 http://dx.doi.org/10.4314/bajopas.v14i1.9S

Bayero Journal of Pure and Applied Sciences, 14(1): 53 - 58
ISSN 2006 – 6996

DETERMINATION OF AFLATOXIGENIC *Aspergillus* AND AFLATOXINS FROM MAIZE, MILLET AND SORGHUM SOLD IN KATSINA METROPOLIS

Zulaihat B. A., Shamsudeen U., Zubairu U. D.
Corresponding Author: Department of Microbiology, Umaru Musa Yaradua University, Katsina-Nigeria.
Email: zulaihat.bashir@umyu.edu.ng, Mobile NO: +2347063264770.

ABSTRACT
Aflatoxins are toxic secondary metabolites produced by some species of Aspergillus particularly Aspergillus flavus and Aspergillus parasiticus. These fungi have been known to contaminate cereal grains, thereby causing both economic and negative health impact. This research aimed at determining aflatoxigenic fungi and aflatoxins in Maize, Millet and Sorghum. A total of 9 Samples were purchased from three different markets in Katsina metropolis. Sample homogenates was cultured on Potato dextrose agar at room temperature for 3-7days. Isolates were identified using phenotypic characteristics (Macroscopic and microscopic features). Fungi presumed to be aflatoxigenic were further subjected to molecular Aflatoxin gene detection using multiplex PCR with 3sets of primers for aflR, aflD and aflP genes. Similarly, Enzyme linked immunosorbent assay (ELISA) was used for quantitative evaluation of aflatoxins in the samples where aflatoxin B1 and Total Aflatoxins (Sum of B1, B2, G1 and G2) were assessed. Fungi isolated from the samples include; Aspergillus niger, Aspergillus flavus, Fusarium, Penicillium and Rhizupus spp. However, subjecting isolates to molecular characterization showed that aflD and aflP were present in three (3) of the isolates while gene aflR was present in four (4) of the isolates. Assessing the total aflatoxin concentration has shown that Sorghum had the highest concentration of 2.07±0.6ppb, while Maize has the lowest concentration of 1.98±0.06ppb. Moreover, Aflatoxin B1 evaluation of the samples showed that Maize had the highest concentration of 20.67±5.34µg/kg , while Millet had the least concentration of 0.67±0.61µg/kg. In summary, the amounts of total aflatoxin in the cereal under this study are within the recommended limit 10 parts per billion while with regards to B1 aflatoxin, maize exceeded the recommended limit of 4µg/kg by European Union and 20µg/kg by Standards Organization of Nigeria. Consequently, it is recommended that sensitization of farmers and vendors about the severity of Aflatoxin contamination of grains and control majors should be done.
Key words: Aflatoxins; Fungal flora; Aspergillus; Enzyme linked Immunosorbent Assay (ELISA)

INTRODUCTION

Maize, Millet and sorghum are one of the most important cereals in the world at large, they are both food and cash crops used for human consumption either taken raw, cooked or processed into flour and as animal feeds (Kumar *et al.,* 2022). Cereals have been widely reported to be prone to contamination by potentially toxigenic fungi (Michael *et al.,* 2019) These fungi can produce over three hundred types of mycotoxins which have detrimental biological and economic impacts (WHO, 2018, Apeh *et al.,* 2016). Aspergillus species produce aflatoxins (AF) and ochratoxin A (OTA); Penicillium species, also produce ochratoxin A(OTA); and Fusarium species, produce deoxynivalenol (DON), zearalenone (ZEA), fumonisins (FB), HT-2 and T-2 (Atongbiik *et al*., 2017). Aflatoxins are low molecular weight toxic protein compounds produced by fungi of the genera *Aspergillus* as secondary metabolites where *Aspergillus flavus* and *A. parasiticus* are reported to be the excellent aflatoxin producers under favourable conditions among the *Aspergillus* species (Salisu and Amajir 2020). *A. flavus* and *A. parasiticus* are excellent aflatoxin producers under favourable conditions (Baha'uddeen *et al*., 2020).

Among all the mycotoxin types affecting food and feed Aflatoxins are the most toxic i.e. it is the major toxin in food and feed that ultimately harm human and animal health (Padeep *et al,.* 2017). Increased exposure to aflatoxins is generally associated with acute or chronic toxicities depending on the amount and length of exposure (Baha'uddeen *et al*., 2020). These aflatoxins generally are carcinogenic, teratogenic, mutagenic, hepatotoxic and immunosuppressive agents that cause significant damage to human and animal health (Sdini *et al.,* 2015). They can also cause jaundice, chronic hepatitis, cirrhosis, genotoxicity, stunted growth and birth defect (Baha'uddeen *et al*., 2020). This study is aimed at comparing the aflatoxin content of maize, millet and sorghum sourced from Katsina Metropolis.

