Prevalence of Aflatoxins B1 in livestock feeds and its awareness among selected small and medium scale farmers in Zambia

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SUMMARY

Aflatoxin B1 (AFB1) is a poisonous and carcinogenic fungal by-product produced by *Aspergillus flavus* and *Aspergillus parasiticus* species, causing acute and chronic toxicity in humans and animals. The global concern surrounding AFB1 arises from its adverse effects on public health, the economy, and social well-being. The aim of the study was to determine the prevalence of AFB1 in feeds and its awareness among small and medium scale livestock farmers. Livestock feed samples were randomly collected and analyzed in the laboratory for fungi known to produce aflatoxins using selective media. The positive samples were subjected to QSight LX50 UHPLC coupled with QSight 220 triple quadrupole Mass Spectrometer analysis to confirm presence of AFB1 and determine the quantity of the levels of AFB1. The questionnaire was employed to assess cognizance of aflatoxin among livestock farmers. On average every 27 out of 100 collected samples were found to be contaminated with aflatoxins. Poultry feeds had the highest contamination followed by feeds for other species. Further analysis revealed a significant association between storage and aflatoxins contaminations (X^2= 10.65; p-value= 0.03). The contamination ranged from 6.5 to70.2ng g^{-1} of AFB1. The study also revealed a varied levels of awareness on AFB1 between small and medium scale farmers with medium scale farmers having a relatively higher awareness on the effect of AFB1 than small scale farmers. The presence of AFB1 shows that it is necessary to implement mitigation measures that focus on reducing the incidence of aflatoxin contamination, and improvement of AFB1 awareness in small and medium scale livestock farmers.

Keywords: *Aflatoxins, prevalence, livestock feeds, small-scale, medium-scale, farmers, contamination.*

INTRODUCTION

Aflatoxin B1 (AFB1) is a fungal by-product that causes acute and chronic toxicity in humans and many other animals. Aflatoxins pose a significant health risk to both livestock and humans. In addition to their toxicological effects on animals, the carry-over of these toxins through animal-derived products, such as meat, milk, and eggs, into the human food can serve as a source of AFB1 contamination. It has been estimated that up to 25% of the world's food crops and an even higher percentage of animal feedstuffs are significantly contaminated by mycotoxins, including aflatoxins (Negash 2018; Nishimwe *et al*. 2019). Aflatoxins represent a global public health concern and are of economic significance (Jallow *et al*. 2021). The toxins have a substantial impact on both human and animal health, leading to significant losses in the egg industry due to their deleterious effects on egg production and food quality (Feddern *et al*. 2013).

Aflatoxins are highly toxic to livestock, poultry and humans (Peles *et al*. 2019). Even low concentrations of aflatoxins consumed by animals can result into death within 72 hours (Cassel and Campbell 2001). In general, at non-fatal levels, the health and productivity of animals fed on aflatoxin-contaminated feed are seriously impaired (Cassel and Campbell 2001). For example, the consumption of aflatoxin-contaminated food may lead to liver cirrhosis and cancer, stunting, reduced immunity, reduced weight gain, and even death (Kachapulula *et al*. 2017). The impact on public health, social life, and the economy, including livestock production is more significant in developing countries located in tropical and subtropical regions (Benkerroum 2020; Njoroge, 2018) due to environmental factors. The production of aflatoxins by aflatoxin-producing fungi is typically stimulated by specific factors during various stages of crop growth and storage. Conditions that favor the growth of *Aspergillus* spp include high humidity levels of around 65% and temperatures between 10°C and 40°C, which are considered ideal for storage fungi (Atanda 2011; Hashemi *et al*. 2020).
The poor feed and feed ingredient handling as well as storage conditions that are favourable for fungal growth are some of the known predisposing factors to aflatoxin contamination (Nakavuma *et al*. 2020). The presence of aflatoxin-producing moulds in feed leads to flavours and odours that reduce the palatability and nutritional value of feed, hence, affecting consumption by livestock (Feddern *et al*. 2013). The quality of animal feedstuff can directly affect animal productivity, and health and can have drastic effects on food that is later consumed by humans as final products (Alvarado *et al*. 2017). Aflatoxicosis can manifest in both acute and chronic forms and may affect various organs, leading to a range of health issues in humans and animals and beyond (Lizárraga-Paulín *et al*. 2011).

