Occurrence of Aflatoxigenic *Aspergillus* Species in Various Varieties of Peanuts Produced in Western Kenya

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ABSTRACT

Aflatoxin contaminates foods including peanuts. Aflatoxin is a carcinogenic toxin mainly produced bty Aspergillus flavus. Other Aspergillus species that rarely produce aflatoxins are A. nomius and A. niger. Aflatoxin is associated with liver failure, hepatocellular carcinoma (HCC) and death. Recent studies have shown that peanuts in Kenya are highly contaminated with aflatoxins but information gaps exist on the characterization of the Aspergillus species that produce aflatoxins in peanuts in Kenya. Therefore, this gap necessitated the determination of the Aspergillus species producing aflatoxins in peanuts from the main growing districts of Busia and Kisii Central districts. One hundred and two (102) peanuts samples were collected from farmers' in each district Aspergillus species were isolated from the peanut samples by using the dilution plate technique on modified Rose Bengal agar. Phenotypical characterization of the identified Aspergillus section flavus isolates from the peanuts samples was determined using the procedure of Mellon and Cotty. This study identified five (5) Aspergillus species as contaminants in peanuts analyzed in this study. They were Aspergillus flavus L-strain, Aspergillus flavus S-strain, Aspergillus parasiticus, Aspergillus niger and Aspergillus tamari. Overall, the occurrence of Aspergillus flavus L- strain and A. flavus S- strain were significantly higher than other species identified (H = 15.55, df = 4, P = 0.004) in peanuts from the two districts. However, A. flavus Sstrain was the most dominant species identified in the study with a mean occurrence of 45.1%. Aspergillus flavus L- strain was the most common isolate (58.8%) in peanuts from Busia district while A. flavus S- strain was the most common strain (60.2%) in peanuts from Kisii Central district. Overall, the occurrence of Aspergillus flavus L strain and A. flavus S strain were significantly higher than other species identified (H = 15.55, df = 4, P = 0.004) in peanuts from the two districts. However, A. flavus S-strain was the most dominant species (F=3.15, df =25, P=0.031) with an overall mean occurrence of 45.1%. The confirmation of occurrence of other species that produce toxins such as A. niger and A. tamarii which also produces cyclopiazonic acid suggests the need to screen peanuts for other carcinogenic mycotoxins.

Keywords: Aflatoxins; Peanuts; Aspergillus species

1. Introduction

Aflatoxins are highly carcinogenic mycotoxins that are produced by *Aspergillus* species, specifically *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins contamination of foods including peanuts are major hazards to human health and has been associated with liver failure, stunted growth in children, hepatocellular carcinoma (HCC) and death [1]. In Kenya, a high incidence rate of liver cancer was reported in the year 2008. The study reported that in every 100, 000 people suffering from liver cancer, 8.5 % were males while 4.9 % were females [2]. Aflatoxins are produced by *Aspergillus* species in food crops such as peanuts when they are poorly dried and stored [3].

Kenya has repeatedly experienced epidemics of acute aflatoxicosis especially in the Eastern province in the years 2001, 2004, 2005 and 2006 [4]. The largest outbreak due to maize aflatoxin poisoning was reported in the year, 2004 where 125 people died out of the 317 reported cases [5]. Studies conducted in other developing countries established a relationship between levels of aflatoxin and *Aspergillus* contamination. While both *A. flavus* and *A. parasiticus* can produce aflatoxin B toxins, *A. parasiticus* exclusively produce the G1 and G2 aflatoxins [3]. Recent studies have shown that peanuts in Kenya are highly contaminated with aflatoxins [6], but information gaps exist on the distribution of aflatoxin producing *Aspergillus* species. Further, few studies have been done to characterize the fungi that produce the different types of aflatoxins. This gap necessitated the determination of the *Aspergillus* species producing aflatoxins in peanuts from the main peanut producing districts of Busia and Kisii Central districts in western Kenya.

2. Materials and Methods

2.1. Study areas

The study was conducted in two districts in western Kenya namely Busia and Kisii Central. These are the main peanuts producing districts in the region and have several peanuts processors as well as a high demand for peanuts and their products [7].

2.2. Study design

A cross-sectional study was adopted among the peanuts farmers in Busia and Kisii Central districts.

