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Assessment of Cereals for Fungal Contaminants and Presence of Aflatoxins from Two Major Markets in Kaduna Metropolis

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ABSTRACT

Aflatoxins are mycotoxins produced by fungi of the genus *Aspergillus* that contaminate food products such as cereals leading to serious health and economic consequences. In order to assess the level of fungal contaminants and the presence of aflatoxins in such products, five (5) different cereal samples comprising of rice, maize, millet, wheat and sorghum were purchased each from two major markets within Kaduna metropolis. The samples were assessed for fungal contaminants using ten-fold serial dilution and cultured appropriate dilutions using pour-plate technique. Aflatoxins were detected using Enzyme-linked Immunosorbent Assay (ELISA). The results obtained revealed viable fungal counts ranging from 3 x 10^2 to 3.2 x 10^3 cfu/g. The fungal contaminants identified from the samples include *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus fumigatus*. The aflatoxins detected from the samples were within the range of 0.3 to 0.6 part per billion (ppb) which is less than the maximum value (10ppb) recommended by regulatory agencies such as NAFDAC. This indicates that the level of aflatoxin in the samples has insignificant risks to consumers. However, the fungal contamination is an indication of high-level contamination of the cereals, the key producers of aflatoxins. The contamination of such products by fungi should be a source of worry and necessitates the need for proper personal and environmental hygiene in the processing of cereals.

INTRODUCTION

Aflatoxin is a mycotoxin that occurs naturally as secondary metabolites in filamentous moulds but its toxicity on humans, animals and other living organisms can lead to death or severe consequences [1]. It is carcinogenic, poisonous when consumed in food or feed making it a food safety concern and why it is strictly regulated in many countries [2]. Fungi of the genus *Aspergillus* are known producers of aflatoxin with several species including *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* among others as the main sources [3]. The toxins they produced are divided into four based on blue (B) or green (G) fluorescence in long-wavelength ultraviolet (UV) light as AFB1, AFG1, AFB2 and AFG2 in their order of toxicity with AFB1 being the most toxic (carcinogenic) [4]. These four are the primary aflatoxins of concern in food and feedstuffs. AFB1 is also the most abundant, potent and the main aflatoxin produced by most toxigenic moulds [5]. It has been classified by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen with hepatotoxic, mutagenic, immunosuppressive and teratogenic properties [6].

The fungi that produced the toxin contaminate crops in a complex process and made its way into the food chain because they are genotoxins when growth conditions are favourable [7]. The contamination affects crops on the farm and after harvest during storage, transport or trading. The contamination affects agricultural sector by undermining food security and safety, the health sector and local/international trade. When contaminated foods are consumed, it causes certain health effects including death [8]. Crops contamination dominates in humid and warm regions where optimal temperature and humidity is high, suitable for the growth of the moulds and toxin production as in sub-Saharan Africa [9]. Other factors contributing to crop

contamination are damages on crop, inadequate storage facilities, exposure to sun or air, and activities of insects [10].

Nigeria lies on the sub Saharan Africa with warm climate and most of the population rely solely on cereals such as maize, rice, millet, wheat and sorghum as a major source of food. The cereals are widely consumed staple foods which necessitate the need for monitoring and strict regulation without which it can lead to a serious public health hazard [11]. Consequently, Nigeria's National Agency for Food and Drug Administration and Control (NAFDAC) has set an action limit on food products likely to contain aflatoxin. All the various type of Aflatoxins (B1, B2, G1 and G2) were reported to be present in cereals by Hussein and Brasel [4] which further explained the need for monitoring and enforced regulation. As a result, large quantities of contaminated crops are wasted every year.

Effective monitoring remains the viable option to minimize and control aflatoxin which requires accurate detection of toxins [12]. Several conventional chromatographic techniques such as thin-layer, high liquid and gas-liquid as well as mass spectroscopy, spectrofluorometry and spectrophotometry are accurately used in most aflatoxin detection [13, 14]. These methods are costly, laborious and time consuming which necessitates the need for easier, fast and effective methods that will detect the toxin no matter how little it is present in food. Immunochemical methods such as Enzyme linked Immunosorbent assay (ELISA) is a highly sensitive technique that meets up the above criteria and are used to examine aflatoxins level [15].

