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Prevalence of Aflatoxin Biosynthesis Genes According to Aflatoxin Levels in Maize of Different Varieties in Kenya

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SC and DO designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors WW and CB reviewed the experimental design and all drafts of the manuscript. Authors SC and JG managed the ELISA aflatoxin analyses of the study. Authors SC and CB identified the fungal isolates. Authors SC and DO undertook molecular assay while author SC performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine aflatoxin biosynthetic genes in fungal isolates in relation to aflatoxin levels in maize grain varieties from epidemiologically aflatoxicosis hot spots and non-aflatoxin hot spots agro-ecological zones in Kenya.
Study Design: Purposeful sampling technique was applied targeting regions of variable aflatoxicoses susceptibilities.

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Place and Duration of Study: Samples were sourced from Kitui/Kibwezi counties, an aflatoxin hot spot, during 2008-2010 Maize growing seasons. Comparative samples were from Uasin-Gishu county and Perkerra irrigation scheme in Baringo County, both with no previous acute aflatoxicosis and practicing commercial cultivation under rain-fed and irrigation farming systems, respectively.
Methodology: Maize samples *(n=295*) and fungal isolates *(n=61)* were analyzed for aflatoxin contamination and presence of aflatoxin biosynthetic genes, respectively. Total aflatoxin quantification was by a commercial Enzyme Linked Immunosorbent Assay (ELISA) kits, Boratest®, while molecular characterization of *Aspergillus flavus (n=40)* and *A. parasiticus (n=21)* isolates applied Quadruplex Multiplex PCR technique encompassing four aflatoxin biosynthetic genes: *nor-1, ver-1, omt-A* and *aflR.* Findings from the study variables were analyzed according to maize variety, agro-ecological origin of maize samples and fungal species besides type of farming system.
Results: Uasin-Gishu maize samples *(n=158)* assayed for aflatoxins belonged to six maize commercial varieties; H614*,* H629, H6213, H6210*,* H613 and H628 alongside an indigenous variety, *Kipkaa.* All Perkerra samples *(n=61)* also belonged to a commercial variety, H513. Contrastingly, all Kitui/Kibwezi samples (*n=76*) belonged to an indigenous variety, *Kikamba (Kinyanya*). The varieties H613, H628 and *Kipkaa* all had the samples (100%) within the Kenyan statutory safe total aflatoxin limit (≤10.0 ppb) whereas *Kikamba* and H513 varieties had 82.9% and 83.6% samples within safety limits, respectively. Similarly, the mean aflatoxin content for all the seven Uasin-Gishu varieties was only 1.62 ppb while *Kikamba* and H513 had means of 14.6 ppb and 15.6 ppb, respectively *(P=0.05).* Positive PCR amplification results were obtained in 96.3%, 84.2% and 80% for Kitui/Kibwezi, Perkerra and Uasin-Gishu isolates, respectively whereas regional distribution of amplicon spectrum was 6, 3 and 2 out of 8, respectively. A similar regional pattern was established regarding prevalence of PCR positive isolates and whose maize samples of origin also tested ELISA-aflatoxin positive, having been 81.5%, 52.6% and 26.7% for Kitui/Kibwezi, Perkerra and Uasin-Gishu. Interestingly, the only isolate PCR positive for all the four genes under assay and whose maize sample of origin had aflatoxins was coincidentally from Kitui/Kibwezi, an epidemiologically aflatoxicosis hot spot agro-ecological zone.

Keywords: Multiplex; PCR; aflatoxin; biosynthesis; genes; maize; varieties; Kenya.

1. INTRODUCTION

1.1 Aflatoxins and Aflatoxin Food Poisoning

Maize is Kenya's principle staple food crop, providing substantial caloric intake to most urban and rural households of diverse socio-economic spectrum. The annual per capita consumption of maize products is approximately of 100 Kg where it is eaten in different dietary forms [1,2,3]. Besides this critical role regarding its direct importance to the national food security, its rich ingredients comprising oils and starch makes it an ideal basic raw material for various industrial processes including: animal feeds, corn oil, starch powder, sweeteners, candies, fermented dairy products as well as both traditional and commercial alcoholic beverages, particularly busaa, changaa and lager beers [4,5,6].

It is on the background of the high per capita consumption and the wide spectrum of maize utilization in Kenya that its mycological and aflatoxin safety is of great public health concern. Among the fungi and mycotoxins that have epidemiological significance with regard to Kenya's public health in the past is aflatoxin producing fungi, specifically, *Aspergillus flavus* and aflatoxins, respectively. The first fatal case was in 1981 when 20 patients, 12 of whom died (Case Fatality Rate; CFR=60%) were diagnosed with hepatitis caused by eating aflatoxin contaminated maize in Machakos district. Maize samples and victims liver tissues indicated high AFB_1 levels of 12,000 ppb and 89 ppb, respectively [7]. In another fatal outbreak never witnessed in the history of aflatoxin food poisoning, 477 cases associated with eating contaminated maize were documented with a case-fatality rate of 40% during the period 2004-2006 where 150 deaths occurred in several districts of Eastern Province including Machakos, Makueni, Kitui and Embeere [8,9]. Aflatoxins occurred in 55% of maize meals at levels greater than the then statutory limit of 20 ppb, with samples recording as high as 8,000 ppb [10]. The same region of lower Eastern Province including the now re-named Makueni and Kitui Counties were the most severely affected [8,11,12].

