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Research Article

Aspergillus species and Aflatoxin Contamination of Pre and Post-Harvest Maize Grain in West Gojam, Ethiopia

Masresha Ahmed Assaye[1]*, Negero Gemeda[2] and Geremew Tassew weledesemayat[3]

[1]*Department of Internal Medicine, College of Health Sciences, Addis Ababa University, King George VI St, Addis Ababa, Ethiopia*

[2]*Ethiopian Public Health Institute, Addis Ababa, Ethiopia*

[3]*Food, Medicine and Health Care Administration and Control Authority of Ethiopia, Addis Ababa, Ethiopia*

Abstract

Contamination of maize by aflatoxins is of major concern because of the health hazards associated with it. Therefore, the present study was designed to assess the level of *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize grain. Results indicated that from fifteen pre and fifteen post-harvest maize samples 77.7% of pre-harvest with level range from 3.13 to 63.66 µg/kg and 80% of post-harvest sample with level range from 9.02 to 139.8 µg/kg were contaminated by total aflatoxin. The mean total aflatoxin contamination was 18.38 µg/kg for pre-harvest and 43.36 µg/kg for post-harvest. Aflatoxin B1 was detected in 66.7% of pre-harvest maize with the mean level of 5.00 µg/kg and in 87.7% in post-harvest maize with the mean level of 9.86 µg/kg. To be precise, paired t-test statistical analysis for total and aflatoxin B1 in maize samples were showed that both total and aflatoxin B1 increased significantly from pre-harvest to post-harvest maize ($p<0.05$). About 66.7% of pre-harvest and 86.7% of post-harvest maize samples were exceed the acceptance limit of total aflatoxin and aflatoxin B1 recommended by European Union maximum limit. *Aspergillus* species contaminations in pre-harvest maize 53.3% of samples were contaminated by *A. flavus* = 26.7%, *A. parasiticus* = 13.3% and *A. niger* group = 13.3% and in post-harvest maize 79.9% of samples were contaminated by *A. flavus* = 46.6%, *A. parasiticus* = 20.0% and *A. niger* group = 13.3%. In conclusion, the results of the present study revealed that although it was expected that pre-harvest maize to have minimal *Aspergillus* and aflatoxin contamination, the contamination was high in pre harvest and significantly increase from pre-harvest to post harvest.

Keywords: Aflatoxin; *Aspergillus* species; Pre and post-harvest; Maize, Mycotoxin

***Corresponding author:** Assaye MA, Department of Internal Medicine, College of Health Sciences, Addis Ababa University, P.O. Box 2380, Addis Ababa, Ethiopia, Tel: +251 911347186; mail: masreshaman1@yahoo.com; masresha.ahmed@aau.edu.et

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Introduction

Maize is the third most important crop after rice and wheat cultivated in the world. It is a food that is part of the staple diet in Sub-Saharan Africa [1]. Maize is Ethiopia's leading cereal in terms of production, with 6 million tons produced in 2012 by 9 million farmers across 2 million hectares of land. Over half of all Ethiopian farmers grow maize, mostly for subsistence, with 75% of all maize produced being consumed by the farming household. Currently, maize is the cheapest source of calorie intake in Ethiopia, providing 20.6% of per capita calorie intake nationally. Maize is thus an important crop for overall food security and for economic development in the country [2]. However, the grain is vulnerable to degradation by mycotoxigenic fungi which include *Aspergillus, Fusarium* and *Penicillium* [1].

Aspergillus species are the most common toxigenic species in various grains, legumes, oil seeds and foods and feeds. The two most agriculturally important species are *Aspergillus flavus* and *A. parasiticus,* which are found through-out the world, being present in both the soil and the air [3]. Among the 18 different types of aflatoxins identified, the major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2). Aflatoxin B1 is found widely and in greater concentrations than other naturally occurring forms of aflatoxin throughout the world in foods such as maize, peanuts and peanut products, cotton seed and its extractions, and to some extent, chillies, peppers, and pistachio nuts [4].

