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ORIGINAL RESEARCH

Occurrence of Aflatoxins in Poultry Feed in Selected Chicken Rearing Villages of Bishoftu Ethiopia

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Background: Aflatoxins (AFs) are major contaminants of feed used in the poultry industry that negatively affect animal and human health. In Ethiopia, previous studies on AFs mainly considered cattle feed and milk but scarce information exists for poultry feeds.

Methods: The aim of this study was to determine the occurrence of AFs in poultry feed in selected chicken rearing villages of Bishoftu. The study was conducted from December 2018 to May 2019. Thirty-three compound poultry feed samples were collected and analyzed for aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and total AFs (AFT) using high performance liquid chromatography (HPLC). The moisture content of the samples was also determined.

Results: The result indicated that 31 (94%) from a total of 33 samples were contaminated with AFs. The mean levels of AFB1, AFB2, AFG1, AFG2 and AFT were 70.11 µg/kg, 13.50 µg/kg, 88.55 µg/kg, 18.00 µg/kg and 190.18 µg/kg, respectively. This study found AFs at a level above the limit of FDA regulatory levels of 20 µg/kg in 25 (72.75%) samples for AFT and 22 (66.67%) samples for AFB1. The analysis of moisture content of the samples, ranges from 7.33% to 11.17%, indicating all were at optimal value (<12%).

Conclusion: The study showed the high contamination of AFs in poultry feeds with optimal moisture content and hence further investigations are needed to address the cause. The study also supports the need for preventive strategies of AFs contamination in poultry feeds in Bishoftu.

Keywords: aflatoxins, Bishoftu, HPLC, poultry feeds

Introduction

Aflatoxins (AFs), a class of mycotoxins produced mainly by *Aspergillus flavus and A. parasiticus*, are major contaminants of common feed ingredients used in poultry rations.[1] There are four major naturally produced AFs known as AFB1, AFB2, AFG1, and AFG2. Also, when mammals feed on AFB1 contaminated feedstuff, the AFB1 is metabolized and aflatoxin M1 (AFM1) is excreted in their milk.[2] The International Agency for Research on Cancer (IARC) indicated AFs (including AFB1, AFB2, AFG1, AFG2 and AFM1) were classified as carcinogenic to humans (Group 1).[3] Contamination by AFs can take place at any point along the food or feed chain under a wide range of climatic conditions.[4]

In developing countries, including Ethiopia, the poultry industry offers an opportunity to feed the rising human population and to provide income for farmers.[5] However the emergence of AFs poses a negative impact on the poultry industry.[6] When AF contaminated feed is consumed by chickens, physiological parameters such as weight gain, feed intake, feed conversion efficiency, and reproductive performance are changed.[7] AFs in poultry also cause changes in biochemical and hematological parameters which enhance susceptibility to diseases.[8]

AFs are not only dangerous for health, but AFs also deteriorate the marketing quality of contaminated products; thus, involving strong economic losses.[9,10] This includes reduced egg quality, egg production, mortality of chickens, increased veterinary treatment costs, challenges in disposal of contaminated feeds or feed ingredients including the possible threat to human health.[11–15]

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Ethiopia is favorable for the growth of aflatoxigenic fungi and hence AF contamination of grains as documented by different studies.[16,17] Studies also showed that liver diseases are increasing in Ethiopia and cause the death of many individuals.[18] AF contamination of animal feeds, livestock and animal products are also increasing in the country.[19] Globally, AFs in poultry feed have been reported by different studies but data on AF levels in poultry feeds remains scarce in Ethiopia; however contaminated food of animal origin is an important source of exposure to the human including poultry products like eggs and flesh.[20–25] Regular monitoring is crucial for ensuring the safety of poultry feeds. In addition awareness of farmers about the risk factors that aggravate contamination of animal feed by AFs in Ethiopia is very poor.[26,27] Reports indicate that Ethiopia has small to large intensive poultry farms which are located in and around Addis Ababa, of which most are located in Bishoftu.[28,29] The farms have a market network with hotels, restaurants, supermarkets and shops in Bishoftu and other parts of the country. Therefore the current study investigated the occurrence of AFs levels on poultry feed samples from selected chicken rearing villages of Bishoftu, practicing small and medium scale chicken production.