2.3. Study population

The study comprised of peanuts producers in Busia and Kisii Central districts. The number of farmers/producers growing groundnuts varied between different divisions within each district. The divisions were determined based on altitude, mean annual rainfall, temperature and probability of successful growing of peanuts in that zone. The peanuts production statistics of farmers were obtained from the division's agricultural offices.

2.4. Sampling technique

Peanuts farmers' households were sampled from the divisions. In Busia, farmers in the leading groundnut producing divisions identified as Butula, Matayos, Funyula and Budalangi were purposively selected. The other division in Busia (Township) had only small areas under groundnuts cultivation and was therefore, not included in the study. In Kisii, farmers in the four leading peanut producing divisions; Keumbu, Masimba, Suneka and Mosocho were also purposively sampled. The other divisions in the district (Kisii Town and Marani) were excluded from the study due to small areas under groundnuts production. Within the divisions (agroecological zones) selected in both Busia and Kisii Central districts, peanuts farmers households were randomly selected by staggering every fourth household within the division administrative boundary, the starting point being the fourth household from the division's agricultural office in the particular divisions. The sampling interval was obtained based on the approximate peanuts farmers' population of 408 in the study areas [8] divided by the sample size (102).

2.5. Study approval, Ethical consideration and Informed consent

Ethical approval was obtained from Kenyatta University Ethics Review Committee. The study objectives were explained to the peanuts farmers and they were allowed to ask questions. After giving consent, the farmers who were willing to participate signed a consent form and peanuts samples were collected.

2.6. Laboratory Analysis

2.6.1. Sample collection

A total of 102 peanut samples from each peanut farmer's household in each study district were collected using the procedure of Whitaker, 2006 [9]. Peanuts stored in sacks or boxes were sampled from different parts using a closed spear driven through the top and sides of each sack or box to obtain a total of 0.5 kg of sample. Each of the 0.5 kg samples of unsorted peanuts was put in clean polyethylene bags, sealed, labeled and transported in cool boxes to Bora Limited Laboratory, Nairobi and University of Nairobi, Department of Food Science, Nutrition and Technology and they were stored at 4°C until the time of analysis for incidence, types and levels of aflatoxins in peanuts.

2.6.2 Aspergillus species culture and identification

Twenty (20) grams of each peanut sample was mixed thoroughly by shaking and grounded using a dry mill kitchen grinder (Kanchan multipurpose Kitchen machine, Kanchan International Limited Mumbai, India). *Aspergillus* species were isolated from the peanut samples by using the dilution plate technique on modified Rose Bengal agar using the procedure of Probst *et al.*, 2007 [10]. Briefly, 10 grams of each grounded sample was added to 90 ml of 0.1% peptone dissolved in water. This mixture was shaken on a rotary shaker for approximately 15 minutes and diluted 10^2 , 10^3 and 10^4 . Aliquots of 0.1 ml of each dilution was spread (in triplicate) on the surface of the Dichloran Rose Bengal Chloramphenicol agar medium [11]. All plates were incubated for 3-7 days at 28° C in the dark and under room temperature. All the three sets of dilutions averaging between 10 and 60 colonies per petridish were counted and an average was obtained. The results were computed and expressed as colony forming units per g (cfu/g) of peanuts.

The colonies of *Aspergillus* species were sub-cultured on 9 cm diameter petridishes containing 20 ml of Malt Extract Agar (MEA) and Czapek-Dox agar (CZ), and then incubated for 7 days in the dark at 25°C. They were subsequentially examined for colony colour, presence and size of sclerotia, head seriation and conidial morphology. Identification was performed according to Klich, 2002 [12]. All isolates were also cultured on *Aspergillus flavus parasiticus* agar (AFPA) for 3-5 days at 25°C in the dark to confirm group identification by colony reverse colour. All isolates were subsequently cultured on CZ agar at 42°C and colony diameters measured after 7 days of incubation [13]. Identification of species isolates was done according to Klich, 2002 [12], and by comparison with reference strains obtained from Dr. Bruce Horn (USDA National Peanut Research Lab, Dawson, Georgia, United States of America).