The safety of food and feed for human and animal consumption should be of top most priority with regards to the regulation of agricultural and food industries [5]. Mycotoxin attracts worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and trade [6]. Aflatoxin contamination is a serious food safety problem throughout the world [3] and it is not only a potential source of health hazards but is also involved in the spoilage of agricultural commodities [7]. The regulations on the import and sale of aflatoxin-contaminated food products results in huge losses each year to the agriculture and feed industries [4]. Maize is one of the major cereals crops of global importance, and has always been an important commodity to be traded overseas as food, feed and an industrial grain crop in several countries. Unfortunately, it is also vulnerable to the growth of aflatoxigenic fungi, resulting into subsequent aflatoxin production which causes major yield and economic losses [6]. The Food and Agricultural Organization (FAO), estimates that between 25% and 50% of agricultural crops worldwide is contaminated by mycotoxins [8]. The estimated value of maize lost to aflatoxin is $225 million per year, out of the $932 million due all the mycotoxins in the United States [6].

In recent years, data on mycotoxins of maize in Africa have begun to accumulate with reports, for instance, from Kenya [1], Nigeria [9] and Benin [10]. In Ethiopia there are some reports but they have limited information on the occurrence of *Aspergillus* species and aflatoxins in pre and post-harvest maize. Habtamu Fufa [11] has done on most commonly consumed agricultural commodities in some part of Ethiopia and Amare Ayalew [12] has done some work on mycotoxins

and surface and internal fungi of maize in three cities (Dire Dawa, Adama and Ambo). In general there is limited works has been done in pre-harvest and post-harvest maize in the world. Therefore the present study is designed to assess the level of *Aspergillus* and Aflatoxin contamination in pre and post-harvest maize products in west Gojam Ethiopia.

Materials and Methods

Study site

Mirab Gojjam (or "West Gojjam") is a zone in the Amhara region of Ethiopia, with an area of 13,311.94 square kilometers. West Gojam of the Amhara region is the highest maize-producing zone not only in the region but also in Ethiopia. Based on the five years data the average annual production of maize this zone is 3,209,274 quintals. This amount represents 46% of the regions and 10% of the national average production. The study was conducted in three woredas: Burie (Latitude (N)10°42′, Longitude (E) 37°4′E, Altitude 2091 m, annual average temp. 14-24°C, annual rain fall 1200 mm. Population 23292; Finoteselam (Latitude (N) 10°42′, Longitude (E) 37°16′, Altitude 1917 m, annual average temperature 9.8-23.5°C, annual rain fall 1250 mm. Population 25913); Jabitehnan (Latitude (N), 10°41′53″ Longitude (E) 37°10′35″, Altitude 1500-2300 m, annual average temperature 14-32°C, annual rain fall 1250 mm, Population 231,232) [2,13].

Sample size and sampling method

A total of 30 samples of 15 for pre-harvest collection in December 2014 and 2015 for post-harvest were collected from farmers in 3 woredas of West Gojam Zone (Burie, Finote Selam and Jabitehnan). Five for pre-harvest and five for post-harvest samples from each woredas were collected. For pre-harvest samples were collected through Systematic random sampling in which maize grown and storages within 10 km radius of the respective woredas and samples for post-harvest were collected from the same farmers.

Samples collection and transportation

For pre-harvest, 1 kg of each maize samples were collected from the standing maize in the field from each farmers immediately visually assessed for insect damage, discoloration due to fungal agents and these samples were taken immediately to the laboratory, hand-shelled and sun dried for 2-3 days and the samples were then divided into two; one third of the sample for mycological analysis and two-thirds for aflatoxin analysis packed and stored in bags at 4°C in the laboratory until they were be analyzed.

For post-harvest 1 kg of each sample was collected after 3 month of harvest during March 2015, the collected maize samples were placed in clean polyethylene bag with hermetic sealing, labeled and shipped to the laboratory of Food Science and Nutrition Program, Addis Ababa University. The samples were then divided into two; one third of the sample for mycological analysis and two-thirds for aflatoxin analysis packed and stored at 4°C in the laboratory until they were analyzed.

Laboratory studies

Determination of moisture content: Moisture contents of the maize samples were determined according to using the official method 925.05 [14].

HPLC-FLD determination of aflatoxins in maize sample: Validation for HPLC-FLD detection of aflatoxins in maize samples was made in accordance with method validated by Faculty of Pharmaceutical Science Laboratory of Food Analysis Ghent, Belgium, Europe for the determination of aflatoxin in maize sample. This method has been widely used for the determination of aflatoxins in food; no post column derivitization needed Robust; easy and relatively fast procedure for sample preparation; Quick, Easy, Cheap, Effective, Rugged, and Safe.

Sample preparation: Samples were homogenized prior to milling and a representative sample were taken and milled to a desired particule size (0.5 mm - 1.0 mm).