Detection of these aflatoxins could mostly be done by culture on the fungal media such as SDA and AFPA to detect presence of AFB1 producing fungi and quantification of be achieved through different methods including the use of Enzyme linked Immunosorbent assay (ELISA) and High Performance Liquid Chromatography (HPLC) (Rajendran *et al*. 2022; Khayoon *et al*., 2010). When the levels of AFB1 are above the legal limits, the consequences can be far-reaching to livestock farmers, who probably have little or no knowledge effect of the aflatoxins (Nguyen *et al*. 2018; Anjum *et al*. 2012). This study sought to investigate aflatoxin in livestock feeds and awareness among the small and medium-scale livestock farmers and assist to inform mitigation measures that can be put in place to prevent or minimize effects of aflatoxins.

MATERIALS AND METHODS

Study site and sample size

The study was conducted in Chilanga district located between Latitude 15º34'12.85"S and Longitude 28º16'12.73"E with an elevation of 1,215 meters (3986 feet) above sea level. The district shares its boundaries with Kafue district on the South-East, Mumbwa district on the West, Lusaka district on the North and Chibombo district on the North-western direction. The district consists of five (5) veterinary camps namely; Kasupe, Namayani, Chilanga, Mwembeshi and Nakachenje. The veterinary camps are designated areas within a district, and each camp is supervised by a Veterinary para-professionals (VPP) to safeguard the welfare of livestock and provide essential veterinary services to farmers and livestock owners in the community. Three (3) veterinary camps were randomly selected which are: Kasupe, Namayani and Nakachenje for the current study (Fig. 1). The climatic conditions of the district feature hot summers and warm winters with cold conditions in June and July while the average daily temperatures reach around 32ºC (90ºF). Majority of farmers in this district practice crop and livestock farming at a commercial or subsistence scale.

The district serves as a model for other geographical areas of Zambia and beyond with similar characteristics in terms of climatic and farming practices.

