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Research Application Summary

Fungal contaminants and aflatoxins in maize from lower eastern region in Kenya

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Abstract

Aflatoxin outbreaks have been reported in the lower eastern region of Kenya since 1978. These outbreaks have caused a lot of concern as some have led to loss of lives. This study was conducted to assess the extent of fungal contaminants in maize in the region and more importantly the exposure to aflatoxicosis due to *Aspergillus* spp contamination. Seventy two maize samples were purchased from farmers (24 samples from each county) interviewed in the survey that was conducted in October 2012. The samples were first assessed for general fungal contaminants and then for *Aspergillus* spp. contaminants. *Aspergillus* spp. were isolated using Czapek Dox agar medium. Fusarium, *Aspergillus* and *Penicillium* were the predominant fungal species that were isolated and for the *Aspergillus* species, *A. niger* and *A. flavus* were most predominant. Aflatoxin quantification was carried out using LC-MS analysis. The findings showed that aflatoxin B1 was more predominant than aflatoxin B2, aflatoxin G1 and aflatoxin G2. More than 70 % of the samples were fit for human consumption since they had aflatoxin levels less than 10ng/g while the highest had 128ng/g. Poor post-harvest practices might be the main source of Aspergillus contamination which leads to high level of aflatoxin as revealed in this study. Although most farmers are aware of the aflatoxin problem, continuous mycotoxin awareness campaign by Government and other stakeholders is recommended until everybody along the maize farming and trading value chains is aware. This will significantly contribute to eradication of this perennial aflatoxin problem in the region.

Key words: Aflatoxin, *Aspergillus,* Kenya, maize contaminants

Résumé

Des cas élevés d'aflatoxines ont été signalés dans la région du sud-est du Kenya depuis 1978, et ont suscité beaucoup d'inquiétude car certaines ont entraîné des pertes en vies humaines. Cette étude a été menée pour évaluer l'étendue des contaminants fongiques dans le maïs dans la région et, plus important encore, l'exposition à l'aflatoxicose due à la contamination par *Aspergillus spp*. Soixante-douze échantillons de maïs ont été achetés auprès des agriculteurs interrogés dans une enquête menée en octobre 2012. Les échantillons ont d'abord été étudiés pour y évaluer les contaminants fongiques généraux, puis *Aspergillus spp. Aspergillus spp*. a été isolé en utilisant du milieu gélose

Czapek Dox. *Fusarium, Aspergillus* et *Penicillium* étaient les espèces fongiques prédominantes qui ont été isolées et pour les espèces *Aspergillus, A. niger* et *A. flavus* étaient les plus prédominantes. L'évaluation de l'aflatoxine a été réalisée à l'aide d'une analyse LC-MS. Les résultats ont montré que l'aflatoxine B1 était plus prédominante que l'aflatoxine B2, l'aflatoxine G1 et l'aflatoxine G2. Plus de 70% des échantillons étaient sains pour la consommation humaine car ils avaient des niveaux d'aflatoxine inférieurs à 10 ng / g tandis que le plus élevé avait 128 ng / g. De mauvaises pratiques après récolte pourraient être la principale source de contamination par Aspergillus, ce qui conduit à un niveau élevé d'aflatoxine, comme le révèle cette étude. Bien que la plupart des agriculteurs soient conscients du problème des aflatoxines, une campagne continue de sensibilisation aux mycotoxines par le gouvernement et d'autres parties prenantes est recommandée jusqu'à ce que tous les acteurs de la chaîne de culture et de commercialisation du maïs en soient conscients. Ceci contribuera de manière significative à l'éradication de ce défi persistant causé par les aflatoxines dans la région.