Materials and Methods

Description of the Study Area

The study was conducted in Bishoftu, Oromia Regional State which is found about 47 km south east of Addis Ababa, the capital city of Ethiopia. Bishoftu is located at 8° 43′–8° 45′ N latitude and 38° 56'E longitudes at an altitude of 1850 meters above sea level in the central high land of Ethiopia. It has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26 °C and 14 °C respectively, with mean relative humidity of 61.3%.[30]

Study Design and Sample Size Determination

A cross sectional study design was used to determine the level of AFs in poultry feed during December 2018 to May 2019. Large commercial poultry farmers were unwilling to provide samples because of the fear of risks of contamination of their farm and the regulatory actions that may be taken by the government authorities if the publication of the research results is unrewarding. As a result small to medium scale poultry farms organized in micro-enterprises in Bishoftu town were used as a target of the study in collaboration with the Bishoftu town municipal office by advising the microenterprise members about the importance of the study.

The market channels for these farms are primary collectors who have market networks with hotels, restaurants, supermarkets and shops in Bishoftu. The marketing share of these farms is generally informal and difficult to state in figures.

Based on the secondary data obtained from Bishoftu municipal office there were a total of 36 small and medium scale poultry farm micro-enterprises actively functioning in 2019. To take the proper sample size first sample size was determined according to Thrusfield formula (Thrusfield, 2005) $n = (Z_\alpha{}^2 \times pq)/L^2$. Where, $Z_\alpha = 1.96$, p = expected contamination, q = 1-p = 0.5, $L^2 = 5\% = 0.05$. Since there was no prior study in the study site an expected occurrence of AFs 50% (p = 0.5) was used for the calculation and this yields n = 384. Because the sample is to be taken from a relatively small population (N = 36, ie <10,000), the required minimum sample obtained from the above was estimated to be adjusted using finite population correction $n' = 1/(1/n+1/N)$.[31] Thus, the desired sample size was $n' = 33$.

Sample Collection and Sampling

Poultry feed samples were collected from five villages of Bishoftu based on the accessibility of small and medium scale poultry farms, namely Air force (n = 3), Kajima (n = 7), Hora (n = 8), Cheleleka (n = 8) and Babogaya (n = 7). Since AFs occur in heterogeneous fashion in feed, to make a composite sample consisting of subsamples from every part of package in store, sack, or unit of feeds incremental and aggregate sample was taken. Based on the observation of this study farmers store/place feeds in different schemes. We operationally categorized the feed store/places of farms as sack, unit or store. Store (10–20 packages filled with feed), unit of feeds (2–10 packages filled with feed) and sack (1 package filled with feed). For sampling we classified the feed store/place in farms to 4 parts (lots) based on no of height/layers as: 1

sack/layer (very small height), 2–4 sacks/layers (small height), 5–9 sacks/layers (medium height), and 10–20 sacks/layers (large height). Then according to Commission Regulation No 401/2006 each lot has to be sampled separately.[32] Therefore 100 g of sample was randomly collected from one of the lots in the farm. Incremental samples were taken at various places in the lot and in the case of lots more than one package aggregate sample was taken.

Analytical Procedure

The AFs content in the feeds and feed ingredients was determined at the Veterinary Drug and Animal Feed Administration and Control Authority, Addis Ababa, Ethiopia, using HPLC and the operation of the instrument is done as per the manufacturer's Instructions.

Chemicals

All chemicals and reagents were of analytical grade and HPLC standard. The working solutions of AFB1, AFB2, AFG1 and AFG2 standard (each 50 µg/kg were purchased from Sigma-Aldrich (St. Louis, MO, USA) and prepared according to the AOAC procedure).[33]

Instrumentation

Quantitative analyses of the AFs were carried out using a HPLC unit that consisted of a pump and quaternary gradient system. A fluorescence detector was used for the quantitation under the following conditions: 360 nm excitation and 440 nm emission. The analytical column was a ZORBAX SB-C18, 150×4.6, 3.5 µm particle size. All HPLC analysis was carried out under isocratic conditions using a mobile phase of water: acetonitrile: methanol (60:25:15) and the flow rate was fixed at 1.0 mL/min. The injection volume was 20 µL and it was a standard injection with needle wash. Stop time for quaternary pump was 10 min and the temperature of the column was 35 °C on both sides (Shimadzu, USA).