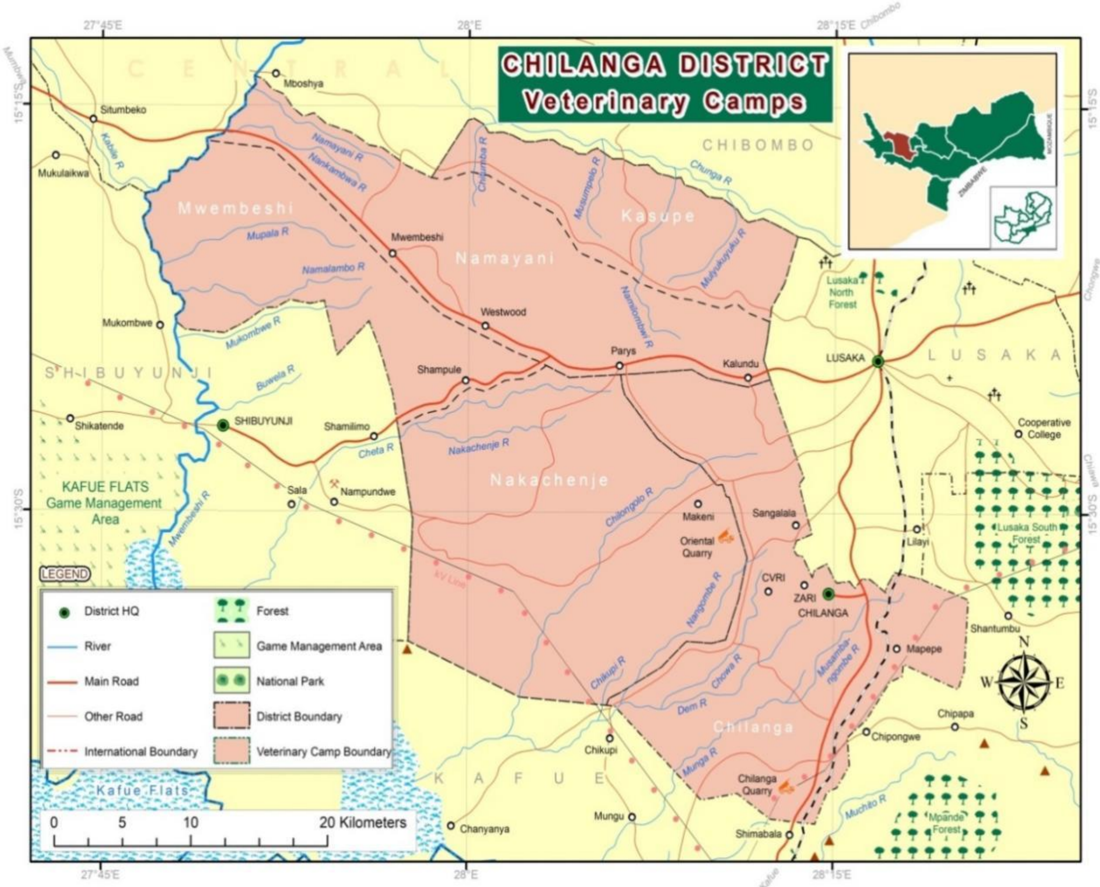


Figure 1. Veterinary camps in Chilanga district(Veterinary Department 2020)

***Sample* population**

For the sample population, the 95% level of confidence and absolute precision of 5% was used. Since there was no previous prevalence report of aflatoxin contamination in feeds in the Chilanga district, the expected prevalence of 60% from the previous study was used (Equation 1). The following formula for sample size was used (Naing *et al*. 2006):-

$$n' = \frac{NZ^2P(1-P)}{d^2(N-1)+Z^2P(1-P)}$$

Where: - n'= Sample size with finite population correction, N=Population size (140 farmers), Z= Z statistic for a level of confidence (1.96), P= Expected proportion (in a proportion of one) (60%=0.6) (Anjum *et al*. 2012), and d= Precision (in a proportion of one) (0.05). The calculated minimum sample size for the study was 101 samples.

Animal feed sampling and laboratory analysis

Approximately 50 g of feed samples were randomly collected from opened feed bags and the feeders after obtaining verbal consent from the farmers. Total of 100 samples were collected from three (3) veterinary camps within the study area, and the spatulas were used to collect the samples into sterile and air tight sample bags. The samples were labeled, and temporarily stored a cooler box and transported to the laboratory for analysis at the Central Veterinary Research Institute (CVRI). The feed samples were tested for fungal growth on sabourauds dextrose agar (SDA) and *Aspergillus flavus* and *Aspergillus parasiticus* agar (AFPA). 10 grams of each sample of animal feed was weighed and transferred into 50 mls centrifuge tubes, where forty (40) ml of 0.1% w/v peptone water was added and homogenized for 5 minutes using a vortex mixer at 300 rpm. One (1.0 ml) of the homogenized sample mixture was then added to nine (9 mls) of 0.1% w/v peptone water. The samples were cultured on SDA and incubated at room temperature (25-28ºC) for 7 days. The colonies grown on the SDA were gram stained. (The colony was identified and picked with a sterile wire loop to make a smear in the drop of normal saline and air dried. This was followed by fixing of the smear by passing through the flame three times.

The smear was then flooded with crystal violet for 15 seconds, then washed and flooded with the Iodine mordant solution for 30 seconds. This step was followed by the addition of acetone, and 10% carbol fuchsin for 15seconds and washed. After a wash the smears were air dried and examined under a microscope. Samples that showed fungal appearance, their colonies were subcultured on *Aspergillus flavus* and *Aspergillus parasiticus* selective agar (AFPA). The colonies from SDA were transferred onto the AFPA to isolate the Aspergillus spp. This was done by subculturing the colonies from each sample onto the surface plate of *Aspergillus flavus* and *Aspergillus parasiticus* agar (AFPA). AFPA plates were incubated at 28ºC and the results were read after 5 days. The colonies on the AFPA agar were gram stained and examined under the microscope as stated above.