Mots clés: Aflatoxine, *Aspergillus,* contaminants du maïs

Introduction

The safety of food and feed has been a major concern in most countries in the last years as more knowledge is being generated on the occurrence of natural toxins in foodstuffs and edible plant species. Among these natural toxins are mycotoxins which are toxic metabolites of fungi contaminating food and feed, phytotoxins produced by algae contaminating fishery products and plant toxins in edible plant species (Sheila and Margret, 2012). Among the three natural toxins named above, mycotoxins have been given more attention due to their hazardous effect both to animals as well as human beings (Miller, 1991).

Mycotoxins are toxic secondary metabolites produced by certain fungi in agricultural products that are susceptible to mould infestations (Bennett and Klich, 2003; Wagacha and Muthomi, 2008; Morenoa *et al*., 2009). The most important mycotoxins are aflatoxins, ochratoxins, deoxynivalenol, zearalenone, fumonisin and T-2 toxin. There are more than 300 known mycotoxins produced naturally of which Aflatoxins is the most widely studied (Sheila and Margret, 2012).

Aflatoxins are natural metabolites produced by Aspergillus species of fungi primarily *Aspergillus flavus* and *Aspergillus parasticus*. *Aspergillus* fungi are found in air, soil, seeds and plant debris and can contaminate maize, peanuts, peanut meal, cotton seed, cotton seed meal and beans. According to Nassir and Jolley (2002), aflatoxins are primarily associated with maize and maize products than any other crop. There are more than 13 known different types of aflatoxins of which the most common ones produced in maize are AFB1, AFB2, AFG1 and AFG2. It is appreciated that aflatoxin contamination only becomes dangerous for human consumption after certain levels are exceeded. These levels are normally specified for Aflatoxin contamination of type B1 (AFB1) and total aflatoxin contamination. The most conservative levels are specified by the European Union as 2ng/g and 4ng/g for AFB1 and total aflatoxin, respectively (Rahmani, *et al*., 2010). In Kenya, the acceptable level of AFB1 was initially 20ng/g (Onsongo, *et al*., 2004) but has now been revised to 10ng/g for total aflatoxin (Muthomi *et al*., 2012).

Aflatoxin outbreaks in Kenya have been reported over years but the largest outbreak reported in the history was in 2004 when 317 cases were reported with 125 deaths in Makueni, Kitui and neighboring districts (Lewis *et al*., 2005). Maize from the affected areas contained as much as 4,400ng/g aflatoxin

B1, which is 440 times greater than the 10 ng/g tolerance level set by the Kenya Bureau of Standards (KEBS). It is believed that most of the aflatoxin poisoning outbreaks occurred in remote villages and, therefore, the number of people affected could have been higher than reported (Lewis *et al*., 2005).

The aflatoxin contamination in maize has been associated with drought combined with high temperature as well as insect injury (Betran and Isakeit, 2003). Genotype, soil types, drought and insect activity are important in determining the likelihood of pre-harvest contamination (Cole *et al*., 1995). Poor harvesting practices, improper storage and less than optimal conditions during transport and marketing can also contribute to fungal growth and proliferation of mycotoxins (Bhat and Vasanthi, 2003; Wagacha and Muthomi, 2008).The major factors that have been reported to contribute to aflatoxin production in maize include moisture content (Manoch *et al*., 1988), relative humidity and temperature in storage (Moreno and Kang, 1999), storage period (Liu and Yu, 2006) and storage types (Roy and Chourasia, 2001).

Therefore, the objectives of this study were to assess farmers' practices during harvest and post-harvest handling of maize that may lead to aflatoxin contaminations in the lower eastern region of Kenya. The study was also undertaken to determine the distribution of fungal contaminants with the main focus being *Aspergillus* spp. and associated aflatoxin contamination on maize seeds in the region.

Materials and methods

Evaluating farmers' practices that may lead to aflatoxin contamination. A survey was carried out using an open and closed questionnaire in lower eastern regions in Kenya where cases of aflatoxin contaminations on maize had been reported. The regions surveyed include Machakos county, Kitui county and Makueni county. From each county two agro-ecological zones were identified, i.e., LM4 and LM5 from which 12 farmers were randomly identified and interviewed. From each farmer, one kilo of maize seed was purchased and packed in a khaki paper bag and delivered to the University of Nairobi microbiology laboratory for isolation of *Aspergillus*. The remainder of the grain was stored at 4^0C for further analysis.