Mycotoxin standards and chemicals: The chemical and reagents used for aflatoxin analysis were HPLC grade acetonitrile, methanol, n-hexane, $MgSO_4$ anhydrous salt, NaCl, aflatoxin standard and deionized water. The AFG1, AFG2, AFB1 and AFB2 standards were obtained from Sigma-Aldrich. The pure reference standards were stored in dark place at 4°C. The *Aspergillus* species isolation and identification equipments and chemicals utilized were incubator, petridish and Potato Dextrose Agar (PDA), 10% sodium hypochlorite solution, and ethanol absolute (99.7%).

Calibration curve: Five-point calibration curve graphs were obtained using concentration of 1-16 ppb of AFG2, 5-80 ppb of AFG1, 1-16 ppb of AFB2 and 10-160 ppb of AFB1.

Extraction and clean-up: Sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 ml) and 5 ml de-ionized water was added and vortex briefly, and then the samples were allowed to stand for 30 min. But spiked samples were left for equilibration for 30 mins before addition of water. After 30 min, 5 ml of extraction solvent (100% ACN) was added and briefly mixed using a vortex mixer and then it was shacked for 30 min at position 7 using an end-over-end shaker, subsequently, $MgSO_4$ anhydrous salt 2.0 ± 0.05 g and NaCl 0.5 ± 0.01 g were added, shaken briefly to prevent agglomeration of the salts and was mixing using a vortex for 2 min. Afterwards, the tube was centrifuge at 4000 x g for 15 min. Then 4 ml of the top organic layer was transferred to a new tube and evaporated under N2 at 40°C. To the residue 200 µl of injection solvent (A : B 50/50) and 200 µl of n-hexane was added and dissolved using a vortex, filtered with micro filter and Centrifuge for 10 min at 10,000 x g. Finally 150 µl was collected from the lower phase into HPLC vials.

Chromatographic conditions: The HPLC analyses were carried out with Agilent 1100 system, consisting of a degasser, binary pump, auto sampler, column oven and a fluorescence detector. The chromatographic separation was performed with the Zorbax SB RP C18, 150 × 4.6 mm, 5 µm chromatographic column. The mobile phase was of A: Milli Q water (100%) and B: MeOH/ACN (71.5/28.5, v/v) With the gradient elution profile as follows: 25% B at 0 min, 25% B at 1 min, 40% B at 12 min, 50% B at 25 min, 100% B at 28 min, 100% B at 30 min, 25% B at 32 min and 25% B at 35 min. The column temperature was 30°C at the flow rate of 1.5 ml/min to achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 20 µl for both standard and sample solutions.

Isolation and identification of fungi: Fifty seeds per sample were surface sterilized with 10% Sodium hypochlorite (NaOCl) solution for 1 min, followed by immersion in sterile distilled water for 1 min. Surface sterilized seeds were then placed on freshly prepared Potato Dextrose Agar (PDA) plates (five seeds per plate) within which 0.05 mg of streptomycin sulphate has been added to suppress the growth of bacteria in petri plates and incubated for three days at 25°C. Pure cultures of different out growing fungi were obtained by transferring

fungal colonies to new PDA plates using sterile toothpicks, and incubating the plates for 5-7 days at 25°C. Pure cultures of each isolate were then stored at 4°C in vials containing 2.5 ml of sterile distilled water for further use.

Species identification: Isolates were identified to a species level based on morphological (phenotypic) features as described by Abdi et al., Negero et al. and Cotty et al. [15-17]. For this purpose: Isolates representing each pure culture were grown on PDA at 25°C for 5-7 days. Variations in growth rate and thermo-tolerance were also used in identification of *Aspergillus* species. *Aspergillus* colonies are downy to powdery in texture. *Aspergillus fumigates* is a thermo-tolerant fungus and grows well at temperatures over 40°C. This property is unique to *Aspergillus fumigatusis* among the *Aspergillus* species. *Aspergillus flavus* can be readily distinguished from other *Aspergillus* species by lack of growth at 5°C, by rapid growth at both 25 and 37°C and by the production of a bright yellow-green conidial color [16].

Data processing and analysis

Data obtained from laboratory were entered into Microsoft Excel sheets and exported the software SPSS version 20.0 for analysis. Chi-squared or Fisher Exact tests, when appropriate, were used to verify the statistical significance. Student's t-test for paired samples was used to see if there was significance difference in aflatoxin level between pre and post-harvest samples. The data were also analyzed by using one way Analysis of Variance (ANOVA) and Least Significant Difference (LSD). P-value of <0.05 was considered to be significant.