MATERIALS AND METHODS

Sampling site

The samples were bought from three (3) different markets within Katsina Metropolis of Katsina State. Samples were taken specifically from Central market, Kofar Marusa market and Yarkutungu market.

Sample collection and processing

A total of nine (9) samples, three (3) each of maize, millet and sorghum were collected from Central, Yar kutungu and Kofar Marusa markets of katsina Metropolis. Samples were collected randomly from vendors in sterile polythene bag and brought to the Laboratory for further processing. The samples were ground to powder in a sterile dry laboratory blender and were packaged in sterile dry and well labeled containers and were kept at room temperature.

Isolation of Fungi

Fungal Isolation was carried out using the procedure adopted from Salisu and Almajir (2020) with some modifications. One gram (1g) of the milled sample was weighed into a sterile test tube, suspended in 9ml of sterile distilled water. The suspension was serially diluted to 10^{-5} of the original concentration. Half a milliliter (0.5ml) of each dilution was poured in sterile plates followed by molten Potato Dextrose Agar (PDA) which was aseptically prepared according to the manufacturer's instructions and supplemented with 0.01mg/ml chloramphenicol (Fidson chloramphenicol). The plates were incubated at 37^{o}C for 3days.After incubation the plates were observed for fungal growth. Pure cultures were obtained by picking individual species observed and were inoculated on a freshly prepared PDA for identification and further screening.

Identification

All the pure isolates obtained were subjected to presumptive identification by observing macroscopic features involving pigmentation, colonial form and microscopic features such as type of hyphae, presence of sporangia or conidia. Results were compared with Fungal Atlas by David *et al*., (2007). Isolates presumed to be *Aspergillus Flavus* were subjected to molecular detection of aflatoxin gene using Multiplex PCR.

Aflatoxin Gene Detection

Fungal DNA Extraction

The genomic DNA was isolated from the fungal mycelia, harvested from freshly growing cultures in potato dextrose agar. The fungal tissue was transferred to a mortar and ground vigorously with a pestle. The lysis buffer [1M Tris-HCl (pH 7.5), 0.05M EDTA, 0.9M NaCl, and 1% sodium dodecylsulfate] was added to the mixture and placed in a water bath at 65°C for 30 min. The resultant suspension was centrifuged for 10 min at 10,000 rpm, and the supernatant that is the crude lysate was transferred to a fresh, labeled microfuge tubes. Precipitation of the DNA in the crude lysate was done using chloroform: isoamyl alcohol (24:1) method. The procedure was followed by centrifugation at 10,000 rpm for 10 min. The supernatant was then transferred to a fresh microcentrifuge tube, and an equal volume of ice cold 95% ethanol was added and the mixture was kept at -20^{o}C for one hour. Centrifugation followed at 10,000 rpm for 10 min., the supernatant was removed, and the pellet was suspended again in 100 µl nuclease free water. This protocol was adopted from Rahimiet *al*., 2016.

Polymerase Chain Reaction (PCR) Amplification

For the amplification of clustered pathway genes in aflatoxin biosynthesis, the PCR reaction was performed following the method described by Rahimi *et al*., (2016) with some modifications. All of the three genes were amplified by multiplex reactions following optimization. A reaction mix was prepared in a 0.2mL microtube with the following components; 12.5µL Biolabs Master mix (New England,UK), 1µL of each forward and reverse primers, 4.5uL of nuclease free water and 5µL of DNA template.

The tubes were transferred to Applied Biosystems 9700 thermocycler programmed with the following cycling conditions; Initial Denaturation at 94°C for 30seconds followed by 40 cycles of denaturation at 94°C for 2 minutes, annealing at 60°C for 45 seconds, extension at 68°C for 2 minutes, followed by final extension at 68°C for 7 minutes.

Agarose Gel Electrophoresis

The amplified products were subjected to electrophoresis using 1% agarose gel electrophoresis, pre-stained with ethidium bromide. The samples were loaded in allocated wells alongside 100-bp DNA ladder (Biolabs, New England, uk) and electrophoresis was carried out for 1hr at 100 volts and visualized under ultraviolet light using a gel documentation system (BioRad Gel Doc XR).

