels in feed for regulatory bodies to initiate strategic policies as control measures. The present study aimed to estimate the level of aflatoxins (AFB1, AFB2, AFG1, AFG2) in the bovine feed samples that were collected from in and around Lahore and their comparison was made with the given standards.

MATERIALS AND METHODS

Types, collection and categorization of samples

Different samples of bovine feed were collected from local markets of Raiwind and Bedian road in sterilized plastic polythene bags. The total 50 samples were categorized into two groups: Group A that was composed of 30 feed samples obtained from Raiwind and Group B which was composed of 20 feed samples obtained from Bedian road.

Treatment, Preparation and extraction of samples

All the collected feed samples were dried and finely ground into powder with pester and mortar. 50 gram of each sample was measured and kept into 500 ml conical flask. Then, 225mL of chloroform and 25mL of water was added into the flasks. The samples were placed on a wrist action shaker (Burrell Scientific wrist Action TM Model 75) for 35 minutes. The samples were filtered with Whatman filter paper and filtrate remained with 50 mL of volume. The filtrates were placed on a steam bath for evaporation and leftovers allowed to cool down following the method of Harris, (2010).

Thin Layer Chromatography (TLC)

Spotting of 25 µL of test solution on TLC plate with micro syringe was done. Standard spots of 5 or 10 µL of aflatoxin (B1, B2, G1, and G2) were also spotted on the same plate as internal standard.

The TLC plate was then subjected to two TLC tanks of mobile phases. The TLC plate was developed with anhydrous ether in the first TLC tank up to till the mark. After development, the plate was removed from the tank and then dried. Then,

the plate was redeveloped and placed in the second TLC tank with acetone-chloroform concentration of 1:9. TLC plate was removed and left for drying (Fig. 2). TLC was performed using methods described for aflatoxin determination by Vladimir, (1985) and Wacoo *et al*. (2014).

For the detection and estimation of aflatoxins in the test solution spots, the plate was observed under UV light Scanner. Fluorescing intensities of sample spots were compared with those of standard aflatoxin spots. In the case of the fluorescing spot of sample lied between the standard spots, the average values of two standard spots were considered. Moreover, a very important step known as fluorescing sample spots for the aflatoxin analysis was performed. For this TLC plate was evenly sprayed with aqueous sulphuric acid (50/50 v/v) allowed to dry and then viewed under UV light (365 nm) (Fig. 1).

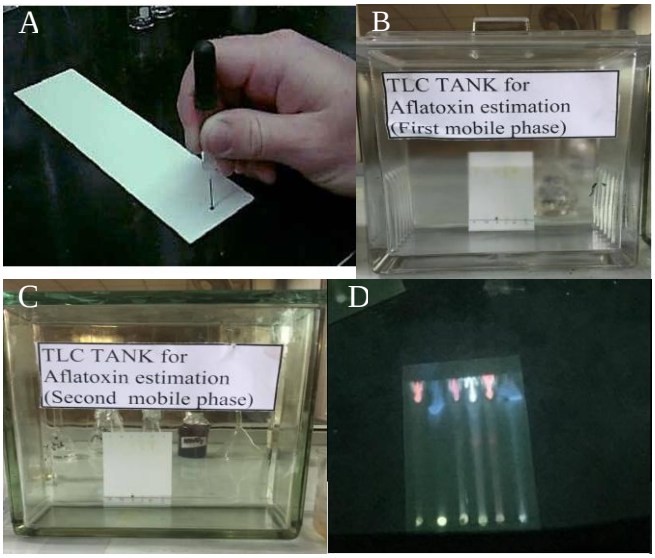

Fig. 1: A: Spotting on TLC plate, B: TLC (First mobile phase, C: TLC (Second mobile phase), D: Observation of TLC under UV light Scanner

RESULTS

The present study was conducted for estimation of aflatoxins in bovine feed. 50 samples of feed were collected from two different localities of Lahore (Raiwind, Bedian Road). The samples were analyzed for Thin Layer Chromatography (TLC).

It was noticed that out of total 50 samples, 20 samples (40%) were found as fit as aflatoxins (B1, B2, G1, G2) were not detected. Moreover, 30 samples (60%) were found contaminated with either B1 or B2 aflatoxins. B1 aflatoxin was found in 30 samples (60%) whereas B2 aflatoxin was found only in 10 (20 %) samples. However, a low level of total aflatoxin (B1, B2, G1 and G2) was found as 28.6 ppb and the highest level was recorded as 394.3 ppb (Table 1).

Furthermore, the other 30 feed samples of site A (Raiwind) and site B (Bedian) 15 (50%) had range from 20.1- 200.0 ppb for total aflatoxins and these were labeled as contaminated. Moreover, 15 samples (50%) were found with a very high level of aflatoxin (B1 and B2) ranging from 216.7-361.43 ppb and these were labeled as unfit. Ratio of fitness (F) versus contaminated (C) versus unfit (U) F: C: U was found in total 50 samples as 20:15:15, respectively. Ratio of F: C: U in 30 samples collected from Raiwind (Site A) was 11:9:10 (36.7 %, 30% and 33.3%), whereas ratio of F: C: U in 20 samples collected from Bedian road (Site B) was found as 9:5:6 (45%, 30 % and 25 %) respectively, (Table 2).