Results and Discussion

Moisture content of pre and post-harvest maize

Moisture level of pre-harvest maize varied between 12.99-15.71%, while the post-harvest sample recorded moisture levels of 10.23-13.81%. The average moisture content of pre and post-harvest maize was 14.38 ± 0.95 and 12.40 ± 0.94, respectively.

Crop is usually physiologically mature 7-8 weeks after flowering, at which time the grain contains 35-40% moisture and has maximum dry weight. This is the time at which the crop should be harvested to avoid unnecessary losses in the field. However, in Ethiopia, the time and method of harvesting maize depend on weather conditions, the size of the crop and how quickly the farmer wants to utilize the crop. In this study, samples were collected 2-3 weeks before harvest but the maize aged more than 4 month after flowering and average moisture content of 14.38 ± 0.95 which was unacceptable. Timing of harvest greatly affects the extent of aflatoxin contamination. Extended field drying of maize increased insect infestation and fungal contamination. Aflatoxin levels increased by about 4 times by the third week and more than 7 times when maize harvest was delayed for 4 weeks [18].

Validation of HPLC-FLD method for aflatoxins analysis in maize sample

Validation for HPLC-FLD detection of aflatoxins in maize samples was made in accordance with method validated by Faculty of Pharmaceutical Science Laboratory of Food Analysis Ghent, Belgium, Europe for the determination of aflatoxin in maize sample. The analytical methodology was validated in terms of linearity, reproducibility, repeatability; percent recovery Limits of Detection (LOD) and Quantification (LOQ). Retention times in naturally contaminated samples and standards containing aflatoxins AFG2, AFG1, AFB2 and AFB1 were: 19.228, 21.428, 23.343 and 25.81 min, respectively (Figure 1).

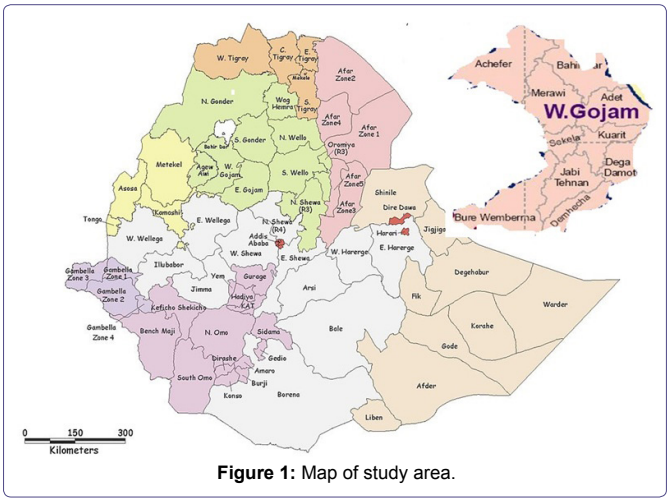

Figure 1: Map of study area.

Five-point calibration curve graphs were obtained with concentration of 1-16 ppb of AFG2, 5-80 ppb of AFG1, 1-16 ppb of AFB2 and 10-160 ppb of AFB1. Calibration graphs were drawn by linear regression of the least-squares method using the peak area of standard as response versus concentration. The correlation coefficients were >0.998, which was considered as evidence of an acceptable fit of the data to the regression line [19].

The test for the precision of the method was checked and verified by repeatability, inter-day and intra-day precision as shown in table 1. It was checked by injecting Aflatoxin (G2=1, G1=5, B2=1 and B2=10) at the low level concentration and Aflatoxin (G2=8, G1=40, B2=8 and B2= 80) at the high level concentration for 5 times on the same day for inter day precision. Similarly, for the inter-day precision similar concentration was analyzed on different days.

As shown in table 1, Relative Standard Deviations (RSDr) for within-day samples for peak area at the low level assayed concentration were G2=3.26, G1=0.75, B2=0.82 and B1=3.39 and G2=3.26, G1=0.75, B2=0.82 and B1=3.39 at the high level. Similarly, for retention time Relative Standard Deviations (RSDr) for within-day samples at the low level assayed concentration were G2=0.05, G1=0.06, B2=0.05 and B1=0.05 and G2=0.23, G1=0.24, B2=0.21 and B1=0.19 at the high level.