Table 1 Specifications and Nucleotide Sequences of the primer used

Gene name	Sequence	Gene Length	Melting Temperature	Source
afl D (Nor-1)	F-5′-	702	62.8	(Nooshin *et al.*,
afl D (Nor-1)	CTCATCACACGCAGGCATCGG-3′		63.1	2018).
	R-5′-AGATGCCTGCCACACTGTCT-3′			
afl P (Omt-1)	F-5′-CCCATCTCGATAGCGCCTG-3′	611	61.7	(Nooshin *et al.*,
afl P (Omt-1)	R-GCCACCCATACCTAGATCAAAGC-3		60.1	2018).
afl R	F- 5'-	1032	60.7	(Gbolagade *et al.,*
afl R	TATCTCCCCCCGGGCATCTCCCGG-3'		61.1	2020)
	R- 5'-CCGTCAGACAGCCACTGGACACGG-3'			

Aflatoxin Quantification

The Total and B1 aflatoxin were quantified according to the manufacturer's instructions using RIDA screen Aflatoxin total (Art No. R4701) Immuno Assay kit and Sigma Aflatoxin B1 ELISA kit respectively.

RESULT

Identification of the Fungal Isolates

A total of 18 organisms were isolated of which only five (5) isolates were identified as *Aspergillus flavus* from CMI, CMMZ, YMMZ, KMMZ and KMS. The table below show the macroscopic and microscopic examination of the fungal genera isolated.

Table 2: Isolates Identified based on Macroscopic and microscopic examination

Sample code	Name of organism
CMZ-1	*Aspergillus niger*
CMZ-2	*Aspergillus flavus*
YMZ-1	*Aspergillus flavus*
YMZ-2	*Fusarium* spp.
KMZ-1	*Aspergillus flavus*
KMZ-2	*Fusarium* spp.
CMI-1	*Aspergillus flavus*
CMI-2	*Aspergillusniger*
YMI-1	*Rhizopus* spp.
KMI-1	*Aspergillus niger*
KMI-2	*Fusarium* spp.
KMI-3	*Penicillium* spp.
CS-1	*Aspergillus flavus*
CS-2	*Fusarium* spp.
YS-1	*Aspergillus flavus*
YS-2	*Aspergillus Niger*
KS-1	*Penicillium* spp.
KS-2	*Fusarium* spp.

Key: CMZ -Central Market Maize**, KMZ**-Kofar Marusa Market Maize**. YMZ**-Yrkutungu Market Maize**, CMI**- Central Market Millet**, KMI**-KofarMarusa Market Millet**, YMI**-Yrkutungu Market Millet**, CMS**-Central Market Sorghum**. KS**-KofarMarusa Market Sorghum**, YS**-Yrkutungu Market Sorghum. 1, 2, 3 signifies number of isolates

Aflatoxin Gene detection

The five *Aspergillus Flavus* isolates were subjected to aflatoxin gene detection using multiplex PCR, figure 1 depicts the PCR products obtained from each gene fragments. Where aflD, aflR and aflP genes were targeted. The separate bands of aflD, aflP, and aflR gene fragments could be seen at 702, 611, and 1032 bp, respectively. Gene aflD and aflP were likely to be present in three (3) of the isolates while gene aflR was likely to be present in four (4) of the isolates. CMZ-II was positive for all the targeted genes, CMI-II was negative for aflP and positive for aflD and aflR, KMZ-I was positive for both aflD and aflP but negative for aflR, YMZ-I was negative for both afl D and afl P and positive for afl R. YS-I however was negative for aflD and positive for both aflP and aflR.

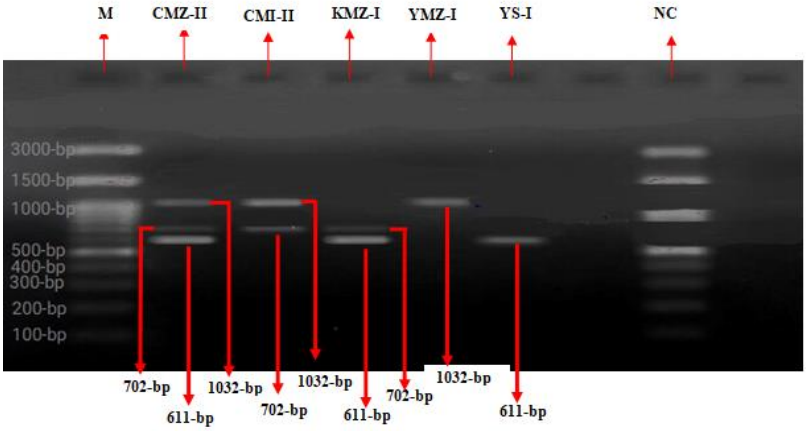

Figure 1 Distribution of aflR, aflP and aflD genes with respect to the sizes of the genes as detected by gel electrophoresis.