Presumptive colonies of aflatoxigenic fungi from the AFPA agar were examined under the UV light (365 nm) for illumination from the 7th day through to the 10th day of incubation to screen for the presence or absence of blue fluorescence in the agar surrounding the colonies observed on the reverse side of the culture in glass petri dishes under fluorescence light, as described by (Khan *et al*., 2020). Positive colonies were selected for AFB1 quantification using Ultra-High Performance Liquid Chromatography (UHPLC)

Quantification of AFB1

The UHPLC system utilized in this study comprised QSight LX50 quaternary pumps, inbuilt UHPLC precision sampling injector, and QSight 220 3Q Mass spectrometer. A guard column SSP Brownlee (2.7 um C18 2.1 x 5mm) (PerkinElmer, USA) was positioned between the auto injector and the separative column, PerkinElmer Bromnlee SSP Phenyl-Hexyl (2.7 µm, 2.1 x 100 mm, part No. N9308485) (SSP Brownlee PerkinElmer, USA), and simplicity 3Q software as an integrator employed. Clean-up of extracts from animal feed was conducted using SPE columns (Waters, Ireland). The SPE columns used were AflaTest WB immunoaffinity columns (Vicam, Watertown, MA, USA) designed for detecting aflatoxins and have a total volume of 3 mL. The SPE columns employ highly sensitive monoclonal antibodies that isolate and purify targeted mycotoxins, including aflatoxins B1 (AFB1).

UHPLC-grade organic solvents from Merck-Cica and HPLC grade water were used. Methanol UHPLC grade 99.95% was obtained from Merck-Cica, while aflatoxin B1 standard was purchased from Sigma Chemical Co. Aflatoxin stock standard solutions (1 µg/ml) were prepared in methanol and stored at 4°C in the dark. A test sample was homogenized using the high shear blender in 20 ml of 80% methanol. After filtration, a 2 ml portion of the filtrate was loaded onto an Oasis prime HLB SPE column. Elution of aflatoxins was achieved with a 4:1 mixture of methanol and water. One (1 ml) of filtrate was put into 2 ml UHPLC vial for analysis. For aflatoxin separation, the mobile phase comprised methanol-water (80:20). The flow rate was 0.3 ml/min, and QSight 220 Mass Spectrometer (MS) Transition ions for Aflatoxin B1 313.3-241.3 and 313.3-285.2 were targeted for qualification and quantification respectively. Qualitative analysis employed a mobile phase of methanol-water (4:1) on SSP Brownlee columns (2.7 um C18 2.1 x 5mm and i.d 0.127mm) maintained at 35°C while that of the guard column was 2.1 x 5 mm. Aflatoxins were detected using the previously mentioned MS parameters and the injection volume was 5 µl. This is a validated in-house method tailored to the matrix and target analyte in this study, taking into account linearity, precision, limit of detection (LOD), limit of quantification (LOQ), and recovery.

Awareness on aflatoxins in the feeds

Face-to-face interviews were conducted with owners of the livestock using both open and close-ended structured questionnaires. The questionnaire survey was designed to collect information on the level of education of the participants, farm type, livestock kept by type and breed, the type of feed used, whether homemade or commercial, the storage of livestock feed used at their respective farms, the awareness on aflatoxin contamination in animal feeds and their effects on livestock productivity (Nguyen *et al*. 2018).

Data analysis

The collected data were compiled and entered in Microsoft Excel and analyzed using the EPI Info statistical package 7.2.0.1 (Communicable Disease Centre (CDC), GA, USA). Qualitative content analysis (QCA) methods of data analysis were used on qualitative data. In QCA, all the concept with the similar themes were coded with the same identity and were analysed quantitatively. Chromatographic separation of AFB1 was conducted by a PerkinElmer UHPLC system and analyte determination was achieved using a PerkinElmer QSight™ 220 triple quadruple Mass Spectrometer detector with a dual ionization source. Since the aflatoxins are positively charged, the mass detector was set in positive mode. All instrument control, data acquisition and data processing was performed using Simplicity 3Q™ software.