Aflatoxins are toxic secondary fungal metabolites produced by species within genus *Aspergillus*, section *Flavi.* which include *A. flavus, A. parasiticus* and *A. nomius* [13]. Biosynthesis is genetically encoded by clustered genes whereby disease and death in humans and other animals can occur upon ingestion even at low concentrations. The most potent aflatoxins of great relevance regarding food safety and human health are: Aflatoxin B_1, B_2, G_1 and G_2. These aflatoxins are produced mainly by the two species: *A. flavus* (AFB_1, AFB_2) and *A. parasiticus* (AFB_1, AFB_2, AFG_1 and AFG_2 [14]. Regarding toxicity, Aflatoxin B_1 (AFB_1) is considered the most potent of all, with the levels of carcinogenicity in declining order being AFB_1>AFG_1>AFB_2>AFG_2 [15,16]. The International Cancer Research Institute (ICRI) identifies aflatoxins as a Class 1 carcinogen, resulting in regulation of these toxins to very low concentrations in traded food commodities [17,18]. The national statutory maximum limit for total aflatoxin levels for maize and maize products in Kenya is stipulated at 10 ppb and 5 ppb for AFB_1, having been upgraded from previous 20 ppb for total toxins following the 2004-2006 tragic aflatoxicosis outbreak [19,20].

The data based on recurrent outbreak of acute aflatoxin poisoning in Kenya together with the widely documented dietary contamination profiles of staple foods, particularly maize as regards incidence of aflatoxin producing fungi and aflatoxins at unsafe levels indicate the profound magnitude of the threat that aflatoxins pose to the National food safety and Public health in Kenya. The favorable regional agro-ecological conditions for fungal proliferation and unsound agronomic practices including use of maize varieties susceptible to aflatoxin producing fungi could be some of the factors that enhance development of aflatoxins along the maize food value supply chain [21,22].

Research targeting finding sources of human aflatoxin exposure include those that established home-grown maize was the primary source of contamination during the 2004-2006 aflatoxicoses in Eastern Province [8,9,23]. In other comparative studies, factors including high levels of aflatoxigenic fungi in soils and the semi-arid climatic conditions of Kitui, Machakos and Makueni counties were found to correlate with the high levels of aflatoxins in maize in these regions compared to areas with humid and abundant rainfall including Uasin-Gishu, Nandi, Trans-Nzoia, Nyeri, Bungoma and Siaya counties where contamination levels were lower [24,25,26,27].

1.2 Incidence of Aflatoxins According to Maize Variety

The various studies in Kenya on aflatoxin prevalence have hardly addressed its incidence in relation to type of maize varieties other those by Mutiga [28]. Majority of farmers in Eastern Province use indigenous varieties, particularly *Kikamba (Kinyanya)* that are locally sourced and its use is popular farming practice due to lower pricing, quick maturity and perceived high yielding under the local semi-arid agro-ecological conditions. The seeds are sourced either from their own grain reserves of previous crop, from their neighbors, or mostly from village-level cereal seed-grain markets [29]. This practice has however, been found to encourage cross-transfer of toxigenic strains from one infected crop to another in the maize farm ecosystem in studies from Uganda and Nigeria [30,31]. Further, the *Kikamba* has big sized cobs, with ears that do not droop at maturity instead it remains upright thereby predisposing the maize ears to birds' damage and fungal infection [32].

Contrastingly, the commercial certified seed varieties established popular with farmers in high maize productivity agro-ecological zones of Western Kenya, including H614 and H513 have robust qualities amongst: disease resistance, sweet taste, good standability and complete husk cover [33,34]. The ears also droop at physiological maturity hence avoiding pre-harvest fungal contamination [35,36]. The husk on the ear constitutes a major physical barrier to infection of kernels by *A. flavus* [37]. However, multi-colored indigenous maize varieties locally called *Kipkaa* are still preferred by some farmers in Uasin-Gishu County due to their early maturity and sweet taste. This variety, like the *Kinyanya* does not droop at physiological maturity and is popularly used for brewing traditional beer, busaa [38]. In Mexico, these multi-colored maize varieties are used to make popular pasta diets, tortillas, whose consumption has unfortunately been linked to cervical cancer [39].

1.3 Aflatoxin Biosynthesis Genes

The understanding of the conditions under which the aflatoxin biosynthetic genes are expressed enables prediction and control of the conditions enhancing aflatoxin secretion in foods [40]. It has been established that expression of aflatoxin

biosynthetic genes does not occur during active growth phases but is instead common during growth repression and stressful periods, particularly high temperatures and prolonged low moisture content [41]. These conditions are coincidentally prevalent in the regions of Kenya associated with recurrent acute aflatoxicosis and those under irrigation farming system [24].

The biosynthetic pathway of aflatoxins as a secondary metabolites derived from a polyketide comprising of 27 enzymatic steps, about 30 genes and more than 15 structurally defined aflatoxin intermediates [42], is via the conversion process:

acetate → polyketide → anthraquinones → xanthones → aflatoxins [43,44],

In aflatoxin producing *Aspergillus* spp., cluster genes in aflatoxin pathway contain three structural genes namely norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1),* sterigmatocystin O-methyltransferase (*omt-A*), and aflatoxin regulatory gene (*aflR*). These genes constitute complete aflatoxin machinery and are involved in the biosynthesis of AFB_1, AFB_2, AFG_1 and AFG_2, [45], as illustrated in (Fig. 1).