Similarly, Relative Standard Deviations (RSDr) for inter-day samples for peak area at the low level assayed concentration were G2=1.37, G1=2.18, B2=0.1.39 and B1=5.5 and G2=0.85, G1=5.04, B2=1.48 and B1=5.25 at the high level. Similarly, for retention time Relative Standard Deviations (RSDr) for inter-day samples at the low level assayed concentration were G2=0.15, G1=0.15, B2=0.16 and B1=0.15 and G2=0.04, G1=0.03, B2=0.03 and B1=0.03 at the high level (Table 1). Relative standard deviations for retention time and peak area of five replicates were <0.2 and <6% respectively, which showed high precision [19,20].

Limit of Detection (LOD) is the concentration of analyte which induce signal (S) that is 3 times higher than the background noise level (N). S/N=3 The detection limits in this study were 0.03, 0.3, 0.014 and 0.15 µg/kg for aflatoxins B1, B2, G1 and G2, respectively, calculated based on three times the standard deviation of the noise (Table 1).

The Limit of Quantitation (LOQ) of the aflatoxins in sample is the concentration of analyte which induce Signal (S) that is 10 times higher than the background noise level (N). S/N=10. The Limit of Quantitation (LOQ) were 0.11, 1.28, 0.05 and 0.5 µg/kg for aflatoxins B1, B2,

	Spike level (µg/kg)	Area and Retention Time	AFG2	AFG1	AFB2	AFB1
Limit of detection (µg/kg)			0.03	0.3	0.14	0.15
Limit of quantification (µg/kg)			0.11	1.28	0.05	0.5
Repeatability, RSDr (%)	G2=1, G1=5	Area	3.26	0.75	0.82	3.39
	B2=1 and B1=10	Retention time	0.05	0.06	0.05	0.05
	G2=8, G1=40	Area	0.5	5.13	1.12	5.3
	B2=16 and B1=80	Retention time	0.23	0.24	0.21	0.19
Reproducibility, RSDr (%)	G2=1, G1=5	Area	1.37	2.18	1.39	5.5
	B2=1 and B1=10	Retention time	0.15	0.15	0.16	0.15
	G2=8, G1=40	Area	0.85	5.04	1.48	5.25
	B2=16 and B1=80	Retention time	0.04	0.03	0.03	0.03

Table 1: Validation of Method for Analysis of *Aflatoxins*.

G1 and G2, respectively, calculated based on 10 times the background noise level (Table 1).

The accuracy of the method was measured from analytical recovery in duplicate sample at spike levels of aflatoxin (G2=4, G=5, B2=4 and B=40 *µ*g/kg). Recovery percentage of each spiked sample was calculated as recovery = (amount found/amount added) x 100. The average recovery were AFG2=92.1%, AFG=82.8%, AFB2=97.5% and AFB1=98.3%. The average recoveries for spiked samples ranged from 82.8-98.3% (Table 2). The recoveries obtained were within the range of 70 to 125%, which were acceptable according to AOAC International guidelines for method validation [20].

Aflatoxin	Aflatoxin concentration in sample (µg/kg)	Aflatoxin level added (µg/kg)	Result found (µg/kg)		Replicate % recovery	Average recovery %	RSD %	
			1	2	1	2		
AFG2	0	4	3.81	3.56	95.25	89.03	92.14	4.8
AFG1	12.28	5	14.05	14.56	81.31	84.26	82.79	2.5
AFB2	0	4	4.051	3.75	101.3	93.75	97.53	5.4
AFB1	13.878	40	53.357	52.53	99.03	97.5	98.3	1.1

Table 2: Recovery test of aflatoxin from maize sample.

Levels of aflatoxin in maize samples from West Gojam

In the present study, 30 samples consisting of 15 pre and 15 post-harvest maize samples were collected from farmers' fields of West Gojam. Samples were analyzed in duplicate for AFG2, AFG1, AFB2 and AFB1 contamination by HPLC-FLD. Aflatoxin contamination in maize was observed in 77.7% of pre-harvest by level range 3.13-63.66 µg/kg and 80% by level range 9.02-139.8 µg/kg of post-harvest sample. The samples showed that the mean total aflatoxin level of 18.38 and 43.43 µg/kg for pre and post-harvest maize, respectively (Table 3).

Type of Sample	Number of Samples	Mean (µg/kg)	Mean of Difference	SD	Std. Error Mean	95 % CL for Mean	t-test	P-value
Pre-harvest	15	18.38	25.0	26.7	6.9	10.2-39.8	3.6	0.003
Post-harvest	15	43.4						

Table 3: Paired comparison of the mean difference of total aflatoxin level in pre and post-harvest maize.