Sample Preparation

Sample preparation was conducted using the method of AOAC Official method 950.02 for animal feed.[34] To achieve the maximum particle size reduction and thoroughness of the mix the entire lots of samples were ground through hammer mill and passed through a number 14 sieve split sample sequentially in sample splitter. The coarse portions were re-ground.

Extraction

20 g of ground sample was added into a beaker with 2.0 g of NaCl. Then extraction was done with 100 mL methanol/water (4/1, v/v) and 50 mL of n-hexane in a blender jar at high speed for five minutes. The extract was then passed through a pleated filter. Fourteen milliliters of the purified extract was added to 86 mL PBS buffer (pH 7.2). Finally the evenly mixed sample was filtered using a Buchner funnel and a filter paper.

Clean-Up

The AflaCLEAN^{TM} immunoaffinity column (LC Tech GMBH, Germany) was used for cleanup purposes. After opening the column, the storage buffer was drained until the level reaches the upper frit. The sample was passed through a 0.2 µm syringe filter to remove residual turbidity. Then 25 mL of the diluted extract was taken and passed through the AflaCLEAN^{TM} column with a flow rate of 2 mL/min (1 drop/sec). Then the sample was allowed to drain through the column until there is no more sample in the column. The column was then washed with 10 mL of distilled water and the residual water removed from the column by applying the gentle pressure of a vacuum pump. Finally the elution was done using 1 mL of methanol at least 2 times. The first addition of methanol was left for 5 minutes in order for the methanol to act on the column to break the AFs-antibody bond.

Calibration

The calibration curve was prepared using the working calibration solutions prior to analysis according to the method and check the plot for linearity. These solutions cover various ranges of AFs concentrations. To establish a calibration curve, 8 calibration points of a mixed AFs standard containing various concentrations per injection solvent (0.5, 1.25, 2.5, 5, 7.5, 10, 15, 20 µg/kg) prepared. Linear regression was performed using a statistical program from the amount of AFs and area under the peak as indicated in Table 1. The correlation coefficient of the calibration curve is an indicator of method

Table 1 Retention Time (RT), Level (Lvl), Amount (Amt), Areas Under Peaks and Response Factor for AFs Extracted from the Calibration Curves

No	RT	Signal	Compound	Lvl	Amt (ng/ul)	Area	Rsp. Factor	Ref.	ISTD
1	5.398	FLD1A	G2	1	0.500	0.19461	2.569	No	No
				2	1.250	0.47078	2.655		
				3	2.500	0.91999	2.717		
				4	5.000	1.824	2.740		
				5	7.500	2.691	2.787		
				6	10.000	3.510	2.849		
				7	15.000	5.130	2.924		
				8	20.000	7.004	2.856		
2	6.250	FLD1A	G1	1	0.500	0.091543	5.642	No	No
				2	1.250	0.19611	6.374		
				3	2.500	0.39042	6.403		
				4	5.000	0.77678	6.437		
				5	7.500	1.146	6.545		
				6	10.000	1.485	6.732		
				7	15.000	2.167	6.921		
				8	20.000	2.912	6.868		
3	6.758	FLD1A	B2	1	0.500	0.499952	1.001	No	No
				2	1.250	1.174	1.065		
				3	2.500	2.328	1.074		
				4	5.000	4.606	1.086		
				5	7.500	6.736	1.113		
				6	10.000	8.917	1.121		
				7	15.000	13.115	1.144		
				8	20.000	18.018	1.110		
4	7.970	FLD1A	B1	1	0.500	0.20940	2.388	No	No
				2	1.250	0.49687	2.516		
				3	2.500	0.95024	2.631		
				4	5.000	1.878	2.663		
				5	7.500	2.744	2.733		
				6	10.000	3.665	2.729		
				7	15.000	5.415	2.770		
				8	20.000	7.467	2.679		