2.6.2.1 Aspergillus flavus strains characterization

Phenotypical characterization of the identified *Aspergillus* section flavus isolates from the peanuts samples was determined using the procedure of Mellon and Cotty, 2004 [14]. Briefly, up to 10 colonies of identified isolates of *Aspergillus flavus* were aseptically transferred onto 5/2 agar (5% V-8 juice and 2% agar, pH 5.2) and incubated for 5 - 7 days at 31 C. The isolates were then classified on the basis of colony characteristics and conidial morphology at x400 magnification using a high resolution microscope. Colony radius was measured in millimeter (mm) and the colony color of isolates determined using Methuen color book [15]. Isolates with abundant small sclerotia (average diameter < 400mm) were classified as strain S of *A. flavus* [16]. Isolates with smooth conidia and large sclerotia (average diameter over 400mm) were classified as the L strain of *A. flavus* [16].

3. Results

3.1. Peanuts varieties from Busia and Kisii Central districts

A total of 204 peanut samples of different varieties were collected in the two districts. In Busia district, the 102 peanuts were of four different varieties; Valencia red, Uganda local, Homabay local and Local red. The 102 peanuts from Kisii Central district were of three different varieties; Valencia red, Uganda local and Homabay local. In both districts, Valencia red variety had the most number of the samples, 59 and 89 from Busia and Kisii Central districts respectively which were significantly different from the other varieties ($\chi^2 = 12.00$, df = 9, P = 0.02). There were more samples of Uganda local red (21) and Homabay local (20) varieties from Busia district compared to those from Kisii Central district. Local red variety had only 2 samples in Busia and none in Kisii Central (**Table 1**).

Table 1. Peanut varieties from Busia and Kisii Central districts.

	Number of peanut samples collected			
Peanut variety	Busia	Kisii Central	Total	
Valencia red ^a	59	89	148	
Uganda local red	21	5	26	
Homa Bay local	20	8	28	
Local red	2	0	2	
Total	102	102	204	

a= peanut variety with a significantly higher number of samples in the study.

3.2 Occurrence of Aflatoxins producing Aspergillus species in peanuts

Five (5) Aspergillus species were identified as contaminants in peanuts analyzed in this study. They were Aspergillus flavus L- strain, Aspergillus flavus S- strain, Aspergillus parasiticus, Aspergillus niger and Aspergillus tamarii. Overall, the occurrence of Aspergillus flavus L- strain and A. flavus S- strain were significantly higher than other species identified (H = 15.55, df = 4, P = 0.004) in peanuts from the two districts. However, A. flavus S-strain was the most dominant species identified in the study with a mean occurrence of 45.1% (Table 2). Aspergillus flavus L-strain was the most common isolate (58.8%) in peanuts from Busia district while A. flavus S-strain was the most common strain (60.2%) in peanuts from Kisii Central district (Table 2).

Table 2: Occurrence of *Aspergillus* species isolated and identified in peanuts samples in the two study Districts

	Busia		Kisii Central		
Aspergillus species isolated	n	%	n	%	
A. flavus L- strain	60	58.8	22	21.8	
A. flavus S- strain	30	29.4	62	60.2	
A. parasiticus	7	6.9	12	12.0	
A. niger	2	2.0	4	4.0	
A. tamarii	0	0.0	2	2.0	
Negative for Aspergillus species	3	2.9	0	0.0	

Aspergillus parasiticus was the third most common isolate in peanuts from both districts at 12% and 6.9% in peanuts from Kisii Central and Busia districts respectively. Other species including Aspergillus niger was isolated at 2% and 4% in peanuts from Busia and Kisii Central districts respectively. Aspergillus tamarii was the least occurring species at 2% in peanuts from Kisii Central district and none from peanuts from Busia district (Table 3). The mean occurrence for Aspergillus tamarii was 1% in both districts (Table 3). Only, 2.9% of peanut collected from Busia district were negative for Aspergillus species contamination while all peanut from Kisii Central district were contaminated with at least one aflatoxin producing species (Table 3).