However, not many studies on the molecular characterization of mycotoxigenic fungi specifically, *Aspergillus* and *Fusarium* spp*,* in relation to their capability to produce aflatoxins and fumonisins, respectively have been undertaken in Kenya apart from those by Okoth et al*.* [25] and Probst et al*.* [46], and Njoroge, [47]. In these studies, only one gene (*AflR*) and two genes *AflD* (*nor-1*) and (*AflQ*) were used by Probst et al*.* [46], and Okoth et al*.* [25], respectively. Unlike bacterial toxins which are primarily peptides hence are encoded by a single gene, fungal toxins including aflatoxins are multi-ring structures requiring a sequence of genes for their biosynthesis. Therefore no single specific PCR tool exits for any one of the four aflatoxins [48]. The major advantage of the molecular methods involving a combination of a set of several gene primers controlling aflatoxin biosynthesis in a single PCR reaction is the early detection of the contaminating fungi prior to the actual toxin secretion [45,48]. In a multiplexing PCR assay, more than one target sequence can be amplified by using multiple primer pairs targeting multiple gene templates in a single PCR reaction mixture. As an extension to the practical use of PCR molecular tool, this technique has the potential to produce considerable savings on time and costs whereby all the reagents are put in one reaction tube therefore is ideal for conserving especially on the costly *Taq* polymerase [49,50].

In multiplex PCR assays where four different sets of species-specific aflatoxin-gene-primers, comprising the three structural genes (*nor-1, ver-1* and *omt-A)* and one regulatory (*aflR)* gene, the PCR products are a quadruplet banding pattern for aflatoxin producing fungal isolates [49,50]. Therefore, the multiplex PCR is a powerful diagnostic tool that is species-precise, rapid and cost-effective applicable in detecting aflatoxin production capabilities of fungal isolates contaminating in foods [51,52,53,54,55,56,57,58] and feeds [59]. It also been used in the molecular screening of pathogenic clinical isolates [60,61], besides environmental quality assessment for aflatoxin producing fungal isolates [62].

However, recent findings demonstrate the mere presence of the genes as established through cultural, physiological and molecular reflects only the potential of the fungus to produce aflatoxins because toxin secretion is highly variable depending on medium and microbial conditions including: composition, temperature, humidity, growth phase and age of the culture [48]. Therefore, instead of testing for only the potential toxigenicity of the fungal isolates, the aflatoxin levels in the natural food samples should be quantified as well. This method when combined with multiplex-PCR will simultaneously determine the safety levels of the foods and establish presence of genes controlling aflatoxin biosynthesis in the fungal isolates. This study therefore aimed to establish the prevalence of aflatoxin biosynthesis genes in *A. flavus* and *A. parasiticus* isolates in relation to aflatoxin levels in maize of different varieties by applying polyphasic approach involving multiplex PCR consisting of four aflatoxin biosynthesis genes *(nor-1, ver-1, omt-A* and *AflR)* in combination with ELISA-aflatoxin quantification in natural maize grain samples of isolates' origin.

2. MATERIALS AND METHODS

2.1 Sampling Sites and Sample Collection

The study was conducted in two regions historically associated with aflatoxin outbreaks in lower Eastern Province namely Kitui and Kibwezi counties. Comparative regions were Uasin-Gishu county and Perkerra irrigation scheme (Baringo County), both with no previous acute aflatoxicosis and practicing commercial

cultivation under rain-fed and irrigation farming systems, respectively. The three study regions were chosen purposefully on the basis of their history of aflatoxicoses, agro-ecological and farming system differences. Uasin-Gishu district is among the High altitude maize cultivation agro-ecological zone in Kenya with altitude ranges of 2100-2700 metres above sea (7000- 9000) feet. It has a cool and temperate climate with temperature range of 8.4°C to 27°C and annual rainfall ranging from 900 mm to 1,200 mm. The maize varieties cultivated are high yielding and the region is ranked second in maize output in Kenya. On the other hand, while Kitui/Kibwezi and Marigat region, the location of Perkerra irrigation scheme both have semi-arid climatic conditions, no aflatoxin outbreaks have been documented in both Uasin-Gishu and Baringo counties, unlike Kitui/Kibwezi.

Sampling of maize was done during the 2008-2010 growing seasons whereby a total of 158, 76 and 61 samples were obtained from Uasin-Gishu, Kitui/Kibwezi districts and Perkerra irrigation scheme, respectively. The preferred maize varieties were established in each zone. A representative minimum working sample of 2-Kg was obtained at each sampling stage. The samples were packed in paper bags and stored at 4ºC until further analysis which was carried out within 72 hours. Aflatoxin analysis was done at BORA Biotech Ltd laboratories, while fungal isolation and molecular screening of isolates was done at Centre for Biotechnology and Bioinformatics, University of Nairobi, Kenya.