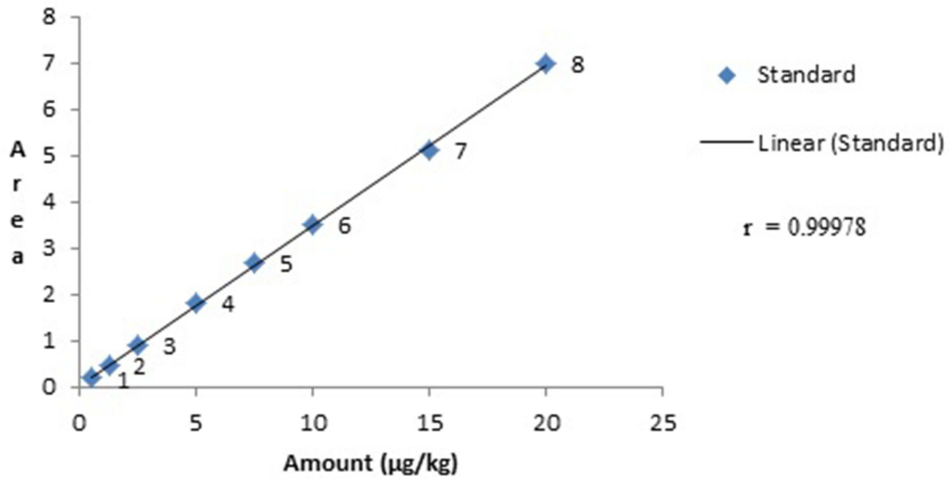

Figure 1 Calibration curve for eight calibration points of AFs standards containing various concentrations per injection solvent (0.5, 1.25, 2.5, 5, 7.5, 10, 15, 20 µg/kg), r = 0.99978.

quality. The correlation coefficient should be greater or equal to 0.995 to run the samples with it.[35,36] Accordingly the calibration found 0.99978, which secured to run the analysis as indicated in Figure 1.

Accuracy

The accuracy of the method was determined by spiking known concentration of AFs standard (10 µg/kg) to the spike feed sample and injected in duplicate to HPLC. The percentage recovery was calculated and the result was 110%. It is evident that the method is accurate within the desired recovery range as per the AOAC guideline for the acceptable recovery at the 10 µg/kg is 70–125%.[37]

Repeatability

Precision of the method was also assessed through the repeatability of the method by assaying ten duplicate injections of AFS spiked samples during the same day under the same experimental conditions. It demonstrated an acceptable RSD% with a value of 5%. The AOAC guidelines for acceptable repeatability (RSD) at 10 µg/kg is <15%.[38]

Determination of Moisture Content

Moisture contents in poultry feed samples were determined using official methods of AOAC. The samples were dried at 105 °C to constant weight, and the average content was calculated as a percentage on wet basis.[39]

Statistical Analysis

For data analysis, Microsoft Excel 2013 and IBM SPSS Statistics version 20 software were used. One-way analysis of variance (ANOVA) was performed to evaluate the mean levels of all AF types between the study villages. A P-value of less than 0.05 was considered as statistically significant.

Results

A total of 33 compound poultry feed samples were analyzed to quantify AFs using HPLC following validation of the measurement procedures as described in methodology. The results revealed that 94% of samples (n = 31) were contaminated with at least one of AFs. Moisture content range from 7.33% to 11.17% with a mean of 9.13% (Table 2).

The mean of AFB1, AFB2, AFG1, AFG2 and AFT in the samples from all villages were 70.11 µg/kg, 13.50 µg/kg, 88.55 µg/kg, 18.00 µg/kg and 190.18 µg/kg respectively. As summarized in Table 2 individual AFs as well as total AFs concentrations in samples from Air Force and Hora sites are higher than the rest of study villages. The ANOVA results revealed that, there was no statistical significant mean differences in AFT and as well as individual AFs among the five study sites (Air force, Cheleleka, Kajima, Babogaya and Hora) at level of significance 0.05 (Table 3).