Table 3: Mean occurrence of different Aspergillus species in peanuts from the two districts

Aspergillus species isolated	n	%
A. flavus L- strain	82	40.2
A. flavus S- strain	92	45.1
A. parasiticus	19	9.3
A. niger	6	2.9
A. tamarii	2	1.0
Negative for Aspergillus species	3	1.5

3.2.1 Aspergillus species in the different varieties of peanuts

All the varieties of peanuts sampled from both Busia and Kisii Central districts were contaminated with at least one or more of A. flavus L- strain, A. flavus S- strain, A. parasiticus, Aspergillus niger and A. tamarii species. Overall, in establishing the most prevalent Aspergillus species isolated in peanuts from Busia and Kisii Central districts, incidences of the five Aspergillus species were compared using One-way Analysis of Variance (ANOVA). The result showed that the incidence of Aspergillus flavus S-strain was significantly higher than other Aspergillus species identified (F = 3.15, F = 0.031).

Aspergillus flavus L- strain was the most highly detected strain (60.6%) in all the peanut varieties from Busia district compared to the other Aspergillus species isolated (H = 10.03, df = 3, P = 0.018). The species was mostly found in Homabay local variety peanuts from Busia at 33.3% from Busia (Table 4). Aspergillus flavus S- strain was the most abundant species in peanuts of the Homabay local from Busia district at an incidence of 40%. Aspergillus parasiticus was also found to be contaminating all the peanut varieties from the study district but was isolated highly in peanuts of local red, Valencia red and Uganda local varieties at an incidence of 28.6%. Aspergillus niger was only detected in all the peanuts of Local red variety while A. tamarii was not detected in any peanut varieties from Busia district (Table 4).

All the strains of Aspergillus except A. tamarii were isolated in all the varieties from Kisii district. However, Aspergillus flavus S-strain had higher occurrence at 60.8% compared to other species identified in peanuts from the district (H = 12.28, df= 4, P = 0.015). Aspergillus flavus S-strain was highly detected in samples of Valencia red variety with incidence of 79% compared to Aspergillus flavus L- strain at 54.6%. Aspergillus parasiticus species was found at an incidence of 41.7% in Homabay local variety while Aspergillus tamarii species was detected in Uganda local red and Homabay local peanut varieties at similar rates of 50% (Table 4).

Table 4: Aspergillus species isolated from the different varieties of peanuts

		Aspergillus species isolated				
District	Peanut variety	A. flavus L-strain	A. flavus S-strain	A. parasiticus	A. niger	A. tamarii
Busia	Valencia red	19(31.7%)	7 (23.3%)	2 (28.6%)	0 (0.0%)	0 (0.0%)
	Uganda local red	19(31.7%)	9(30.0%)	2(28.6%)	0(0.0%)	0(0.0%)
	Homabay local	20(33.3%)	12(40.0%)	1(14.2%)	0(0.0%)	0(0.0%)
	Local red	2(3.3%)	2(6.7%)	2(28.6%)	2(100%)	0(0.0%)
	Total	60(60.6%)	30(30.3%)	7(7.1%)	2(2%)	0(0)
Kisii Central	Valencia red	12(54.6%)	49(79.0%)	4(33.3%)	1(25.0%)	0(0.0%)
	Uganda local red	5(22.7%)	5 (8.1%)	3(25.0%)	2(50.0%)	1(50.0%)
	Homabay local	5(22.7%)	8(12.9%)	5(41.7%)	1(25.0%)	1(50.0%)
	Total	22(21.6%)	62(60.8%)	12(11.7%)	4(3.9%)	2(2%)

The rate in percentage of each species was calculated based on the total number of isolates of each species in each district of study.

4. Discussion

4.1. Peanuts varieties from Busia and Kisii Central districts

In both districts, peanuts of Valencia red variety had the highest number of samples, 59 and 89 from Busia and Kisii Central districts respectively which were significantly different from the other varieties. This could have been attributed to the fact that Valencia red variety is the most common improved variety of peanuts planted by farmers [17] compared to other varieties in the study areas. The variety tends to be preferred due to higher yield and resistance to diseases by *Aspergillus* species compared to the local varieties [17]. The results of this study are in line with other studies that documented a higher number of peanuts of Valencia red variety than the other varieties in the study areas [17].

