Research article
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Determination of Aflatoxins contamination in Corn (*Zea mays.*) used for animal feed

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Abstract
This research study aimed to estimate the total aflatoxins in corn used for livestock feed intended for breeding cattle, beef, and mature poultry. Total 60 corn samples for livestock feed were collected from different markets and feed shops in Lahore. Total aflatoxin AFB1, AFB2, AFG1, and AFG2 were estimated using Thin Layer Chromatography (TLC) and quantitative ELISA methods. According to the Food and Drug Administration (FDA), the daily intake of aflatoxin in livestock feed is a 100ppb threshold. Total 60 samples were examined; 20% were contaminated with aflatoxin B1 and B2, and the rest of 55% samples were contaminated only with aflatoxin B1. Aflatoxin G1 and G2 were not detected in any feed. The results showed that 20% of samples were contaminated within the permissible limit, 55% were unfit due to having above the allowable limit, and 25% were fit, which did not show any aflatoxin. The current study has demonstrated the easy way of determining aflatoxin in corn used for livestock feed.

***Keywords*:** Aflatoxin, Corn grain, Livestock feed, Permissible level

1. Introduction

Corn (*Zea mays.*) is the 4th significant crop harvesting in Pakistan. It plays a vital role in Pakistan's economy by providing the source of food for human consumption and feed industry. The climatic conditions in Pakistan are usually hot and humid, which ultimately promote the development of various varieties of toxigenic fungi (Francis and Burgess, 1975; Martinez et al., 1981; Zummo and Scott, 1992; Widstrom, 1996; Abdullah et al., 1998; Cardwell et al., 2004). Mostly *Aspergillus flavus*-fungus is more toxigenic as it produces some secondary metabolites named as Aflatoxins (Patterson, 1977). Aflatoxins are comprised of potent mutagenic chemical substances produced from *Aspergillus flavus* recognized by olive green color, and *Aspergillus parasiticus* by gray-green color on corn, as shown in Figure 1. Aflatoxins are divided into different types like AFB1, AFB2, AFG1, AFG2, and AFM1 (hydroxylated metabolite). Among them, AFB1 is the most toxigenic and causes different health problems like stunted growth and liver damage and may lead to cancer disease (Peles et al., 2019; Raduly et al., 2019). Also, AFB1 is the leading cause of AFM1 formation.

Figure 1: Aflatoxins present in Corn (Zea mays.)

Aflatoxins are usually found in the meat of cattle, beef, and poultry chicken or in their eggs if they consumed aflatoxins-contaminated feed and may cause serious health problems in animals and humans (Aquino and Correa, 2011). Fungal attack or aflatoxins production may have been encouraged by various environmental factors, including humidity and temperature during storage (Khan *et al*., 2005). Livestock feed is usually associated with contaminants produced or transferred from molds present in the corn to other cereals and plant proteins and causes the deterioration of feeds and foods for livestock feed intended for breeding beef, cattle, and mature poultry. Although corn plays a vital role in the feed source for livestock animals, it increases the growth of livestock animals with higher efficacy. In Pakistan, corn grain usually comprises about 60-85% of total animal's diet feed, with 15-40% diet made up of hay (roughages & forages), vitamins & minerals, and other byproducts of forages such as distiller grains. Corn grain also provides a source of energy for livestock animals as the energy obtained from grasses is not sufficient to provide enough energy to animals. Despite improving the growth efficiency, corn grain in livestock feed also increases the deposition of fats, ultimately improving the carcass quality; as a result, a consumer may obtain their desirable products.

Despite of corns abundance advantages, corn grain in livestock feed is susceptible to deleterious fungus-aflatoxins, which cause harmful diseases in humans and animals when consumed with these aflatoxin-associated feed. To prevent the aflatoxins level in corn used for livestock feed intended for breeding cattle, beef, and mature poultry by regularly monitoring the aflatoxins levels, especially AFB1 levels in livestock feed is essential (Rosa et al., 2006). The objective of this research study is to analyze the total aflatoxins in corn used for livestock feed using Thin Layer Chromatography (TLC) and quantitative ELISA methods intended for breeding cattle, beef, and mature poultry.