AF=Aflatoxin, SD=Standard Deviation of the mean difference, CI=Confidence Interval

About 77.7% total aflatoxin contamination in pre-harvest maize obtained in this study is consistence to the result of Ayyathurai et al., [4] who reported that the total aflatoxin contamination about 79.7% of pre-harvest maize in India but the results of this study is higher than that of reported in Benin, 42.5% in 1994 and 30% in 1995 in pre-harvest maize [10]. These results were also higher than reported in Kenya which was 22.97% of total aflatoxin contamination in pre-harvest maize [21]. The high total aflatoxin contamination in pre-harvest may be due to delayed harvesting (high likelihood of kernel damage by pests, insect injury), differences in temperature, humidity, using crop resistance variety, crop rotation system and maintaining adequate irrigation schedule [18]. Genotype, soil types, drought and insect activity are also important in determining the likelihood of pre-harvest contamination [1].

Aflatoxin contamination of post-harvest maize (80%) obtained in this study is consistence to result previously reported in Ethiopia, where aflatoxin contamination of post-harvest maize was 88% [12]. However the result of this study is remarkably higher than that of reported in Malaysia which was 40% [20] and 58% in Nigeria [22]. This variation of percentage total aflatoxin contamination in post-harvest may be due to difference in handling process from the time of harvest to the time of consumption. The other reason may be due to once the crop becomes infected under field conditions, the fungal growth continues with increasing at post-harvest and storage conditions [23]. It is also well known that growth of *Aspergillus* spp. and subsequent production of aflatoxins in maize is dependent on a number of factors such as temperature, humidity, insect injury, handling during pre-harvest, harvest and storage [4,21].

According to the present study, in pre-harvest maize sample the average aflatoxin G2, G1, B2 and B1 concentration were 2.10 µg/kg , 10.10 µg/kg , 1.17 µg/kg and 5.00 µg/kg respectively. In post-harvest maize sample the average aflatoxin G2, G1, B2 and B1 concentration in the maize sample were 8.14 µg, 18.11 µg/kg, 7.2 µg/kg and 9.86 µg/kg, respectively (Figure 2).

Figure 2: Chromatogram of (a) Blank sample, (b) Standard calebration curve and (c) Naturally contaminated fungal sample (elution order AFG2, AFG1, AFB2 and AFB1).

Among the naturally occurring aflatoxins (AFG2, AFG1, AFB2 and AFB1), AFB1 is usually predominant and is the most toxic aflatoxin. In this study the most frequently identified was aflatoxin B1 which was 66.7% in pre-harvest and 87.7% in post-harvest. The highest level of AFB1 (29.5 µg/kg) was recorded in post-harvest maize kernel sample. The levels of AFB1 ranged from 3.13 to 27.31 µg/kg and 2.83 to 29.5 µg/kg in pre and post-harvest sample respectively, AFB1 was detected in 60% of pre-harvest samples and 80% in post-harvest sample. The samples showed the mean aflatoxin B1 level of 5.00 µg/kg and 9.86 µg/kg for pre and post-harvest maize respectively (Table 4).

The current prevalence of AFB1 (66.7%) in pre-harvest and (87.7%) in post-harvest was consistent to results previously reported in India which was 79.7% in pre-harvest maize [4] and in Iran which was 77% of post-harvest samples [24] but higher than reported by Muthusamy et al., [21] which was 22.97% of pre-harvest maize and 53.93% post-harvest maize by Ayyathurai et al., [4] which was 22.97% of pre-harvest and 53.93% post-harvest maize. This variation may be due to number of factors such as temperature, humidity, insect injury, handling during pre-harvest, harvest and storage [4,21].

Comparison of the means of total aflatoxin level in pre and post-harvest maize

Table 3 showed that the mean total aflatoxin level in post-harvest is much higher (43.43 µg/kg) than in pre-harvest maize (18.38 µg/kg). Paired t-test statistical analysis for mean of total aflatoxin level in pre and post-harvest maize sample is also showed that there was a significant difference in total aflatoxin level between pre and post-harvest maize (t=3.6, p=0.003) which was less than 0.05. Which means total aflatoxin level was significantly increase from pre-harvest to post-harvest pre-harvest maize. The reasons for high increment of aflatoxin level in post-harvest may be poor harvest and post-harvest practices of the maize [18,21] and once the crop becomes infected under field conditions, the fungal growth continues with increasing at post-harvest and storage conditions [23].