2.2 Determination of Aflatoxin Contamination in Maize Samples

Aflatoxin levels in the maize samples were determined by direct competitive enzyme linked ELISA commercial kits, Boratest®, from BORA Biotech, Kenya. The method has a lower detection limit of ≤2.0 ppb [63]. Briefly, the method involved aflatoxin extraction from a portion of 5 g finely ground sub-sample using methanol/water solution. The extract was then defatted with hexane and centrifuged followed by supernatant recovery and dilution with buffered saline (PBS). The resulting solution was further diluted with methanol-PBS mixture before aflatoxin quantification on ELISA microtiter plates. The wells of ELISA plate were prior coated with anti-aflatoxin antibody, incubated overnight in a moist chamber then emptied. Any free protein binding sites were blocked using bovine serum albumin in PBS followed by plate washing with Tween 20 solution and semi-dried. Volumes of sample extract and equal volumes of AFB1 standards were added into separate wells. Solution of AFB1-enzyme conjugate was simultaneously added to all wells before 2 hour incubation in darkness followed by plate washing and allowing wells semi-dry. A solution of enzyme substrate was added to all wells so as to establish the extent of binding between anti-aflatoxin antibody and aflatoxin-enzyme conjugate whereby upon incubation color develops. The intensity of resulting color both in the sample extracts and standards was determined by reading absorbance at 450 nm using a spectrophotometer ELISA plate reader (UniskanII_Labsystems, Finland [64]. Aflatoxin levels were expressed in µg/kg, equivalent to ppb.

2.3 Isolation and Identification of Aflatoxin Producing Fungal Species

The isolation of *Aspergillus* from the maize samples was undertaken using modified Potato Dextrose Agar, PDA [65,66] enriched with Yeast Extract Sucrose agar (YES). Fungi isolation was by dilution plating technique recommended for isolation of fungi from powdered foods [67]. Triplicate sub-samples each of 10 g maize flour were diluted in 100 ml of sterile double distilled water and vortexed for 1 min. Dilutions of 1 ml suspension were inoculated on a set of triplicate agar plates using sterile pasteur pipette and glass spreader, respectively. Plates were then incubated at 30ºC for 3-7 days. The fungal isolates were identified as *A. flavus* and *A. parasiticus* according to obverse colony color, conidial head and conidia surface texture features [67]. Pure sub-cultures of isolates were preserved in PDA agar slants pending molecular characterization.

2.4 Molecular Characterization of Aflatoxin Producing Fungi

2.4.1 Extraction of genomic DNA

Extraction of fungal DNA was performed according to the method described by Criseo et al. [49,68] with some modifications on annealing temperature. Each isolate was first cultured in a 250 ml conical flask containing YES liquid broth enriched with PDA extract and glucose at 25°C for 72 h under continuous shaking (130 rpm). The mycelium was then aseptically harvested from the broth by filtration using what man filter paper No.1, and then transferred into 15 ml falcon tubes prior to lyophilization.

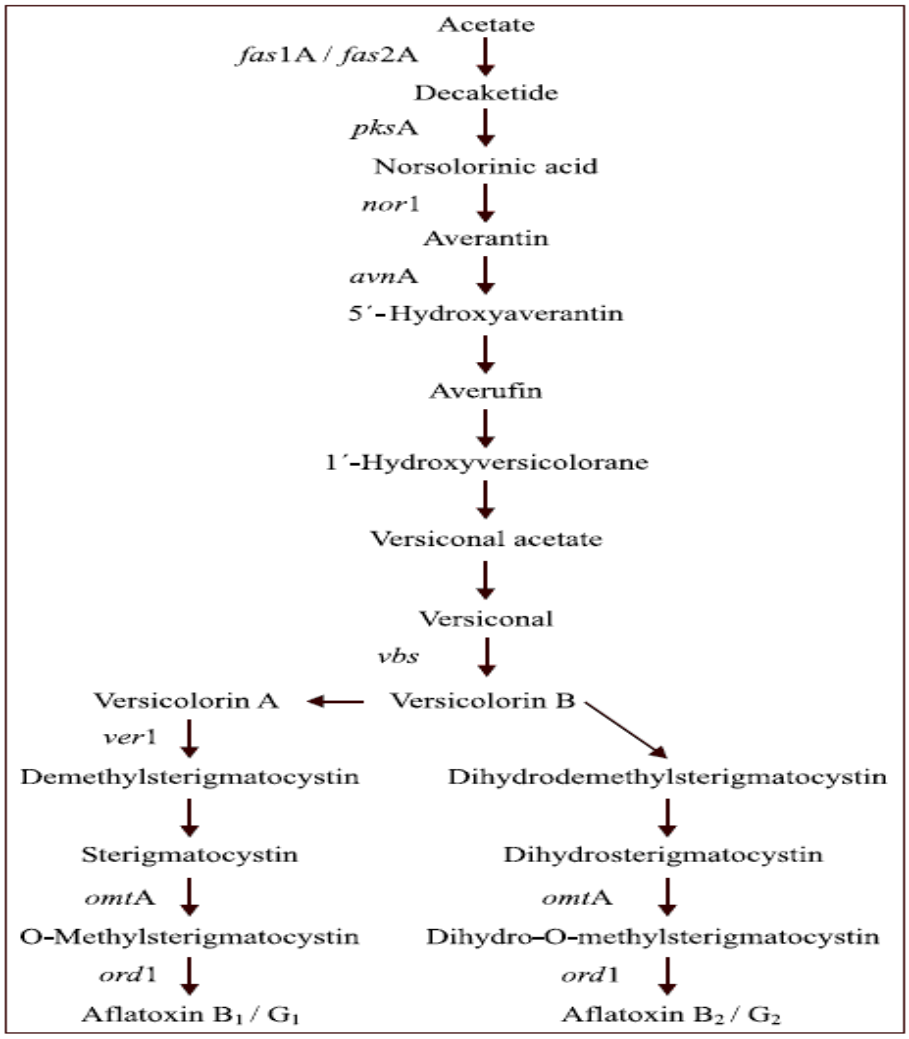


Fig. 1. Biosynthetic pathway of *Aflatoxins* [45]