RESULTS

A total of 100 feed samples were collected from the study site. These samples were collected from three veterinary camps namely: Kasupe (43), Namayani (26) and Nakachenje (31). These samples were collected from twelve (12) categories of livestock farmers. The highest number of feed samples came from poultry farmers 54/100. A total of 27/100 (Chi-square (X^2) = 11.53, p-value = 0.48) samples were positive for aflatoxins. The distribution of positive samples by veterinary camps are: 8/100 Kasupe, Nakachenje 5/100 and 14/100 from Namayani veterinary camps (Table 1). The proportion of positive samples by animal types were 14/27 (51.85%) poultry farmers, 6/27 (22.22%) pig farmers, 4/27 (14.81%) poultry and pig farmers, 2/27 (7.41%) cattle and poultry farmers, and 1/27 (3.70%) cattle, poultry and pig farmers (Table 2) respectively. The results indicate presence of relationship between storage facilities and aflatoxin contamination (x^2 = 10.65, p-value = 0.03). Feed stored in the storage room 10/27 (37%) had a slightly higher proportion of contamination than those stored in the main houses 8/27 (29.6%). The proportion of feed contamination in poultry house was two times higher (6/27) than those in pig house (3/27) (Table 2). Among the 27 positive feed samples tested for aflatoxin, 16 exhibited aflatoxin B1 levels surpassing the recommended threshold of 20ng g^-1, as illustrated in Fig 2. The aflatoxin B1 concentrations varied between 6.51 and 70.2 ng g^{-1}. Notably, 59% of these samples exceeded the legal limits set by several countries, where the regulatory cap is typically 20 ng g^{-1} (0.02ppm). The highest number of samples of AFB1 concentration was within 10-19ng g^{-1} followed by 20-29ng g^{-1}. The grouping was based on the maximum residual limits (MRL) of 0.02 ppm. Any concentration less than or equal to 0.02 ppm is compliant and above 0.02 ppm is non-compliant (Fig. 3)

Table 1: Distribution of samples and storage of feed in relation to the aflatoxin contamination

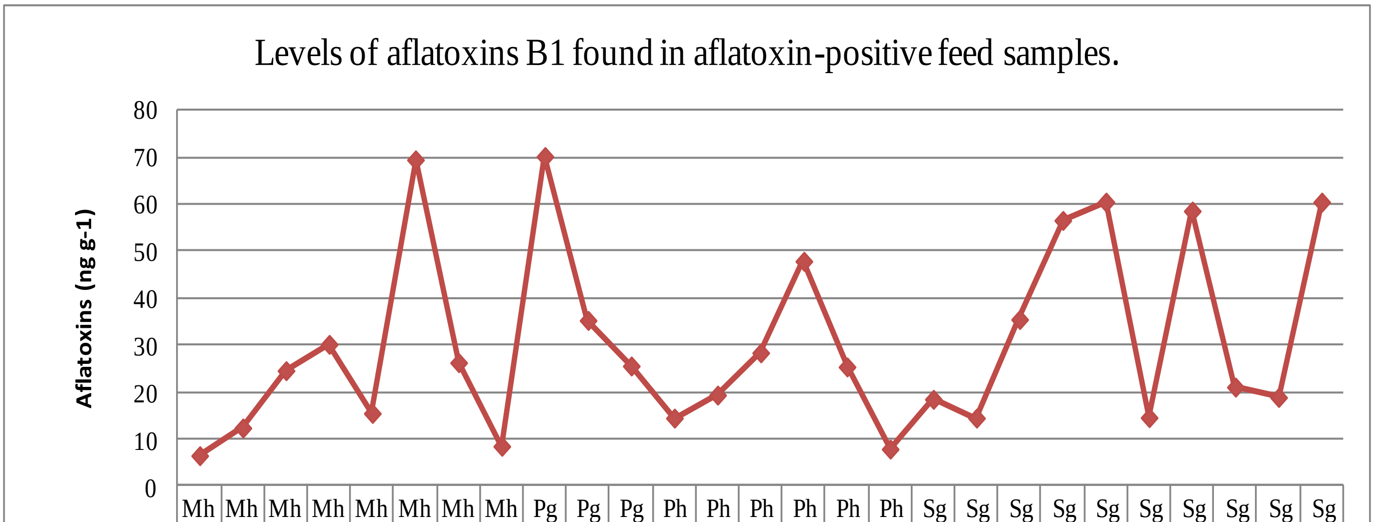
Veterinary camp	No. of samples	Positive samples	Storage facility No. of samples (Positive samples for aflatoxin): Feed room	Main House	Piggery house	Poultry house	Storage room	Chi-square & p-value
Kasupe	43	8	0	12(3)	1(1)	10(1)	20(3)	
Nakachenje	31	5	1(0)	4(2)	0	11(3)	15(0)	X^2 = 10.65
Namayani	26	14	0	6(3)	2(2)	4(2)	14(7)	p-value = 0.03
Total	100	27	1(0)	22(8)	3(3)	25(6)	49(10)	