Isolation and identification of fungi. *Aspergillus* spp. were isolated from whole maize samples collected from farmers. Grains were surface sterilized using 3% sodium hypochlorite. Thirty kernels from each of the sample were plated on Czapek Dox growth medium (agar 20 g, sucrose 30 g, $NaNO_3$ 2 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_47H_2O_2$ 0.01 g, K_2HPO_4 1 g and distilled water 1000 ml) amended with 20 ppm of tetracycline, streptomycin, penicillin and pentachloronitrobenzene (PCNB). The plates were incubated at room temperature (RTP) for up to fourteen days. Kernels with fungal growth were counted. Those with *Aspergillus* spp. growth were separated from the rest for further observation and identification. *Aspergillus* colonies were further sub-cultured in Czapek Dox agar medium for up to 14 days and identified to species level based on both cultural and morphological characteristics.

Analysis of aflatoxin in maize samples. The maize samples were crushed to very fine flour using a Retsch rotor mill (model SK 1, Germany). Ten grams of the flour was weighed into 100 mls falcon tubes. Forty milliliter of acetonitrile: water 84:16 was added and vortexed for five minutes. Forty micro-liters of internal standard griesofluvin 5 mg/ml was added to the mixture and vortexed for

30 minutes and left to settle for another 30 minutes. Six milliliters of the supernatant was drawn and filtered through multistep 228 aflapat column. Four milliliters of the filtrate was evaporated to dryness in the hood. After evaporation, it was re-constituted in 400 micro-liters of methanol: water (20:80), vortexed for five minutes, centrifuged at 10,000 rpm for three minutes and supernatant analyzed using Lc/Ms analysis. LC-MS detection was used in determining the amount of aflatoxins in the samples. The LC-MS consisted of a quaternary LC pump (Model 1200) coupled to Agilent MSD 6120-Single quadruple MS with electrospray sourced from Palo Alto, CA. The system was controlled using ChemStation® software (Hewlett-Packard). Reversed-phase liquid chromatography was performed on an Agilent technologies 1200 infinite series, Zorbax SB C18 column, 2.1 x 50 mm, 1.8 µm (Phenomenex, Torrance, CA).

The sample were dissolved in 100% B (MeOH) (LC-MS grade, Sigma, St. Louis, MO), vortexed and centrifuged at 10,000 rpm to remove insoluble material before analysis by LC-MS. The mobile phase used a gradient program initially 80:20 (A:B), [(A 5% formic acid in LC-MS grade ultra-pure H2O, Sigma, St. Louis, MO] to 0:100 at 10 min and maintained at this solvent proportion for 15 minutes, 80:20 at 26 minutes to 30 minutes which was the run time. The flow rate was 0.7 ml min^{-1}. Injection volume was 10 ∞l and data were acquired in a full-scan positive-ion mode using a 100 to 800 m/z scan range. The dwell time for each ion was 50 ms. Other parameters of the mass spectrometer were as follows: capillary voltage, 3.0 kV; cone voltage, 70 V; extract voltage, 5 V; RF voltage, 0.5 V; source temperature, 110ºC; nitrogen gas temperature for desolvation, 380ºC; and nitrogen gas flow for desolvation, 400 L/h. Injection volume was 10 ∞L.

Results and discussion

Farmers' maize handling practices. A total of 72 maize samples were collected; 24 from each county. All samples were homegrown but in varietal mixtures. Most respondents were owners of the farms and mostly subsistence farmers. This may explain the spread of aflatoxin within the local markets. A number of them dried maize directly on the ground, a practice that may result to contamination with *Aspergillus*. Furthermore, it took most farmers up to two weeks to dry their maize to 13% moisture content required for proper storage. Farmers use change of colour, sound while others bite the maize kernels to determine whether maize is dry and ready for storage.