To extract DNA, small aliquot (5 g) of lyophilized fungal powder was transferred into a 1.5 ml eppendorf tube and 1000µl Extraction buffer consisting of: 1% CTAB, 1.4 MNaCl, 0.1M EDTA, 0.1M TrisHCl at pH 8.0, and 1% Mercaptoethanol added. The mixture was heated in water bath incubation for 1hr, 65°C. The suspension was then centrifuged (15000 rpm, 15 min) with the resulting supernatant transferred to a new eppendorf tube and 400 µl of chloroform: isoamyl-alcohol (24:1) was added. The mixture was vortexed gently before centrifugation (15000 rpm, 15 min). The supernatant was transferred to a new eppendorf tube and 50 µl of 3 M ammonium acetate/sodium acetate added to cause DNA precipitation. To the precipitate, 500 µl of absolute ethanol was added and left overnight to incubate at -20°C. It was thereafter centrifuged (14000 rpm, 5 min) and the supernatant discarded leaving the DNA pellet. Two-fold washing of the pellet with 70% ethanol was then done before a final centrifugation (14000 rpm, 5 min) and resulting supernatant discarded. Finally, the pellet was allowed to air dry prior to resuspension in 50 µl TE buffer to dissolve the DNA which was thereafter stored at -20°C pending further PCR assay.

2.4.2 Multiplex PCR characterization of aflatoxin producing fungi

Multiplex PCR reaction was performed according to Criseo et al. [49,68] with some minor modifications on annealing and extension

Table 1. Details of primers, target genes, primer sequences and PCR product size (ppb)

Primer name	Target gene	Primer sequence (5´-3´)	PCR product size (bp)	Reference
Nor1	*nor-1*	ACCGCTACGCCGGCACTCTCGGCAC	400 bp	[49, 68]
Nor2		GTTGGCCGCCAGCTTCGACACTCCG		
Ver1	*ver-1*	GCCGCAGGCCGCGGAGAAAGTGGT	537 bp	[49, 68]
Ver2		GGGGATATACTCCCGCGACACAGCC		
Omt1	*omt-A*	GTGGACGGACCTAGTCCGACATCAC	797 bp	[49, 68]
Omt2		GTCGGCGCCACGCACTGGGTTGGGG		
AflR1	*aflR*	TATCTCCCCCCGGGCATCTCCCGG	1032 bp	[49, 68]
AflR2		CCGTCAGACAGCCACTGGACACGG		

temperatures. The PCR amplifications was performed on each sample in a total volume of 50µl reaction mix comprising of: 28.4 µl H_2O,5 µl Taq polymerase buffer, 1 µl $DNTP_S$ (10 Mm),1.2 µl of each of the four forward aflatoxin gene primers, 1.2 µl of each of the four reverse aflatoxin gene primers,5 µl DNA template and 1 µl Taq polymerase enzyme.

The Polymerase Chain Reaction (PCR) cycling parameters were programmed as follows; Initial denaturing temp of 95°C for 1minute, followed by 34 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 10 minutes. The total number of cycles was 35, with the final extension of 72°C for 10 minutes. The names of the specific primers used in this study, their respective target genes, their sequences and expected PCR product sizes (bp) are outlined in Table (1). PCR negative control was a PCR mixture consisting of all the components without fungal DNA template whereas PCR positives had fungal isolate DNA (*A. flavus)* that through repeated and independent PCR amplification was established to contain all the four genes under testing this quadruplex PCR. All experiments were repeated twice.

The amplified DNA fragments and DNA Step Ladder of 50-10,000 bp (Sigma-USA**)** were separated using 1.5% agarose gel in TAE buffer and visualized under UV light after staining with ethidium bromide for determination of the four aflatoxin biosynthesis genes alongside their molecular sizes. Gel images were documented in digital camera. All PCR reagents were supplied by F& S Scientific Ltd (Kenya) and *KOBIAN* (Kenya). The primers were designed from OligoSynthesis Unit, (International Livestock Research Institute, ILRI, Nairobi) and all PCR attempts were carried out in GeneAmpThermal Cycler (PCR System 9700-USA).

2.5 Data Analysis

Data analysis was by General Lineal Model (GLM) suitable for unbalanced data using *PASW statistics* 18.0 for Windows software (SPSS Inc.) according to Payne et al*.* [69]. Analysis of variance was performed on mean aflatoxin load at 5% (*α=0.05*) significance level. Pearson's Chi-square test was used to compare aflatoxin contamination and aflatoxin biosynthesis frequencies according to maize variety and agro-ecological zone. Aflatoxin contamination and aflatoxin biosynthesis genes constituted dependent variables while independent variables were maize variety and agro-ecological zone.