The results are statistically significant at p<0.05.

Table 2: Prevalence of aflatoxins in livestock feeds

Type of farm	Total No. of samples collected	No. of samples Positives	% Positivity	Chi-square p-value
Cattle/poultry	3	2	7.41%	
Cattle/poultry/piggery	4	1	3.70%	
Cattle/poultry/piggery/sheep	6	0	0%	
Cattle/sheep	1	0	0%	
Goat/poultry	2	0	0%	
Piggery	14	6	22.22%	$(X^2) = 11.53$,
Poultry	54	14	51.85%	P-value =
Poultry/ducks	1	0	0%	0.48
Poultry/guinea fowl	3	0	0%	
Poultry/piggery	9	4	14.81%	
Sheep/poultry	1	0	0%	
Sheep/poultry/piggery	2	0	0%	
TOTAL	100	27	100%	

The results are statistically significant at p<0.05



	Mh	Mh	Mh	Mh	Mh	Mh	Mh	Mh	Pg	Pg	Pg	Ph	Ph	Ph	Ph	Ph	Ph	Sg	Sg	Sg	Sg	Sg	Sg	Sg	Sg	Sg	Sg
Series1	6.5	12	25	30	16	70	26	8.5	70	35	26	15	19	28	48	25	7.9	19	15	36	57	61	15	59	21	19	61

Figure 2. Levels of Aflatoxin B1 detected in aflatoxin positive feed samples. Units used=ng g^{-1}
Key: Mh=Main house, Pg= Piggery, Ph=Poultry house, Sg=Storage room