The study also revealed that most farmers (more than 50%) stored their maize in plastic bags. Plastic bags can retain moisture and accumulate heat which may promote *Aspergillus* growth and sporulation. At least 20% of the farmers stored their maize in granaries and sisal bags. These are well aerated and could minimize moisture and heat accumulation in grain resulting into reduced aflatoxin contamination.

In terms of awareness, most farmers were already aware of the problems associated with aflatoxicosis. They also could rate the extent of the problem in their respective areas. This is as a result of government and other stakeholders promoting aflatoxin awareness campaigns in the region. The farmers are also aware of practices for mitigating aflatoxin problem. It is worthwhile to note that most farmers were not aware of the tolerant varieties and for those claiming to be aware they were not specific on maize varieties that were tolerant or resistant to aflatoxin contamination. The results show that some post-harvest handling practices by some farmers expose them to aflatoxicosis.

Isolation of fungal inoculum. The most commonly isolated fungus was *Fusarium* spp. This was followed by *Aspergillus, Penicillium* and *Rhizopus* spp. in that order. A small number (<10%) of

samples were clean without any fungal contaminants. The predominant *Aspergillus* species was *A. niger* followed by *A. flavus*, *A. fumigatus* and *A. versicolor* in that order. Results show that *A. niger* had an average occurrence of of 50% and *A. flavus* 45% across the three counties. This was so despite the fact that the two counties Kitui and Makueni have more cases of aflatoxin contamination than Machakos. This also shows that not all of samples with *A. flavus* results in aflatoxin contamination.

The study revealed that post-harvest handling practices contribute to production of aflatoxin for most farmers. The methods used for drying, detecting whether the maize is dry, duration taken to dry the maize and storage are the practices that expose maize to *Aspergillus* contamination. According to Wagacha and Muthomi (2008), there is high frequency of *A. flavus* isolation in soils from different agro ecological zones from the eastern region of Kenya. This indicates that farmers should avoid contact of their maize with soil during harvesting and drying to avoid contamination. Use of plastic bags for maize storage should also be avoided. Mwihia *et al*. (2008) noted that plastic bags accumulate moisture and heat that can promote aflatoxin contamination. Plastic bags are poorly aerated but cheaper and more readily available than the preferred sisal bags or improved granaries (Turner *et al*., 2005).

One of the optimum conditions for growth and subsequent production of *A. flavus* is moisture content above 14% (Mutungi *et al*., 2008). Farmers in these regions have simple methods of determining the moisture content of their maize during and after storage (Muhia *et al*., 2008). They mainly use traditional methods of sound, change of colour and biting with teeth. This may result into storing of wet maize, creating favorable conditions for aflatoxin contamination. The duration of storage has a significant effect on the levels of aflatoxin in maize grain (Sumbali, 2001). Aflatoxin production depends upon storage conditions like moisture content (Manoch *et al*., 1988), relative humidity and temperature (Moreno and Kang, 1999), storage period (Liu and Yu, 2006) and storage facility (Roy and Chourasia 2001). Rewetting of the stored maize should be avoided as Hugh *et al*. (1970) noted that rewetted maize had more contamination of *A. flavus* and other *Aspergillus* spp than freshly harvested maize under similar conditions. In this regard, farmers should make sure their storage facilities are water proof with no leakages from roof tops for those who store their maize in granaries and those who store them in their houses to put their maize on wooden raised pallets and no contact with the ground to avoid rewetting as much as possible.

Most farmers were aware of aflatoxin problems in their respective areas. This can be attributed to the various public awareness campaigns run by Food and Agricultural Organization (FAO) and the Ministry of Agriculture after the 2004 aflatoxin outbreak (Strosnider *et al*., 2006). However, this study indicates that awareness campaigns should continue as contamination level are still high.