3. RESULTS

3.1 Prevalence of Aflatoxins in Maize Grain According to Maize Variety

This study was undertaken to establish whether results on aflatoxin content in maize samples from the three study zones varied according to maize variety. The results on the relationship between these two variables were profiled according to aflatoxin content and the maize varieties whereby three categories of aflatoxin levels were established. These included: ≤2.0 ppb, >2.0≤10.0 ppb and>10.0 ppb (Table 2). The maize varieties from Uasin-Gishu tested for aflatoxin contamination were seven namely: H614 (*n=43),* H629 (*n=31),* H6213 (*n=38)*, H6210 (*n=26),* H613 (*n=5),* H628 (*n=5)* and *Kipkaa (n=9).* Maize samples from both Kitui/Kibwezi and Perkerra regions each belonged to only one variety namely; *Kikamba (n=76)* and H513 *(n=61),* respectively (Table 2).

The incidence of aflatoxins as a dependent variable in relation to maize variety as an independent variable among maize from Uasin-Gishu was such that all varieties besides H613 had aflatoxins in the samples tested. Regarding

the safety of maize varieties on the basis of the statutory maximum total aflatoxin limit of ≤10.0 ppb, out of the seven varieties from Uasin-Gishu, only three varieties namely H628, H613 and the indigenous *Kipkaa* had aflatoxin within the threshold safety levels. In contrast, the proportion of samples that had unsafe aflatoxin levels (**>**10ppb) among H614, H629, H6213 and H6210 were 14.0%, 6.5%, 2.6% and 3.8% of the samples, respectively. Similarly, the proportion of samples with unsafe aflatoxin levels among *Kikamba* variety of Kitui/Kibwezi and H513 variety of Perkerra was not different having been 17.1% and 16.4%, respectively.

The relationship between the mean of aflatoxin levels among Uasin-Gishu maize samples and the maize variety showed no significant difference *(P=0.639).* The mean aflatoxin level for the various Uasin-Gishu maize varieties was 0 ppb, 1 ppb, 2 ppb or 4 ppb depending on specific variety. However, the difference between the aflatoxin level for the seven Uasin-Gishu maize varieties was significantly different from those of both *Kikamba* and H513 varieties whose average aflatoxin levels were 14.6 ppb and 15.6 ppb, respectively, *(P<0.001)*. The difference in the mean aflatoxin level between the *Kikamba* and H513 of Kitui/Kibwezi and Perkerra maize samples was however not significant *(P=0.932)*, notwithstanding (Table 2).

3.2 Multiplex PCR Molecular Characterization of *A. flavus* and *A. parasiticus* Isolates

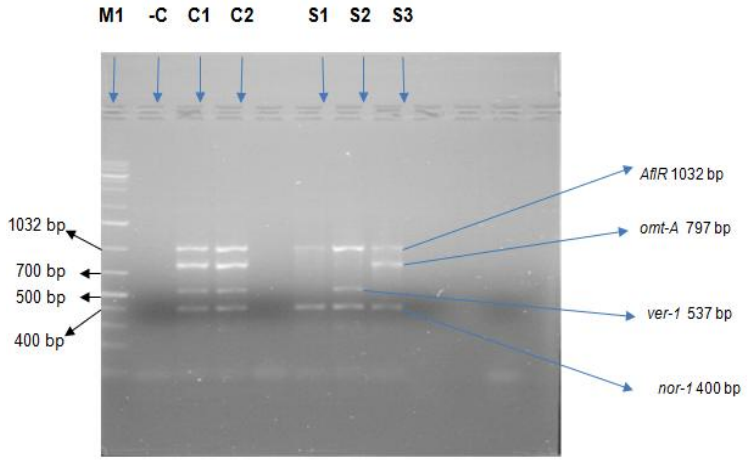
3.2.1 Variation of PCR banding patterns and specific aflatoxin genes in fungal isolates

The Quadruplex Multiplex PCR applied in this study successfully amplified the target genes under test in a total of 61 fungal isolates, *A. flavus (n=41)* and *A. parasiticus (n=20)* resulting in 54 PCR positive isolates (88.5%). These isolates were obtained from maize samples in the three agro-ecological zones of this study namely; Uasin-Gishu (*n*=15), Kitui/Kibwezi (*n*=27) and Perkerra Irrigation Scheme (*n*=19).The expected DNA products fragments showing banding profiles corresponding to genes *aflR, omt-A, ver-1* and *nor-1* genes were visualized in agarose gel at 1032 bp, 797 bp, 537 bp and 400 bp, respectively as estimated from the DNA size marker of 50-10,000 bp (Plate 1).