Comparison of the means of aflatoxin B1 level in pre and post-harvest maize

Paired t-test statistical analysis for mean of B1 aflatoxin in pre and post-harvest maize showed that the mean total aflatoxin level in post-harvest is higher (9.86 µg/kg) than in pre-harvest maize (5.00 µg/kg).

Paired t-test statistical analysis for mean of Aflatoxin B1 level in pre and post-harvest maize sample is also showed that there was a significant difference in total aflatoxin level between pre and post-harvest maize (t=2.28 p=0.039) which is less than 0.05. Which means aflatoxin B1 level was significantly increase from pre-harvest to post-harvest pre-harvest maize (Table 4).

The reasons for high increment of aflatoxin B1 level in post-harvest may be poor harvest and post-harvest practices of the maize [18,21].

Comparison of aflatoxin results with different international standards

National and international institutions and organizations such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have recognized the potential health risks to animals and humans posed by consuming aflatoxin-contaminated food and feed. To protect consumers and farm animals regulatory limits have been adopted. The current Maximum Residue Levels (MRL) for aflatoxins set by the United States' Food and Drug Administration (USFDA) is 20 µg/kg [25] and safe limit of 20 µg/kg is also established by WHO [26]. Maximum level of 2 µg/kg for aflatoxin B1 and 4 µg/kg aflatoxin total has been established in all cereals [27].

A total of 30 samples consisting of 15 pre-and 15 post-harvest maize samples were collected from farmers' fields of West Gojam. Samples were analyzed for AFG2, AFG1, AFB2 and AFB1 contamination by HPLC-FLD. The result presented in table 5 showed that 33.3% of pre-harvest and 73.3% of post-harvest maize samples were exceeded the limit set by US Food and Drug Administration (FDA), the World Health Organization (tolerance limit of 20 µg/kg). The current percentage is lower than reported in Ethiopia cereal and legume samples [11] higher than reported in Kenya stored maize [26] and India [21].

About 66.7% of pre-harvest sample and 86.7% of post-harvest maize samples were exceeded of total aflatoxin levels of European Union (EU) recommended maximum limit 4 µg/kg.

European Union (EU) also established acceptance limit of aflatoxin B1 (<2 µg/kg) but most of samples (pre-harvest = 66.7% and post-harvest = 86.7%) of this study were exceeded the acceptance limit (Table 5).

Type of Sample	Number of Samples	Mean (µg/kg)	Mean of Difference	SD	Std. Error Mean	95 % CL for Mean	t-test	P- value
Pre-harvest	15	5.00	4.86	8.24	2.13	0.29-9.42	2.28	0.039
Post-harvest	15	9.86						

Table 4: Paired comparison of the mean difference of aflatoxin B1 level in pre and post-harvest maize.

AF=Aflatoxin, SD=Standard Deviation of the mean difference, CI=Confidence Interval

Sample	FDA/WHO	EU	
	Total AF <20 µg/kg N (%)	Total AF <4 µg/kg N (%)	AFB1 <2 µg/kg N (%)
Pre-harvest maize sample	5 (33.3)	10 (66.7)	10 (66.7)
Post-harvest maize sample	11 (73.3)	13 (86.7)	13 (86.7)

Table 5: Percentage of maize sample exceeded the limit of aflatoxin level of different international standards.

Isolation and identification of *Aspergillus* species

Aspergillus species were isolated on Potato Dextrose Agar (PDA) and species level were isolated by sub culturing pure cultures of different out growing fungi that were obtained on PDA and by transferring fungal colonies to new PDA plates using sterile tooth picks and incubating the plates for 5-7 days at 25°C. Three different *Aspergillus* spp. were found to be associated with the pre and post-harvest maize samples collected from West Gojam (Figure 3). The first species isolated from the collected samples was *A. flavus*. Colonies of this fungus were characterized by a velvety, yellow to green or the old colony was brown mould with a goldish to red-brown on the reverse (Figure 3). This species also distinguished from other *Aspergillus* species by lack of growth at 5°C, by rapid growth at both 25 and 37°C. The second species were *A. niger* group, the major distinction from the other species of *Aspergillus* is the production of carbon black or dark brown spores of biseriate phialides. The current study also confirmed the production of black or brown-black or black conidia by this species (Figure 3). *A. parasiticus*was the third species isolated from maize samples tested in the current study. Colonies representing this species produced dark green.