The method is highly sensitive, quantitative, simple to perform, can process multiple sample analysis, low sample volume requirements and prepared by directly analysing without the need for initial extract clean-up procedures [16, 17]. In this study, cereals on display for sale in two major markets (Kaduna Central market and Kawo market) in Kaduna Metropolis were selected for the detection of aflatoxin level using ELISA methods. This type of monitoring is necessary to ensure safety of foods consumed and to prevent very difficult to control aflatoxin hazards.

MATERIALS AND METHODS

Sample Collection

Samples of cereals including rice, maize, millet, wheat and sorghum were purchased each during the Summer of 2017 (June - August) from Sheikh Abubakar Gumi Central (Kaduna Central) Market and Kawo market located within Kaduna metropolis. These major markets are strategically located within the city serving large communities and always crowded. The samples were poured into clean polythene bags, tied and transported to microbiology department of Kaduna state university for analysis.

Isolation and Identification of *Aspergillus species*

Viable fungal count was carried out to estimate the number of fungi per sample. To obtain stock solution of the sample, 10 g of each of the samples was weighed using a weighing balance and homogenized in 90 ml of sterile normal saline. This was serially diluted to obtain subsequent dilutions using ten-fold serial dilutions. A 1 ml aliquot from the second dilution (10^{-2} dilution) of each sample was transferred into a labelled petri dish. Sterilized and cooled melted potato dextrose agar (PDA) medium amended with 60 µg/ml of chloramphenicol was poured over the plate aseptically. Each plate was then swirled on the working bench gently to have a proper dispersion between the inoculum and the medium and incubated at room temperature for 4-7 days approximately. The number of colonies on each plate was counted and recorded. To isolate and identify *Aspergillus species* from the different fungi, each unique colony was subcultured into fresh PDA plate in duplicates. The plates were incubated again at room temperature for about 4-7 days approximately [18]. This was followed by macroscopic and microscopic observation to identify *Aspergillus species*. Macroscopy observations were based on appearance, pigmentation, shape and margin of the colonies. For microscopic examination, a small portion of the fungal mycelium was placed on a clean glass slide. A drop of lactophenol blue was added and observed under the microscope. The *Aspergillus* fungi were identified up to species level as described previously by Klich [19].

Detection of Aflatoxin Using ELISA

Enzyme-linked immunosorbent assay (ELISA) technique was used to detect the presence of aflatoxin in the cereal samples. AgraQuant Aflatoxin ELISA test kit (1-20 ppb) was used for the analysis according to the manufacturer's instruction. The aflatoxin test kit comprises of a 96-well plate coated with antibodies to aflatoxin; standard solutions of aflatoxin (0, 1, 2, 4, 10 and 20 ppb); a buffer for diluting standard solutions; a solution of conjugate; a buffer for the conjugate, 96 colour coded dilution microwells, and a reagent for stop solution. The test kit was stored at 4 °C but always brought to room temperature when required for use.

To extract the toxin, each sample was homogenized in a blender after which 5g of the homogenized sample was weighed into a tight capped reagent bottle where 25ml of 80% methanol was added and shaken vigorously in an orbital shaker at 250 rpm for three minutes. It was then allowed to settle, followed by filtration with Whatman No.1 filter paper and the filtrate was collected in a test tube as the substrate. Micropipette was used to dispense 200 µL of conjugate solution each into 6 colour coded dilution wells followed by the addition of 100 µl each of standard (0, 1, 2, 4, 10 and 20 ppb) into the corresponding wells containing the conjugate. Each well was mixed carefully by pipetting up and down 3 times and immediately, 100 µl of the contents was transferred from each mixing well into a corresponding antibody coated well. This was followed by incubation at room temperature for 15 minutes. It was then washed 5 times with distilled water by emptying the content into waste container and washed by filling with distilled water and discarding the water from the antibody coated well. This was repeated 5 times and the antibody coated microwell was dried by hitting it gently against an absorbent (tissue) paper.