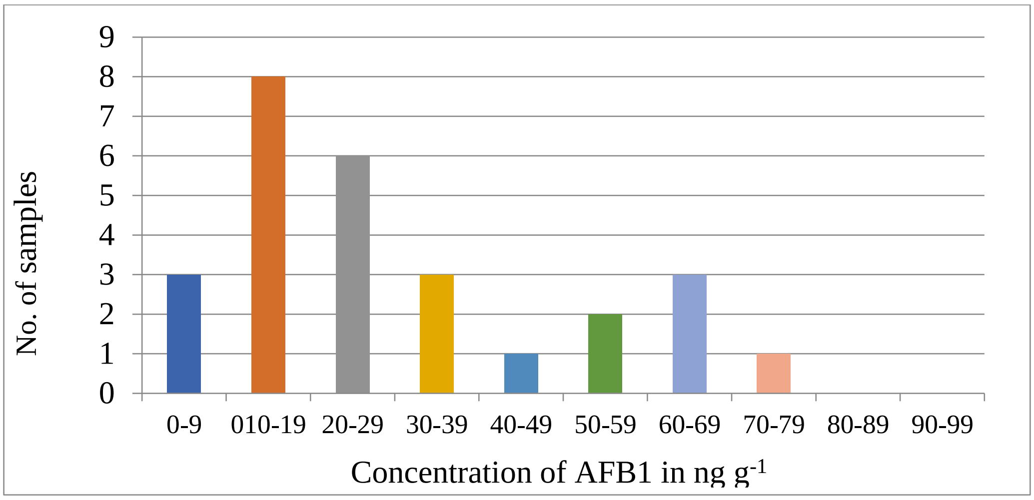


Figure 3. Number of samples for AFB1 concentration within a specified range

Knowledge of farmers on presence of Aflatoxins in the livestock feeds

There were two categories of farmers namely the small (76) and medium (24) scale livestock farmers. A total of 3/100 (3%) of the farmers who knew and understand aflatoxins were medium-scale farmers while for the small-scale farmers, were 11/100 (11%). A total of 21/76 (27.6%) and 65/76 (87.5%) of the medium-scale and small-scale farmers respectively had no knowledge of aflatoxins. Medium-scale farmers never observe any mould growth on their feed while only 2.63% of the small-scale farmers took time to check their feed for fungal growth (Table 3).

Awareness of the farmers on the effects of aflatoxins

The investigation reviewed that 3/24 (12.5%) of the medium-scale farmers and 11/76 (14.47%) of the small-scale farmers who knew the aflatoxins were able to recognise their effects on livestock production. The majority of the farmers did not understand the associated effect of aflatoxins on livestock production (Table 3). In addition, 98% did not know the moulds growing on livestock feeds.

***Table 3**: Knowledge and effect on aflatoxins of the livestock farmers*

Farm type	Total No. of Farmers	Knowledge and effect on aflatoxins	%	Mould observation on feed	% Mould observation
Medium scale	24	3	3%	0	0.00%
Small scale	76	11	11%	2	2.63%
TOTAL	100	14	14%	2	2.63%

DISCUSSION

This study showed that a relatively large proportion of animal feeds are contaminated with Aflatoxins to the levels above the legal limit set by most countries. This might be harmful to the workers and the livestock (Negash 2018). The feed stored in the storage rooms had a higher number of aflatoxin contamination. Storage conditions including humidity and temperature were not established. However, high relative humidity (RH), and poor airflow, which create an environment conducive for fungal growth. Presence of higher proportion of fungal contamination in storage rooms which are smaller than main house increases the likelihood of having favaourable conditions for fungal growth in storage rooms than the main house. The highest proportion of AFB1 contamination was found in poultry feeds (14 out of 27 samples). This could be partly due to the fact that poultry feeds samples were over represented with 54% of all collected samples or famers may be storing poultry feeds in conditions conducive for fungal growth. The higher proportion of AFB1 in poultry feeds are slightly similar to the study reported in Pakistan, where the average contamination was 23.75% (Anjum *et al*. 2012) but was lower than those described by (Naveed *et al*. 2022).

Importantly, this study revealed that, 86% of the farmers are unaware about aflatoxins. It has been reported elsewhere that farmers with limited knowledge about mould in feed, are generally unaware of the presence of aflatoxins (Rajendran *et al*. 2022). Limited awareness on AFB1 may have influenced the way animal feeds are stored, and could increase the risk of fungal contamination and AFB1. These findings align with reports from other regions, indicating that small-scale farmers often do not store animal feeds in suitable conditions (Negash 2018), and may have led to higher livestock mortalities, especially among small-scale farmers (Peles *et al*. 2019).

The existence of AFB1 above the tolerable limit and lack of awareness on AFB1 among farmers underscores the need to intensify educational campaigns to improve the health of both livestock and consumers focusing on raising awareness on AFB1 and preventive measures such as ensuring well-ventilated, temperature-controlled, and moisture-free animal feeds storage facilities. Although the study was limited to farmers, the results could be beneficial to animal feed producers by creating awareness on AFB1 and help to take precautionary measures.

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CONFLICT OF INTEREST

Authors do not have any conflict of interest

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