Table 2. Mean Total Aflatoxins (ppb) and Prevalence (%) in Three Profiles (≤2.00ppb, >2.0≤10.0ppb, >10.0ppb) According to Varieties of Maize Samples from Three Agro-ecological Study Zones: Uasin-Gishu County (H614, H629, H6213, H6210, H628, H613, *Kipkaa*-Indigenous) Kitui/Kibwezi Counties (*Kikamba*-Indigenous) and Perkerra Irrigation Scheme (H513)

Maize variety	Aflatoxin levels (ppb)						
	Mean ppb	≤2.0ppb		>2.0≤10.0ppb		>10.0ppb	
		AF[a]	RF[b]	AF[a]	RF[b]	AF[a]	RF[b]
H614 *n=43*	2.0±1	33	76.7%	4	9.3%	6	14.0 %
H629 *n=32*	2.0±1	26	83.9%	4	12.9 %	2	6.5 %
H6213 *n=38*	1.0±0	35	92.1%	2	5.3 %	1	2.6 %
H6210 *n=26*	2.0±1	21	80.8%	4	15.4%	1	3.8%
H628 *n=5*	4.0±1	0	0%	5	100%	0	0%
H613 *n=5*	0ppb	5	100 %	0	0%	0	0%
Kipkaa *n=9*	1.0±0	7	77.8%	2	22.2%	0	0 %
Kikamba *n=76*	14.6±1	20	26.3%	43	66.6%	13	17.1%
H513 *(n=61)*	15.6±1	25	41%	26	42.2%	10	16.4%

AF[a] Absolute frequency: RF[b] Relative frequency calculated from contaminated samples: n = Number of maize samples analyzed for aflatoxin contamination



*Lane: **M** = DNA Step Ladder Marker (50-10,000 bp)*
*Lane **1** = Negative Control PCR Mixture without fungal DNA*
*Lane **2 &3** =Positive Control:A.flavus isolate: Quadruplicate Banding Pattern (nor-1,ver-1,omt-A,AflR)*
*Lane **5** = A.flavus isolate showing Duplicate gene products (nor-1,AflR)*
*Lane **6** = A.flavus isolate showing Triplicate gene products (nor-1,ver-1,AflR)*
*Lane **7** = A.parasiticus isolate showing Triplicate gene products (nor-1,omt-A ,AflR)*

Plate 1. Multiplex PCR amplification products' profiles on agarose gel showing DNA banding patterns for various genes controlling Aflatoxin biosynthesis (*nor-1, ver-1, omt-A,* and *aflR*) in *A. flavus* and *A. parasiticus* Isolates

The prevalence of various gene amplicons patterns demonstrated that whereas only one isolate (1.6%) showed DNA fragments corresponding to the complete set of all the four genes under assay, the triplicate DNA banding pattern, indicating presence of three genes was the most prevalent amplicon pattern occurring in 31 out of the 61 isolates, with 50.8% representation (Fig. 2). Isolates that exhibited double and single banding patterns, indicating presence of two genes and one gene were 17 and 5, representing 27.9 % and 8.2%, respectively while PCR negative isolates without any of the four genes were seven (11.5%).

The frequency of specific aflatoxin genes revealed that *nor-1* was the most prevalent at 86.9 % representation among the 61 isolates while *omt-A* and *AflR* occurred equally in 52.5 % of the isolates, respectively. The gene *versicolorin A dehydrogenase*, *ver-1,* was the least prevalent, occurring in only 31.1 % of the isolates (Fig. 3).

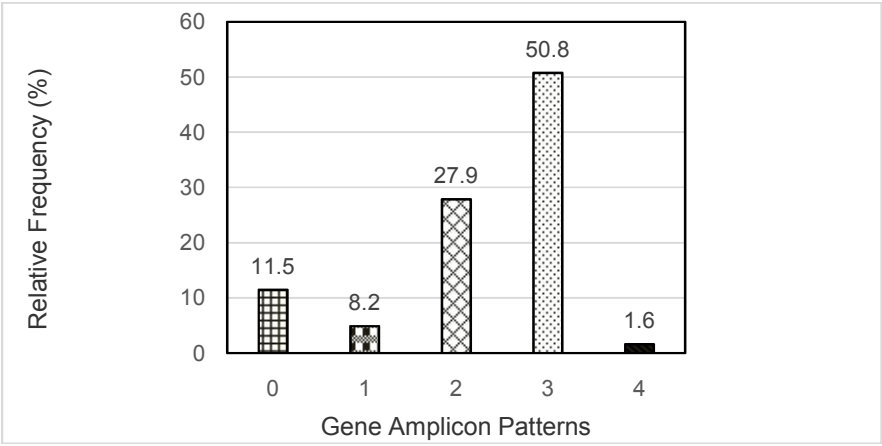


Fig. 2. Frequency of various gene amplicon patterns in aflatoxin producing fungal isolates out of the four genes (*nor-1, ver-1, omt-A,* and *aflR*) used in multiplex PCR assay

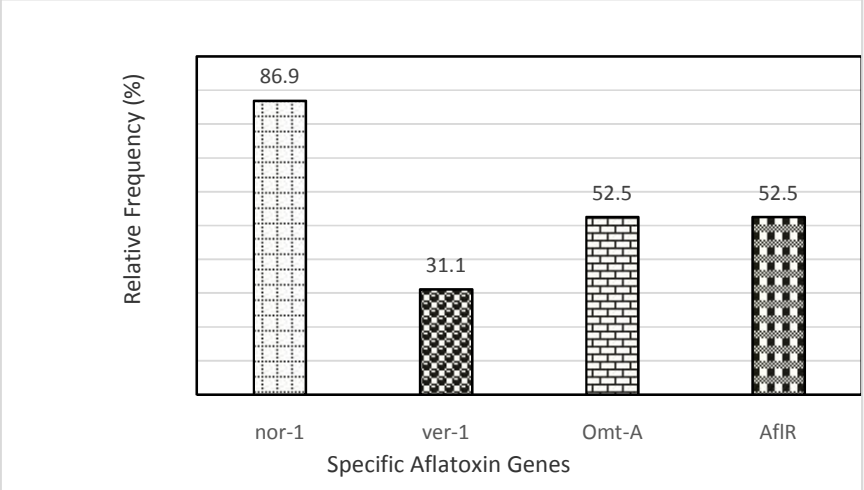


Fig. 3. Frequency of specific aflatoxin biosynthesis genes: *nor-1, ver-1, omt-A* and *aflR* under test in the multiplex PCR assay