In this study, there were few samples of peanuts of local varieties; Uganda local red (26), Homabay local (28) and Local red (2) from the two study districts. It is possible that peanuts farmers avoid planting these varieties because they have low yields and are less resistant to diseases [18]. Moreover, local varieties of peanuts such as Uganda local red, Homabay local and Local red, have been reported to be more susceptible to rosette virus and mould than improved varieties [19], and a positive correlation between the diseases and aflatoxin contamination of peanuts have been documented in other studies [20]. These findings are consistent with those reported in a previous study by Mutegi *et al.*, 2010 [17].

4.2 Occurrence of Aflatoxins producing *Aspergillus* species in peanuts from Busia and Kisii Central Districts.

This study identified the *Aspergillus* species in peanuts from Busia and Kisii Central that are involved in the production of aflatoxins in the peanuts. The predominant *Aspergillus* species across the districts with over 58% incidence were *A. flavus S*- strain and *Aspergillus flavus L*-strain, with an incidence of 60.2% and 58.8% respectively. *Aspergillus flavus* which includes *L* strain and *S* strain have been documented as the common species that grow and produce aflatoxins in foods including peanuts than other *Aspergillus* species [21]. These *Aspergillus* species have been isolated at slightly higher incidences in peanuts in a previous study [17], *S* strain at 78% and *L* strain at 68%. The difference in incidences between the current and the previous studies could have been contributed by difference in sample sizes and the specific study Districts.

Aspergillus flavus L- strain was the most common isolate (58.8%) in peanuts from Busia district while A. flavus S- strain was the most common strain (60.2%) in peanuts from Kisii Central district. This might be contributed by difference in weather conditions between the two study districts. Aspergillus flavus S strain contains aflatoxin Q (aflQ) toxigenic genes which usually produces high aflatoxins in wet conditions while Aspergillus flavus L- strain (contains aflatoxin D toxigenic genes) that produces high aflatoxins in dry conditions [22]. It was noted that the species was isolated at a high incidence in Kisii Central while Aspergillus flavus L- strain was isolated more in Busia district. The results are in line with other studies [23, 17]. Aspergillus parasiticus, Aspergillus niger and Aspergillus tamarii were isolated in this study at overall mean occurrences of 9.3%, 2.9% and 1% respectively in peanuts from the two study districts. The low occurrences of these three Aspergillus species in the study areas is in line with the reports of Horn, 2005 [23], who documented these species in the United States of America and Mutegi et al., 2010 [13] in Kenya at comparable low occurrences.

The high incidence of *A. flavus* S- strain particularly in Kisii Central that produces aflatoxin [13] and in particular, the most potent Aflatoxin B1[13], indicates a risk of aflatoxin contamination of peanuts in areas in the western Kenya with wet climatic conditions which enhances the growth and production of aflatoxins mainly by *Aspergillus flavus*. In as much as the occurrence of *Aspergillus flavus L*- strain was slightly low in peanuts from Busia (58.8%) compared to *A. flavus S*- strain in Kisii Central (60.2%), it did not result to higher level of aflatoxins in the peanuts compared to *A. flavus S*- strain and this could be attributed to the fact that most of the *L*-strains may be atoxigenic. This resulted to low levels of total aflatoxin in peanuts from Busia

compared to those from Kisii Central. A similar trend has been found in other studies whereby *Aspergillus parasiticus* has been found to be the main source of aflatoxin in foods including peanuts [10].

Aspergillus parasiticus was the third most common isolate in peanuts from both districts. The species was isolated in peanuts from Kisii Central district at 12% and 6.9% in peanuts from Busia district. Aspergillus parasiticus is known to be common in wet climatic conditions and such environmental conditions facilitates growth and aflatoxin production especially aflatoxin G1 by the species [13]. This reason could explain the high occurrence of Aspergillus parasiticus in Kisii Central district compared to Busia. The results are consistent with previous study on peanuts by Mutegi et al. 2009 [24] where Aspergillus parasiticus was documented as the third most common Aspergillus species after A. flavus S- strain and Aspergillus flavus L- strain in the production of aflatoxin in peanuts. The confirmation of occurrence of other species that produce toxins such as A. niger and A. tamarii which also produce cyclopiazonic acid [25], suggests the need to screen peanuts not just for aflatoxins but also for other carcinogenic mycotoxins.