Aflatoxin Quantification

Total Aflatoxin quantification

Total aflatoxin concentration of all the samples was determined and all the samples showed some level of total aflatoxin contamination. All the samples have almost the same total aflatoxin concentration (2.01 ± .06 ppb).

Aflatoxin B1 Quantification

All the samples were tested for the presence of Aflatoxin B1. Maize showed the highest concentration (20.67 ± 5.34 µg/g) and Millet showed the lowest concentration (.67 ± .61).

Table 2 Total and B1 Aflatoxin Concentration

Sample	Sample Code	Total Aflatoxin(ppb)	Mean total aflatoxin	Aflatoxin B1(µk/kg)	Mean Aflatoxin B1
Maize	CMMZ	1.9	1.98±0.06	15.0	20.67±5.34
	YMMZ	2.0		25.6	
	KMMZ	2.0		21.4	
Millet	CMMI	2.0	2.00±0.00	0.8	0.67±0.61
	YMMI	2.0		1.2	
	KMMI	2.0		0.0	
Sorghum	CMS	2.0	2.07±0.06	10.5	3.5±6.06
	YMS	2.1		0.0	
	KMS	2.1		0.0	

Statistical Analysis

The statistical analysis (One way ANOVA) with respect to the total aflatoxin of the samples showed that there was no significant difference ($F(2, 6) = .098$, $p > .05$) between the sample while The statistical analysis (Kruskal Wali) with respect to the B1 aflatoxin of the samples showed that there is no significant difference ($F(2, 6) = .061$, $p > .05$) between the sample.

DISCUSSIONS

Different species of molds were observed from the samples, some of which have the potential for mycotoxin production. *Aspergillus flavus* was isolated from all the maize samples this might explain the high aflatoxin B1 observed in maize because *Aspergillus flavus* is known to produce aflatoxin B1.

The same organisms observed in maize in the research were observed in maize by Aklaku *et al*., (2020), Aminu and Keta (2021) and Ekwomadu*et al*., (2018) with the exception of *Rhizupus*. *Aspergilllus flavus* was the predominant organism observed in maize followed by *Fusarium*,this is in line with the study of Shamsudeen *et al*. (2017). Millet had the same frequency of *A. flavus, Rhizupus, A. niger and Penicillium.* The same frequency was also observed in sorghum with the exception of *Fusarium* which had little bit higher occurrence.

Among the numerous genes involved in the aflatoxin biosynthesis pathway, aflD plays an important role in the early conversion of nosolorinic acid into averantin, aflP is involved in the conversion of strigmatocystin into aflatoxin in the late steps and aflR gene has a key role in the regulation of other genes in aflatoxin biosynthetic pathway (Nooshin and Morteza 2018). Results from this study showed that the *A. flavus* isolated from central Market Maize (CMZ-II) was positive for all the targeted genes which shows that CMZ-II is an aflotoxigenic strain of *Aspergillus flavus* this is as reported by Nooshin and Morteza (2018) and Mahror *et al*., (2019). CMI-II was negative for aflP and positive for aflD and aflR which is not enough to conclude that the strain is aflatoxin producing. KMZ-I was positive for both afl D and afl P but negative for afl R which is the regulatory gene that plays an important role in regulating the activity of the structural genes, thus the absence of these gene might be due to mutation which will result in to a failed regulation of the structural gene as a result no aflatoxin will be produced (Mahror *et al*., 2019), hence it is safe to say KMZ-I is a nontoxigenic strain. YMZ-I was negative for both afl D and afl P and positive for afl R. The same strain was reported by Nooshin and Morteza (2018). But the presence of aflR alone is not sufficient to conclude that the isolate is aflatoxigenic (Dehghan *et al*., 2008) YSI however was negeative for aflD both aflP and positive for aflR this might be due to failed amplification, therefore conclusion cannot be drawn regarding its aflotoxin producing ability.

The findings of this study for total aflatoxin (Sum of B1, B2, G1 and G2) concentration of all the samples were positive although the concentration is within the acceptable limits of 10 parts per billion (Sirma *et al.,* 2016). The different cereal types had almost the same total aflatoxin concentration; this might be due to the fact that aflatoxigenic fungi are ubiquitous and can grow in wide variety of food substrate despite the growth condition. Also presence of more than 5%moisture coupled with temperature might be the reason for the presence of aflatoxins in the samples this is in contrast with the study of Michel *et al*., (2019) were maize was most contaminated followed by millet while sorghum was the least contaminated. The results of the statistical analysis showed that there was no significant difference between the sample types.