The maize samples were purely from home grown maize, although a substantial number of farmers (33%) also sell their produce. This likely contributes to aflatoxin spread within the local markets. According to Sheila *et al*. (2012), markets are the major source of chronic aflatoxin exposure to the larger population in Kenya.

The fungi that were isolated from maize included *Fusarium, Aspergillus, Penicillium* and *Rhizopus* spp. *Aspergillus* species isolated were *A. niger, A. flavus, A. fumigates* and *A. versicolor. Aspergillu. niger* was more frequently isolated from the three counties than the rest of the *Aspergillus* spp. The presence of *A. flavus* in some samples across the three counties is a concern as aflatoxins are primarily associated with maize infested with this species of *Aspergillus*.

Aflatoxin contaminations can occur when the crop is in the field or during harvest, drying and storage. Most contamination occurs post-harvest if the produce is not handled properly (Yadgri *et al*., 1970; Wilson *et al*., 1992). From this study, most of the contamination was due to *A. flavus* and may have occurred during the maize handling practices from harvest to storage. The practices like drying maize on the direct ground, storing on plastic bags, and maize taking long to dry are just but among the practices that might have created conducive environment for *Aspergillus* contamination.

Aflatoxin B1 and B2 and G1 were detected in maize samples from Kitui and Makueni but only aflatoxin B1 and B2 were detected in maize samples from Machakos county. The presence of aflatoxin B1 in some samples above 10ng/g is of concern as it is considered to be most toxic to human and even livestock. Cotty and Cardwell (1999) reported presence of aflatoxin B1 and B2 in *A. flavus* but Saleem *et al*. (2012) in addition reported presence of aflatoxins G1 and G2. Although *A. parasticus* was not reported, aflatoxin G1 was detected. Aflatoxin G1 and G2 are associated with *A. parasticus* (Egel *et al*., 1994). Detection of G1 could imply its association with *A. flavus* too. Probst *et al*. (2010) determined aflatoxin content in maize from eastern province of Kenya and found 41% of samples contained levels below 20ng/g. Results from this study indicates a change in the trend; 76.6% of the samples in this study had aflatoxin levels below 20ng/g while 65% were below 10ng/g. Machakos was least affected with 87.5% and 75%, followed by Makueni with 70.8% and 62.5% and lastly Kitui with 70.8% and 58% aflatoxin levels below 20ng/g and 10ng/g, respectively. This drop can be attributed to the various campaigns conducted in the region.

Conclusions and recommendations

Despite the public awareness in the region, aflatoxin contamination still remains a threat to some farmers as evidenced by the high levels of aflatoxin in maize. Some maize samples had levels above ed the maximum limits by both WHO and KEBS of 20ng/g and 10ng/g, respectively. The study reveals that most of the aflatoxin contaminations was as a result of poor handling practices of maize during and after harvest. Most maize farmers in the country are peasants and entirely dependent on nature for drying their maize.

Aflatoxin B1 was predominantly high among the maize samples and exposure to this toxin even in small amounts over a long period leads to chronic aflatoxicosis. This is made worse when contaminated samples from individual farmers get to the market where they are mixed with others and sold to the larger population. It is also worthwhile to note that most of aflatoxin exposure is in homegrown maize from individual farmers within the counties. This is true as maize from individual farmers analyzed in this study had a higher total aflatoxin content of up to 128ppb. Most of the maize that comes from outside the counties especially in form of food aid is usually tested before it gets to the target population.

Good agricultural practices, including both pre and post-harvest practices should be encouraged in the region. New simple and farmer friendly technologies should be developed to help farmers dry their maize, determine when the maize is dry and store their produce without exposing the maize to fungal contaminations. New varieties tolerant or resistant to *Aspergillus flavus* contamination should be developed for farmers in this region. Public awareness campaigns initiated by the Government and other stakeholders should be upscaled to reach the entire population especially farmers far from urban centres who are always hard to reach, yet they supply markets with maize.

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