4.2.1 Distribution of Aspergillus species in the different varieties of peanuts

The results of this study showed that all the varieties of peanuts sampled from both Busia and Kisii Central districts were contaminated with at least one or more of *A. flavus L-* strain, *A. flavus S-* strain, *A. parasiticus, Aspergillus niger and A. tamarii* species. All the *Aspergillus* species were isolated in all the peanuts varieties from Busia district except *A. niger* which was detected in peanuts of Local variety while *A. tamarii* was not detected at all. Overall, *Aspergillus flavus* L- strain was the most highly detected strain (60.6%) in all the peanut varieties collected from Busia district (H = 10.03, df = 3, P = 0.018) followed by *Aspergillus flavus S- strain* at 30.3% occurrence. Previous studies have reported that *Aspergillus flavus L-* strain and *Aspergillus flavus S-* strain are the most common species involved in production of aflatoxins in foods including peanuts [22] and *Aspergillus flavus L-* strain which contains aflD toxigenic genes produces aflatoxin more in dry weather conditions compared to *Aspergillus flavus S-* strain [24].

Aspergillus flavus L- strain was found in peanuts of Homabay local variety at an occurrence of 33.3% while Aspergillus flavus S- strain had 40% in the same variety from Busia. This could have probably been as a result of high susceptibility of the local variety to crop diseases and pests, which result in plant stress thereby predisposing peanuts to the growth of Aspergillus flavus [26] particularly the most toxigenic Aspergillus flavus S strain. Aspergillus parasiticus was also found to be contaminating all the peanut varieties from the study district but highly isolated in peanuts of Local red, Valencia red and Uganda local varieties at similar rates of 28.6%. This could be due to the fact that Aspergillus parasiticus grows and produce aflatoxins even in improved peanuts varieties such as Valencia red. Aspergillus niger was only detected in peanuts of Local red variety from Busia. This indicates that this peanut variety from the area is more highly susceptible to the growth of Aspergillus including the less common species. The results are in line with a previous study [7] that documented that local peanuts varieties such as Local red, Homabay local and Uganda local red are more susceptible to diseases such as stem rot and mould which facilitates the growth of Aspergillus species.

In peanut varieties from Kisii Central district, all the strains; Aspergillus flavus S-strain, Aspergillus flavus L- strain, Aspergillus parasiticus, A. niger except A. tamarii were isolated in all the varieties. However, Aspergillus flavus S-strain had higher occurrence at 60.8% compared to other species identified from the district (H = 12.28, df= 4, P = 0.015). This is because the species better grow in wet weather conditions compared to other Aspergillus species resulting to its high occurrence [19]. This could have contributed to its high detection in peanuts of Valencia red variety (79%). Aspergillus flavus L- strain had low occurrence (21.6%) in all varieties from Kisii Central. However, it's important to note that it was also detected highly in peanuts of Valencia red variety compared to other varieties. This suggests that the variety was more susceptible to Aspergillus species contamination than other varieties. This could probably be contributed by sowing of Aspergillus contaminated Valencia red variety seeds from the supplier in the district which resulted to contaminated harvests.

Aspergillus parasiticus species had a higher incidence of 41.7% in Homabay local variety compared to other varieties. Aspergillus niger had higher occurrence in peanuts of Uganda local red while Aspergillus tamarii was detected in peanuts of Uganda local red and Homabay local varieties at similar rates of 50%. This could be probably due to higher susceptibility of local varieties to crop pests and diseases such as stem rot which facilitates Aspergillus species contamination including the less common species [27]. The result is in line with the study of Mutegi et al. 2009 [24] who showed that peanuts of local varieties have a higher likelihood of being contaminated with aflatoxin than improved varieties. Previous studies have documented higher susceptibility of local varieties peanuts to improved varieties in fungal contamination including Aspergillus species in the United Kingdom [28].

5. Conclusions

Aspergillus flavus L- strain was the most common isolate (58.8%) in peanuts from Busia district while A. flavus S- strain was the most common strain (60.2%) in peanuts from Kisii Central district. Overall, the occurrence of Aspergillus flavus L strain and A. flavus S strain were significantly higher than other species identified (H = 15.55, df = 4, P = 0.004) in peanuts from the two districts. However, A. flavus S-strain was the most detected species (F=3.15, df =25, P=0.031) with an overall mean occurrence of 45.1%.

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