All the samples were tested for Aflatoxin B1 and Maize from all the locations had the highest concentration followed by sorghum while Millet had the least concentration. The contamination observed in sorghum was from central market sorghum and the contamination is attributable to the moisture found in the sample which was more than enough moisture to favor the growth of *Aspergillus* and subsequent aflatoxin production. The high aflatoxin B1 concentration observed in maize might be attributable to the time of harvest, post-harvest handling and temperature. Maize as previously mentioned is harvested during the rainy season, the humidity of the environment during rainy season might cause delayed drying thereby favoring the growth of *Aspergillus* and hence the production of aflatoxin. Poor post-harvest handling such as poor storage and environmental stress such as pest attack might also be the cause of the contamination. These findings are in line with the findings of Michael *et al*., (2019), and Sirma *et al*., (2016) where they reported high aflatoxin B1 in maize

The temperature in Nigeria especially Katsina have an average room temperature that is more than the 20^{o}c required for the growth of *Aspergillus* and the subsequent aflatoxin contamination. This might also be the factor responsible for the contamination of the maize samples and even the other cereal samples

CONCLUSION

The Samples analyzed were contaminated with different species of fungi apart from *Aspergillus flavus* some of which have the potential to produce other types of toxins. The gene detection carried out on the *Aspergillus flavus* isolates however, detected only one aflatoxigenic specie. Conclusively, the findings of this research detected both total and B1 aflatoxin in some of the cereal samples, although most are within the permissible limits. The statistical analysis shows no significant difference between maize, millet and sorghum with respect to total and B1 aflatoxin concentration.

RECOMMENDATIONS

1. Farmers should be trained at local level by appropriate governmental body about mycotoxins in general. Then only farmers with certified training should be allowed to farm grains for the masses.

REFERENCE

Apeh DO, Ochai DO, Adejumo A, Muhammad HL and Saidu A.N, (2016). Mycotoxicological Concerns with Sorghum, Millet and Sesame in Northern Nigeria. *J Anal Bioanal Tech* 7:336

Baha'uddeen Salisu, Siti Marwanis Anua, Wan Rosli Wan Ishak and Nurzafirah Mazlan (2020), Review on the Aflatoxins' Contamination of Foods and Public Health Effects among Nigerian Population, *Umyu journal of microbiological research,* 4(2): 33 – 49

Bahauddeen D.S and Ibrahim R. A (2020) Aflatoxins and aflatoxigenic fungal contamination of common poultry feed products in Katsina State, Nigeria. *Novel Research in Microbiology Journal*, 4(1): 653-665

Michael O. Odo, Fidelis Azi, Ignatius C. Alaka and Veronica N. Nwobasi (2019). Evaluation of aflatoxins levels and molecular identification of toxigenic molds in cereals and cerealderived breakfast foods in Nigeria. *African Journal of Biotechnology,* 18(31): 1025-1030

Pradeep Kumar, Dipendra K. M, Madhu K, Tapan K. M and Sang G. K (2017). Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management, *frontiers in microbiology.* 7(2170).

Sirma A.J., Senerwa D.M1, Grace D., Makita K, Mtimet N., Kang'ethe E.K. and Lindahl J.F, (2016), Aflatoxin b1 occurrence in millet, sorghum and maize from four agro-ecological zones in kenya, *African journal of food agriculture, nutrition and development.* 16(3): 10991-11003

Sudin H., Srilakshmi P.,Krishna K.V. and Samuel M.C (2015). Detection of aflatoxigenic aspergillus by cultural and molecular method: A critical review,*Africal journal of microbiology research*. 9(8): 484-491.

Nooshin S. and Morteza T. (2018). Molecular identification of aflatoxigenic Aspergillus species in feedstuff samples, *Current Medical Mycology*: 4(2): 1-6

Kumar, P., Gupta, A., Mahato, D.K., Pandhi, S., Pandey, A.K.; Kargwal, R., Mishra, S., Suhag,
R., Sharma, N. and Saurabh, V. (2022). Aflatoxins in Cereals and Cereal-Based Products: Occurrence, Toxicity, Impact on Human Health,and Their Detoxification and Management Strategies. *Toxins*, 14(10) 687.

Atongbiik A.M., Nelson O. and Francis K.A., (2017). Aflatoxin contamination in cereals and legumes to reconsider usage as complementary food ingredients for Ghanaian infants: A review, *Journal of Nutrition & Intermediary Metabolism*, 10:1-7

Pradeep K., Dipendra K. M, Madhu K, Tapan K. M and Sang G. K (2017), Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management, *Frontiers in Microbiology.* 7 (2170).