This was followed by the addition of 100 µl of the substrate using a pipette into each antibody coated microwell and incubated at room temperature for 15minutes. At this stage, aflatoxin in the sample extract or control standards can compete with the enzyme-conjugated aflatoxin for the antibody binding sites after which a blue colour developed. A 100 µl of stop solution was dispensed into each of the antibody coated well and the colour changed from blue to yellow. The antibody coated microwell was then read optically using an ELISA reader (Stat Fax 303 - Microstrip Reader) at 450 nm absorbance filter and a differential filter of 630 nm. The optical densities of the samples were compared to that of the standards and the result was interpreted. The results for aflatoxins level are generally the sum of the concentrations of the four toxins.

Statistical Analysis

The data for the aflatoxin level was analysed using one-way ANOVA of variance which revealed insignificant difference of aflatoxin detected from the two markets at $P>0.05$ [20].

RESULTS AND DISCUSSION

Isolation and Identification of *Aspergillus species*

Aspergillus species were isolated from the cereal samples with fungal load as high as $3.2x10^3$ cfu/g as presented in Table 1. This is an indication of gross contamination of the samples by the fungi which could be attributed to the fact that most of the cereals were sold in an open bowl, exposed to air and sunlight in the market making it an easy subject of contact with soil and subsequent attack by fungi. This suggests the need for proper and hygienic practice from harvesting, processing, packaging, storage, transportation, trading to consumption of the crops.

Table 1. Viable fungal count of cereal samples.

Sample	(Sample location CFU/g)	
	Central market (A)	Kawo market (B)
Rice	$3.00x10^2$	$1.900x10^3$
Maize	$1.500x10^3$	$3.200x10^3$
Millet	$2.000x10^3$	$1.300x10^3$
Wheat	$1.800x10^3$	$6.00x10^2$
Sorghum	$1.400x10^2$	$8.00x10^2$

KEY: CFU/g: Colony forming unit per gram, A: Cereal sample obtained from Central market B: Cereal sample obtained from Kawo market.

Species of *Aspergillus* were identified from the isolates obtained from the samples based on their morphological features by comparison to reference strains of *Aspergillus flavus, Aspergillus fumigatus* and *Aspergillus niger*. Other fungi isolated were not identified. The total occurrence of *Aspergillus flavus, Aspergillus fumigatus* is six times each in the 10 samples while *Aspergillus niger* occurs seven times (**Fig.** 1). The occurrences of *Aspergillus* species could be as a result of the prevalence of their spores in the environment which could be easily passed to cereal during processing. The isolation of *Aspergillus* species from cereal can have severe health implication due to toxin production [21]. Most of the species of *Aspergillus* that contaminate cereals are mostly aflatoxin-producing fungi with high frequency of occurrence [22]. Different species of aflatoxigenic moulds have been isolated from cereal but only *Aspergillus flavus* and *Aspergillus niger* were confirmed and identified to be aflatoxigenic moulds [23-26]. The incidence of aflatoxigenic moulds in cereal samples is highly influenced by improper handling methods and inadequate storage facilities.

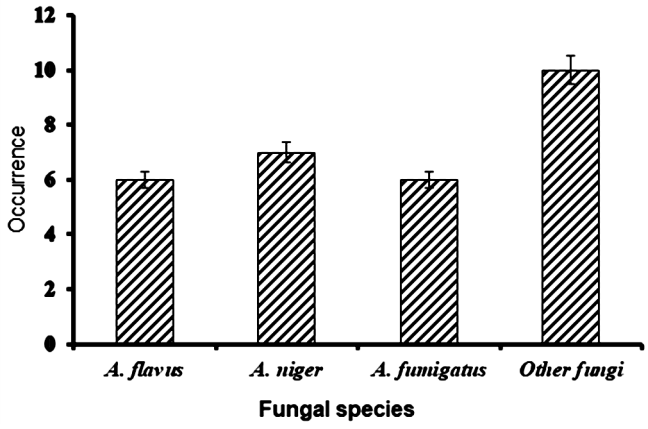

Fig. 1. Occurrence of *Aspergillus* species in cereal samples obtained from the two markets. The samples were prepared using ten-fold serial dilution and cultured on PDA plates in duplicates as described in the methodology. *A. flavus: Aspergillus flavus*, *A. niger: Aspergillus niger*, *A. fumigatus: Aspergillus fumigatus*.