Table 2 Individual Level of AFs in Compound Poultry Feeds Based on Each Study Site

Site	AFB1 (µg/kg)	AFB2 (µg/kg)	AFG1 (µg/kg)	AFG2 (µg/kg)	AFT (µg/kg)	Moisture (%)
Air-force	123.96	21.67	131.95	20.50	298.08	8.67
	58.46	11.20	65.98	13.67	149.31	8.46
	123.22	22.05	187.68	29.39	362.34	8.23
Kajima	102.25	15.84	133.52	22.16	273.77	9.17
	5.54	1.20	31.73	4.62	43.09	9.96
	11.12	1.78	10.40	1.97	25.27	9.40
	2.34	0.06	0.00	0.00	2.40	9.81
	36.49	5.96	25.51	4.75	72.71	8.19
	18.93	2.93	13.61	2.50	37.97	8.65
	29.14	4.41	20.13	3.22	56.90	8.55
Chelekleka	1.06	0.03	0.82	0.00	1.91	11.17
	3.57	0.47	3.61	0.39	8.04	10.56
	171.15	33.93	297.89	57.56	560.53	8.76
	106.23	16.87	131.45	20.86	275.41	9.72
	6.21	1.20	8.65	2.12	18.18	10.62
	10.22	1.30	7.10	0.92	19.54	7.33
	54.08	10.22	27.44	5.87	97.61	9.48
	31.09	4.40	46.65	6.76	88.90	9.17
Babogaya	147.15	29.79	132.32	31.50	340.76	8.27
	29.33	5.94	33.45	6.97	75.69	9.37
	63.30	7.42	40.86	6.86	118.44	9.36
	0.00	0.00	0.00	0.00	0.00	8.97
	0.00	0.00	0.00	0.00	0.00	10.80
	96.72	17.37	106.28	21.73	242.10	9.20
	70.52	12.07	85.56	13.99	182.14	8.73
Hora	55.39	9.99	43.08	9.20	117.66	7.96
	633.94	142.98	921.43	221.43	1919.78	9.19
	98.14	20.12	132.52	27.34	278.12	7.86
	76.71	15.85	89.56	19.54	201.66	9.12
	36.08	7.04	58.26	11.02	112.40	9.33
	21.33	3.71	23.71	4.41	53.16	9.47
	89.19	17.58	110.66	22.78	240.21	8.71
	0.66	0.00	0.22	0.00	0.88	8.99
Mean ± SD	**70.11 ± 112**	**13.50 ± 25**	**88.55 ±164**	**18.00 ± 39**	**190.18 ± 338**	**9.13 ± 0.86**

Table 3 Analysis of Variance for AFs AFT, AFB_1, AFB_2, AFG_1 and AFG_2 by Sites

Type of AFs	Mean AFs (µg/kg) by Villages					Statics	
	Air Force	Kajima	Cheleleka	Babogaya	Hora	F-Value	P-value
AFT	269.91	73.16	133.77	137.02	365.48	0.873	0.493
AFB_1	101.88	29.40	47.95	58.15	126.43	0.855	0.503
AFB_2	18.31	4.60	8.55	10.37	27.16	0.889	0.484
AFG_1	128.54	33.56	65.45	56.92	172.43	0.884	0.486
AFG_2	21.19	5.60	11.81	11.58	39.47	0.949	0.451

Discussion

AFs in poultry feeds affects the poultry production parameters including weight gain, feed intake, feed conversion, reproductive performance of the chickens; moreover it introduces risk to the food chain in humans.[23,40] The present study revealed that 94% (n = 31/33) of samples were contaminated with at least one of the AFs. Comparable to the current finding were studies from Nigeria;[41] central Pakistan;[42] Cameroon[43] revealed 90%, 91.66% and 93.3% of AFs contamination in poultry feeds respectively. Lower findings were reported from Guyana 18.5%[44] and Jordan 23.07%.[45] However reports in the literature showed that contamination of AFs can be as high as 100% in Uganda[27] and 97% in Bangladesh.[46] The direct comparisons of AFs contamination rates are challenging owing to different methodologies to detect AFs, different factors including nature AFs producing fungi, geography, climate, raw materials used to prepare feed and storage conditions of poultry feeds.