2. Materials and Methods

2.1. Sample Collection

Corn samples were arbitrarily purchased and collected from the local grain markets of Lahore city. We have collected 30 different samples of corn in sterile bags made of polythene with proper identification labels on them and transported them to PCSIR laboratories complex for aflatoxins analysis. Among them, 10 corn samples were collected from Garhi Shahu, 10 from Paragon Society, 10 from Allama Iqbal Town, 10 from Johar Town, 10 from Valencia, and 10 from DHA Phase 6. Their locations on global positioning system (GPS) are shown in **Figure 2**.

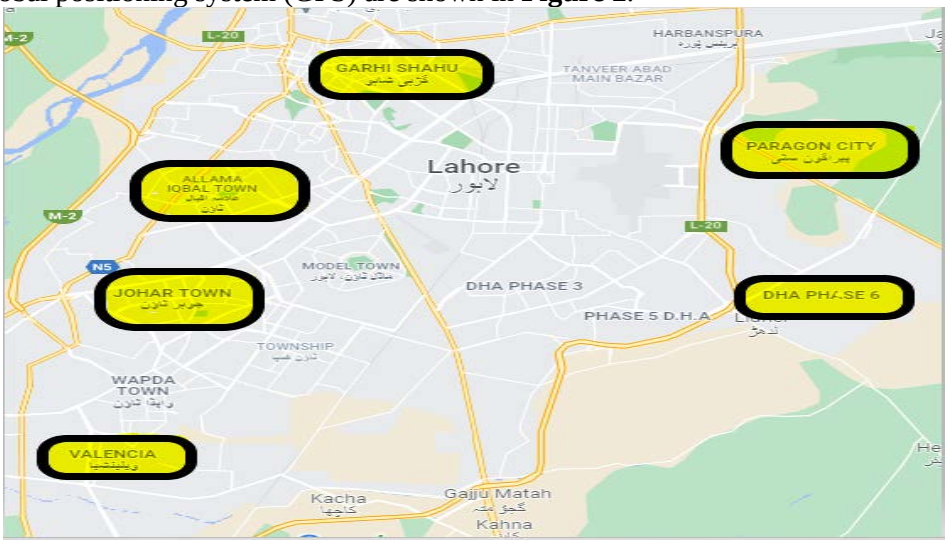

Figure 2: Representing the names and locations of specified areas of Lahore city in global positioning system (GPS) from where samples were collected.

2.2. Chemicals and Reagents

Distilled water (25mL), Diethyl Ether (Tufail Chemical &Surfactant Pvt., Ltd, and Pakistan), Chloroform (150mL) (Pharma Traders Pvt., Ltd., and Pakistan), and Acetone (Malik Textile Industries Pvt., Ltd., and Pakistan), 70%-Methanol, Horseradish peroxidase.

2.3. Glassware and Machinery

Diatomaceous Earth (25mL) (The Planters Pvt., Ltd., Pakistan), Beakers (Paul Marienfeld, Germany), Volumetric flasks, Conical flasks (250mL) (Conical Tool Co., Grand Rapids, MI), Digital Balance, Wrist action shaker (Pharma Traders Pvt., Ltd., Pakistan), Grinding mill (Glen Mills, Inc., Clifton, New Jersey, USA), Hot Plate (PCSIR Laboratories Complex, Pakistan), Filter Paper (Whatman Article No.4, US), Micro syringe 25µL (Mana Life Care Private Limited, Noida), TLC Tank, (CAMAG Chemieerzeugnisse and Adsorptions technik AG, Switzerland), UV cabinet, TLC silica gel 60 F254 plates 20×20cm, Quantitative ELISA assay kits (Neogen Corp., Lansing, MI, USA), Scanning fluorescence detector.

2.4 Assessment of Aflatoxins through TLC Method

The corn samples were passed through a grinding mill to obtain a finely crushed powder. Then sample powder of about 50 grams was added to conical flasks. Conical flasks were marked with samples number, then 25mL of water & 25gram Diatomaceous earth, and about 150mL chloroform were added. The samples were homogenized into a wrist action shaker for about 30 minutes. After that, sample filtrate was obtained in a beaker using What man No. 4 filter paper. Filtrate filled beaker was placed on a hot plate. The aflatoxin sample extract was spotted alongside the aliquots of standard aflatoxin B1, B2, G1, and G2 on percolated TLC silica plate, employing a micro syringe.