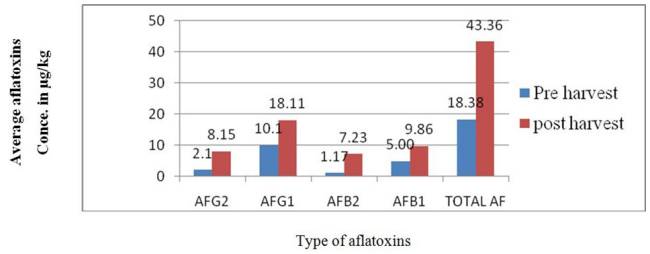

Figure 3: Average concentration of aflatoxin in pre and post-harvest maize.

These observations all the above species were consistent with the findings of [15-17].

From 15 pre-harvest and 15 post-harvest maize samples collected from West Gojam *A. flavus*, *A. parasiticus* and *A. niger group*. *A. flavus* were isolated on PDA. In pre-harvest sample 53.3% of samples were contaminated by *Aspergillus* species (26.7% = *A. flavus*, 13.3% = *A. parasiticus* and 13.3% = *A. niger*) and in post-harvest maize sample were contaminated *Aspergillus* species 80% (46.6% = *A. flavus,* 20.0% = *A. parasiticus* and 13.4% = *A. niger* group) (Figures 4 and 5).

Aspergillus flavus is the main fungal species infecting maize grains. In the current study also the most prevalent *Aspergillus* species in pre-harvest was *A. flavus* (26.7%). This was lower than 80% reported in 1994 and 60% in 1995 in Benin [10] and 65% in Nigeria.

Contamination *Aspergillus flavus* in post-harvest maize was 46.6%. This was lower than reported in Ethiopia 88% from cereal sample 64% from maize sample [28] and in Kenya 78.5% [29] and higher than reported in Iran 27% [24].

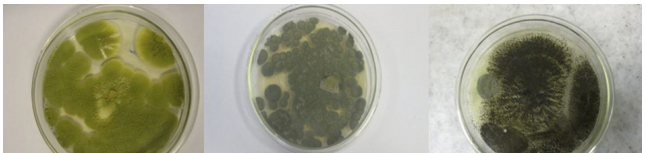

Figure 4: *Aspergillus* spp. isolated from maize sample.

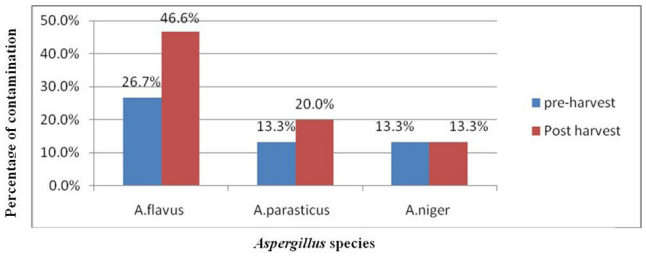

Figure 5: Percentage of *Aspergillus* species pre and post-harvest maize.

Aspergillus species contamination variation in different countries may be due pre and post-harvest practice of the farmers and also different in temperature, humidity, not (using crop resistance variety, crop rotation system, maintaining adequate irrigation schedule) also the cause of this variation [17]. Genotype, soil types, drought and insect activity are also important in determining the likelihood of contamination [1]. Moreover, poor harvesting practices, improper storage, and less than optimal conditions during transport and marketing can also contribute to fungal growth and increase the risk of mycotoxin production [16].

Conclusion

This research has shown that the level of total aflatoxin contamination is very high in pre and post-harvest maize. In addition, the pre and post maize sample also showed that high aflatoxin B1 level in pre and post-harvest sample. Paired t-test statistical analysis for mean of total and aflatoxin B1 in pre and post-harvest maize samples were showed that both total and aflatoxin B1 increase significantly from pre-harvest to post harvest maize. Most of pre and post-harvest samples were exceeded the US Food and Drug Administration (FDA), the World Health Organization (WHO) and European Union (EU) maximum limit of total aflatoxin. In both cases the contaminated maize sample in post-harvest was more exceeded the standards limit than the pre-harvest. This research has also shown that high *Aspergillus* species contamination in pre and post-harvest maize samples. Post-harvest maize samples were more contaminated than the pre-harvest maize.

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