In terms of tolerance limits 72.75% (n = 25) of samples for AFT and 66.67% (n = 22) of samples for AFB1 were above the maximum tolerable level of 20 µg/kg recommended by FAO[47] and USFDA[48] thus making the feeds inappropriate for poultry use. AFs contamination was also found above the acceptable limits in previous investigations such as Kenya 67%[49] and Uganda 66.6%.[50] In terms of dose levels, our research found that practically all values (Table 1) fall within the range of 2 to 3000 µg/kg, which has been demonstrated to reduce poultry productivity.[14]

The AFs contamination rate in terms of tolerance limits in this study (94%) was higher as compared to Guyana 18.5%[44] Bangladesh 21%[46] and Jordan 23.07%.[45] Higher AFs contamination might be as a result of fungal species that are able to grow in optimal moisture content. AFs production with a wider range of moisture contents (5% to 25%) are also documented.[51]

All poultry feed samples moisture content in the study ranged from 7.33% to 11.17% with a mean of 9.13% which is below the moisture content of 12% as benchmarked by the Ghana Standard Authority[52] and the Nigerian industrial standard.[53] The low moisture contents in samples below 12% were in agreement with Nigeria 4%,[54] Peshawar Pakistan 8%[55] and 5.3% in Quetta Pakistan.[56] The results of this study are contrary with the factors that influence AFs production illustrated by different reports.[57,58] However some reports show a negative correlation between moisture and mould[59] and also some fungal species in the *Aspergillus* family have the ability to develop with a lower moisture content.[56,60] Moisture may not be a limiting factor for AFs production, and more research is needed to answer this topic.

Overall, the high levels of AFs contamination in feed should be of concern for the poultry sector, as AFs can seriously impact the poultry industry.[61] In addition eggs, poultry meat, and/or products, are consumed as part of the daily diet for many people and therefore consumers may have a chance of suffering from complications arising from the use of contaminated poultry products.[62–64] It has been mentioned that AF residues in eggs were found following the administration of AF contaminated feedstuffs. According to reports of AFs carryover, between 0.01% and 0.07% of the AFs in feed appeared in eggs.[65,66] Finally, society as a final customer must pay exorbitant costs of increased regulation and research, low exports and high imports and treatments.[67]

Conclusion

This study revealed the occurrence of AFs in poultry samples from Bishoftu Ethiopia. The study documented that 25 (72.75%) samples for AFT and 22 (66.67%) samples for AFB1 were above the limit of FDA regulatory levels of 20 µg/kg for poultry feed. In these regards feed manufacturers should ensure the ingredients and finished feed are to the expected quality. High AFs contamination rates were also found in feeds with optimal moisture and hence further investigations are needed to address the cause. Therefore the study supports the need for preventive strategies of AFs contamination in poultry feed and involvement of regulatory bodies in the poultry industry in Bishoftu town.

Abbreviations

AOAC, Association of Official Analytical Chemists; µg/kg, microgram per kilogram; ANOVA, analysis of variance; FAO, Food and Agricultural Organization; HPLC, high performance liquid chromatography; ISTD, internal standard; USAID, United States Agency for International Development; USFDA, United States Food and Drug Administration.

Data Sharing Statement

All datasets that have led to the drawn deductions in the manuscript are herein presented in the paper.

Ethics Statement

Not applicable. The study does not involve human and animal subjects. It uses feed samples from farms; however verbal informed consent was obtained from the farmers' to collect feed samples as suggested by institutional animal research ethics review committee (Guideline ARERC 2013) at the College of Veterinary Medicine and Agriculture of the Addis Ababa University.

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Author Contributions

All authors contributed to study conception, study design, execution, acquisition of data, and data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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