2.5. Assessment of Aflatoxins through ELISA

Total aflatoxins in corn samples used for livestock feed were determined using quantitative ELISA assay kits (Neogen Corp., Lansing, MI, USA) in which horseradish peroxidase conjugate with 70% methanol from ground samples was mixed and added to antibody-coated micro-wells. After an incubation period of about 15 minutes, the plates were washed & then

enzyme substrate was added and incubated for another 5 minutes. Finally, a stop solution is added, and the intensity of the resulting yellow color is measured using a microplate reader at 450 nm.

3. Result and discussion

For the assessment of aflatoxin, 60 samples were analyzed. It was observed that 75% of the examined samples were contaminated with aflatoxin through TLC method and ELISA's total aflatoxins method (Cardwell et al .,2002). Among them, 55% of samples were contaminated only with aflatoxin B1, while 20% were infected with aflatoxin B1 and B2, and the rest of Aflatoxin G1 and G2 were not detected in any feed. The permissible level FDA of USA, aflatoxin in corn used for livestock feed intended for breeding beef, cattle, and for poultry chicken is 100 ppb (FDA, 2000; Martinez, et al.,1987)). If the amount of aflatoxin exceeds this level, it may cause toxigenic effects on livestock animals and be considered unfit samples (Khan, et al.,2005). If the amount of aflatoxin in feed samples is within the permissible limit, these samples are considered contaminated but have fewer toxigenic effects (Peles et al.,2019). The results showed that 20% were contaminated within the allowable limit, 55% samples were unfit, which were above the permissible range, and 25% samples were fit that didn't show any aflatoxin as shown in **Table 1**.

Table 1: Representing the Aflatoxins level present in Corn intended to use in livestock feed for breeding cattle, beef, and mature poultry.

Corn Samples For Livestock Feed	**Assessment of Aflatoxin through TLC Method**				**Total Aflatoxin**	**ELISA Method**	**Moisture (%)**	**Insect Infestation**
	B1	B2	G1	G2	$B_1+B_2+G_1+G_2$	Total Aflatoxins	10-14%	(Corn Borer)
S 1	95.915	--	ND	ND	95.915	98	11.5%	Absent
S 2	110.732	--	ND	ND	110.732	120	13.5%	Present
S 3	92.912	--	ND	ND	92.912	94	11%	Absent
S 4	93.670	23.320	ND	ND	116.990	120	13.5%	Present
S 5	96.240	22.211	ND	ND	118.451	121	13.5%	Present
S 6	--	--	ND	ND	--	--	12%	Absent
S 7	--	--	ND	ND	--	--	12%	Absent
S 8	86.97	--	ND	ND	86.97	89	11%	Absent
S 9	102.811	35.231	ND	ND	138.042	152	14%	Present
S 10	90.032	--	ND	ND	90.032	93	10%	Absent
S 11	97.451	--	ND	ND	97.451	96	10.5%	Absent
S 12	97.321	--	ND	ND	93.321	92	10%	Absent
S 13	91.723	--	ND	ND	91.723	89	10%	Absent
S 14	99.232	45.431	ND	ND	144.663	155	13.5%	Present
S 15	79.324	44.212	ND	ND	123.536	134	13%	Present
S 16	83.445	24.549	ND	ND	107.994	110	13%	Present
S 17	--	--	ND	ND	--	--	12%	Absent
S 18	--	--	ND	ND	--	--	12%	Absent
S 19	92.710	23.060	ND	ND	115.770	123	14%	Present
S 20	101.976	25.360	ND	ND	127.336	139	13.5%	Present
S 21	156.108	--	ND	ND	156.108	154	14%	Present
S 22	--	--	ND	ND	--	--	12%	Absent
S 23	96.750	--	ND	ND	96.750	95	11.5%	Absent
S 24	100.021	--	ND	ND	100.021	99	11.5%	Absent
S 25	102.473	--	ND	ND	102.473	103	12%	Absent
S 26	106.990	--	ND	ND	106.990	108	12.5%	Present
S 27	--	--	ND	ND	--	--	12%	Absent
S 28	115.221	--	ND	ND	115.221	120	14%	Present
S 29	--	--	ND	ND	--	--	12%	Absent
S 30	--	--	ND	ND	--	--	12%	Absent
S 31	--	--	ND	ND	--	--	12%	Absent