Detection of aflatoxin level in cereal

Aflatoxin was observed in 8 out of the 10 samples analysed representing 80% of the samples. The aflatoxin detected in all the samples ranges from 0.3 to 0.6 part per billion [8] with two samples having a net value of zero as presented in **Fig. 2**. Aflatoxin level detected range from 0.3 – 0.6 ppb in rice, 0 – 0.4 ppb in maize, 0.5 – 0.6 ppb in millet, 0 – 0.6 ppb in wheat and 0.5 – 0.6 ppb in sorghum. According to the markets, Kawo market has a range of 0 – 0.6 ppb while Central market has a range of 0.3 – 0.6 ppb and these results were found to be of no significant statistical difference with P value higher than 0.05.

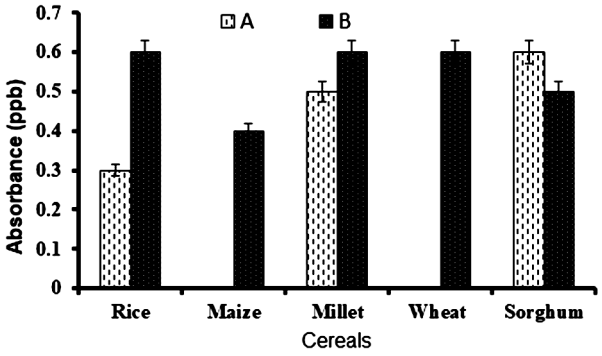

Fig. 2. Concentration of aflatoxin level in cereal samples. The samples were analysed in duplicate using AgraQuant Aflatoxin ELISA test kit according to manufacturer's instruction as stated in the methodology section. A: Cereal sample obtained from Central market, B: Cereal sample obtained from Kawo market.

Although the result obtained is an indication of aflatoxin contamination, the values detected in all the samples were found to be at acceptable limit, less than the maximum limit recommended by NAFDAC of 10 ppb for all the samples analysed [27]. AFB1 which is the most dangerous and often occurring mycotoxin does not pose danger to cereals from the samples analysed. The detection of aflatoxin from samples is an indication of the presence of AFB1 for being the most abundant in cereals and vice versa. Its presence is a major health concern, influenced by conducive growth conditions that support the growth of *Aspergillus species* and control their ability to produce secondary metabolites [28]. The low level of aflatoxin was also reported previously in maize [27], rice [29], wheat and others [30]. High aflatoxin levels were also reported in rice [31] and maize [32].

The observed differences in the aflatoxin concentration between the various samples could be attributed to various factors that can influence fungal growth and subsequent production of aflatoxins. Cereals can be contaminated with aflatoxigenic moulds from the soil during pre-harvest in the farm or post-harvest during transportation, storage or while on display in the markets. The production of aflatoxin is facilitated by such factors as temperature, humidity and both pre and postharvest practices which when it is inadequate, or poor makes the cereals a reservoir for fungi.

The result shows that the samples are safe for consumption based on the low level of aflatoxin detected. However, because of the serious risk posed by mycotoxins in general, adequate food safety measures are required to protect foods from contamination by mycotoxigenic moulds [33]. However, the presence of *Aspergillus spp*. in cereals as the result of this study indicates, does not always implied the presence of the toxin because not all strains produced aflatoxin and other factors are involved in its production [34, 35]. The absence of any aflatoxigenic mould

from samples equally does not rule out the possibility of the presence of the toxin because the fungi may have died but leaving behind an active toxin [5, 36].

CONCLUSION

From the result of this study, it can be concluded that the level of aflatoxin contamination of cereal sold in Kaduna Central market and Kawo market is low and within the acceptable limit. Quantitatively, it does not pose any serious risk of aflatoxin. However, the high fungal count obtained, and *Aspergillus* species isolated from the cereal samples is a source of concern and worry as the product might have severe health implication because *Aspergillus* species is occasionally isolated from human clinical specimen. This necessitates the need for efficient and hygienic care of cereals during both pre-harvest and post-harvest handling. Monitoring of cereals in markets is one of the possible ways to prevent the consequences of aflatoxin contamination. ELISA method is an easy to perform, fast and cost-effective method for the detection of aflatoxins in cereal and will be a major player in the monitoring of aflatoxin contamination in many samples in a cost and time effective manner to ensure compliance with enforced regulations.

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