Table I: Amount of Aflatoxin (ppb)in the Samples collected from Site A (Raiwind) Site B (Bedian)

Site A (Raiwind)		Site B (Bedian)	Total (Site A+ Site B)
No. of samples	**30**	**20**	**50**
Aflatoxin B1			
Positive samples (%)	19 (63.3)	11(55)	30 (60)
Average ± SD	195.32 ± 83.24	248.6 ± 109.3	214.9 ± 95.4
Range (Min-Max)	40.2-329.3	24.1- 361.4	24.1-361.4
Aflatoxin B2			
Positive samples (%)	8 (26.7)	2 (10)	10 (20)
Average ± SD	48.3 ± 25.2	38.8 ± 48.4	46.4±27.8
Range (Min-Max)	16.5-81.3	4.5-73	4.5-81.3
Total Aflatoxin B1+ B2 + G1+ G2			

Positive samples (%)	19 (63.3)	11 (55)	30 (60)
Average ± SD	214.7±92.41	255.7 ± 115.2	229.7± 101.4
Range (Min-Max)	40.2-362.4	28.6-394.3	28.6-394.3

Table II: Number, % and Rage in ppb of different samples of two sites categorized as Fit, contaminated and unfit

Site A (Raiwind)			Site B (Bedian)		
Fit (%) Range	**Contaminated (%) Range**	**Unfit (%) Range**	**Fit (%) Range**	**Contaminated (%) Range**	**Unfit (%) Range**
11 (36.7)	9 (30)	10 (33.3)	9 (45)	6 (30)	5 (25)
ND	(20.1- 200.0)	(216.7 -361.4)	ND	(20.1-200.0)	(216.7-361.4)

DISCUSSION

The present study was conducted to quantify the aflatoxins (AFB1, AFB2, AFG1 and AFG2) in cattle feed samples. It was noticed that 40 % of the feed samples were fit as no aflatoxins detected while 60 % were contaminated with aflatoxins (AFB1 and AFB2). In a study carried out by Gizachew *et al*. (2016), 156 feed samples were used and all the samples were contaminated. Out of 156 feed samples only 16 (10.2%) contained AFB1 at a level less than or equal to 10 ppm. At the same time, 41 (26.2%) of the feed samples contained AFB1 at a level exceeding 100 ppm. While, a study conducted by Sohail *et al.* (2020) with 120 feed samples collected from the District Mansehra, it was reported that almost 92.5% of samples were contaminated with aflatoxin. Similarly, Aman Ullah *et al*. (2016) described in a study that 33 (83 %) out of 40 samples were contaminated for aflatoxin B1. However, a real time UHPLC-MS/MS technique was used for detection of B1, B2, G1 and G2 aflatoxins and only B2 aflatoxin was recovered in two samples out of 19 samples by Lopez Grio *et al*. (2010). Khayoon *et al*. (2010) investigated 42 animal feed samples with HPLC and aflatoxins B1, B2, G1 and G2 were detected with recovery of 98 ± 0.7%, 95 ± 1.0%, 94 ± 3.6% and 97 ± 4.3%, respectively. It was noticed in the present study that the recovery rate for B1 and B2 was 60 % and 20%, respectively; Han *et al.* (2013) recovered low level for AFB1 (42 %) but high amount of AFB2(36 %). Similarly, the study of Mngadi *et al*. (2008) was in line and reported that most of the cattle feed samples were with high levels of mycotoxins. Binder *et al*. (2007) reported that more than half feed samples were contaminated from 2753 in Europe while one third of the samples were found contaminated in Asian-pacific region out of 6391.

Becha and Devi, (2013) analyzed 709 samples for aflatoxins and levels of total aflatoxin widely varied from 1 to 400 ppb in feeds. While, in the present study out of total 30 positive aflatoxin feed samples, 15 (50%) had 20.07 ppb to 200.0 ppb levels of aflatoxins (B1 and B2) and 15 samples (50%) were found with a very high level of aflatoxin (B1 and B2) ranging from 216.86 ppb to 361.43 ppb and these were labelled as unfit (Table 2). The Food and Drug Administration (FDA) tolerance level of aflatoxin for breeding cattle feed was 20-300 ppb as reported by Park & Liang, (1993). According to this our study showed that collectively 73.3 % (22 out of 30) samples from both sites were in range of tolerance level but 26.6 % (8 out of 30) samples were above the tolerance limit. Liu *et al*. (2002), reported that excessive levels of these aflatoxin in feed are generally responsible for various human and animal diseases. Animals feed contaminated with aflatoxins is an important source of acute (lethargy, reproductive problems) or chronic (reduction in feed efficiency and milk production) mycotoxicoses in animals. After ingestion of contaminated feed inherent and acquired physiological properties of different animals work differently for the degradation and biotransformation of these aflatoxins. The development of particular symptoms of aflatoxicosis depends on various factors such as dose, duration of exposure, and even susceptibility of animals (Peles *et al.,* 2019). Ingestion of contaminated feed in animals has a correlation to produce contaminated milk (Gizachew *et al.,* 2016). Milk might be a possible source for aflatoxicosis in humans as it travels via food chain.

In the present study, thin-layer chromatography (TLC) method was used however, some other methods like high-performance liquid chromatography (HPLC), mass spectroscopy, enzyme-linked immune-sorbent assay (ELISA), and electrochemical immune sensor are used for the

estimation of aflatoxins in bovidae and equidae feed as described by Wacoo *et al.* (2014). Even though, each of these methods has advantages and limitations in aflatoxin analysis. However, TLC also gives reliable results that have been narrated in various studies Shannon *et al*. (1983); Storm *et al.* (2014); Wacoo *et al.* (2014).

CONCLUSION

It was finally concluded the percentage of aflatoxins was comparable to literature but the detected amount was found very high. This showed that the present study is very important and sort of a good contribution to stockholders such as public health and the Ministry of Agriculture. The study will create awareness among food and feed handlers against poor practices that contribute to aflatoxin contamination. It also focused to the need for better food storage facilities within the open air markets.

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