S32	109.231	--	ND	ND	109.231	111	13.5%	Present
S33	99.092	23.061	ND	ND	122.153	123	14%	Present
S34	89.932	--	ND	ND	89.932	90	10%	Absent
S35	75.567	--	ND	ND	75.567	78	10%	Absent
S36	99.587	--	ND	ND	99.587	98	10.5%	Present
S37	--	--	ND	ND	--	--	12%	Absent
S38	96.657	--	ND	ND	96.657	97	12%	Absent
S39	79.989	--	ND	ND	78.989	79	11.5%	Absent
S40	97.453	--	ND	ND	97.453	99	11.5%	Present
S41	--	--	ND	ND	--	--	12%	Absent
S42	--	--	ND	ND	--	--	12%	Absent
S43	--	--	ND	ND	--	--	12%	Absent
S45	89.787	10.341	ND	ND	100.128	101	11%	Present
S46	78.982	15.672	ND	ND	94.654	96	11%	Absent
S47	102.789	21.976	ND	ND	124,765	130	14%	Present
S48	--	--	ND	ND	--	--	12%	Absent
S49	--	--	ND	ND	--	--	12%	Absent
S50	93.126	19.920	ND	ND	113.046	115	13.5%	Present
S51	105.767	12.564	ND	ND	118.331	119	13.5%	Present
S52	100.010	28.984	ND	ND	128.994	130	14%	Present
S52	121.567	14.789	ND	ND	136.356	140	14%	Present
S53	96.091	--	ND	ND	96.091	97	12%	Absent
S54	94.098	--	ND	ND	94.098	96	11.5%	Absent
S55	86.901	17.967	ND	ND	104.868	106	13.5%	Present
S56	100.891	--	ND	ND	100.981	99	12%	Present
S57	101.213	18.902	ND	ND	120.115	121	13.5%	Present
S58	99.901	--	ND	ND	99.901	100	13%	Present
S59	112.905	23.123	ND	ND	136.028	140	14%	Present
S60	104.673	23.342	ND	ND	128.015	132	14%	Present

- 15 fit samples
- 20 contaminated samples within the permissible limit
- 25 unfit samples above the permissible range

*Insect Infestation: (Corn borer & Corn earworm may transmit aflatoxins to corn grains).

4. Conclusion

The present research study concluded that the levels of aflatoxins in the given corn samples used for livestock feed intended for breeding cattle, beef, and mature poultry samples were 75%. Among them, 20% were contaminated with aflatoxin B1 and B2, and the rest of 55% samples were contaminated only with aflatoxin B1. Aflatoxin G1 and G2 were not detected in any feed. The permissible level of U.S. Food and drug administration of aflatoxin in livestock feed intended for breeding cattle, beef, and mature poultry is 100ppb. The present study is concluded that this level of aflatoxins mostly above the permissible limit. Thus, consuming average amounts of these feeds poses a health risk for the livestock animals and the consumer because it may lead to the main cause of cancer diseases in animals and in humans who consume them.

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List of Abbreviations

AFB1: Aflatoxin blue fluorescent
AFG1: Aflatoxin greenish blue fluorescent
ELISA: Enzyme-linked immunosorbent assay
FDA: Food and drug administration
TLC: Thin layer chromatography
ND: Not detected

5. Declaration

i. Ethical approval

All work has been done in PCSIR Labs. Complex LLC. With the prior permission of the competent authority.

ii. Consent for publication

This is original research work and has not been submitted and published elsewhere

iii. Competing interests

There is no conflict of interest among authors.

iv. Authors'hors contributions

All authors contributed as per their expertise

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