3.2.2 Pattern of PCR aflatoxin genes amplification products according to agro-ecological origin of isolates and specific fungal species

The variation in the prevalence of the genes controlling aflatoxin biosynthesis among the isolates according to the agro-ecological zones revealed that isolates obtained from Kitui/Kibwezi maize samples had the highest prevalence of 96.3% of PCR positive isolates, whereby out of the 27 isolates tested, 26 indicated positive amplification. The prevalence of PCR positive isolates obtained from Perkerra and Uasin-Gishu maize samples were 84.2% and 80%, respectively (Table 3).

When PCR positive isolates were profiled according to various gene combinations, five (V) clusters were obtained, thereby demonstrating five strains of aflatoxin producing *A. flavus* and *A. parasiticus* spp. Group I had a four amplicon pattern comprising all the four targeted genes, namely: (*nor-1, ver-1, omt-A, AflR),* whereby only an isolate from Kitui/Kibwezi maize samples had this gene combination. Group II had a three amplicon pattern of various gene clusters: (*nor-1,ver-1,AflR*), (*nor-1,omt-A, AflR*), and (*nor-1,ver-1,omt-A*).

Group III of isolates were characterized by presence of a two amplicon pattern comprising (*nor-1, omt-A*) and (*nor 1, AflR*), each contained by 15 and 2 isolates, respectively. Group IV of isolates had only a single amplicon pattern including *(nor-1)* and *(omt-A)* which had representation of 4 and 1 isolate/s, respectively. Interestingly, the gene *(nor-1)* was not only the most prevalent single gene but it also occurred in all the eight PCR positive amplicon patterns obtained in this study. Group V cluster of isolates comprising 7 isolates tested PCR negative, indicating total absence of any of the four genes controlling aflatoxin biosynthesis (Table 3).

The amplicons' spectrum among the PCR positive isolates according to agro-ecological origin of maize source demonstrated that Kitui/Kibwezi isolates had a comparatively wider spectrum of a 6 amplicon pattern out the total 8 obtained in this study. Contrastingly, Uasin-Gishu and Perkerra isolates each had 3 and 2 amplicon patterns, respectively (Table 3).

The relationship between prevalence of these aflatoxin genes and agro-ecological source of isolates on one hand and with the specific aflatoxin producing fungal species, on the other hand demonstrated *A. flavus* isolates had a comparatively wider amplicon spectrum irrespective of the agro-ecological zone than *A. parasiticus*. However, a different phenomenon was observed among isolates from Perkerra whereby whereas *A. flavus* (*n=3*) had wider amplicon spectrum than *A. parasiticus*, *(n=1*), both fungal spp. each had equivalent prevalence of isolates *(n=8*) that tested positive for the PCR assay (Table 3).

Table 3. Regional prevalence of PCR positive isolates, aflatoxin amplicon patterns and PCR/ELISA results according to fungal spp. for Uasin-Gishu, Kitui/ Kibwezi counties and Perkerra irrigation scheme fungal isolates

Region	Number and % of PCR +VE Isolates	Amplicon combination								
		Group I *n=1*		Group II *n=31*		Group III *n=17*		Grp IV *n=5*		Grp V PCR -ve
		nor1, ve-1, omt-A, Afl R	*nor1,ver1 AflR*	*nor1,omt-A Afl R*	*nor1, ver1, omt- A*	*nor1, omt-A*	*nor1, AflR*	*nor1*	*omt-A*	
Uasin-Gishu *(n=15)*	*A.parasiticus* ***n=1***	-	-	-	-	-	-	1	-	2
	A.flavus ***n=11***	-	-	9	-	-	1	1	-	1
	Total ***n=12 (80%)***	**-**	**-**	**9**	**-**	**-**	**1**	**2**	**-**	**3**
Kitui/ Kibwezi*(n=27)*	*A.parasiticus* ***n=6***	-	3	1	-	2	-	-	-	1
	A.flavus ***n=20***	1	13	3	2	-	1	-	-	-
	Total ***n=26 (96.3%)***	**1**	**16**	**4**	**2**	**2**	**1**	**-**	**1**	**1**
Perkerra (*n=19)*	*A.parasiticus* ***n=8***	-	-	-	-	8	-	-	-	2
	A.flavus ***n=8***	-	-	-	-	5	-	2	1	1
	Total ***n=16 (84.2%)***	**-**	**-**	**-**	**-**	**13**	**-**	**2**	**1**	**3**
Total ***(n=61)***	**Grand Total** ***n=54(88.5)***	**1**	**16**	**13**	**2**	**15**	**2**	**4**	**1**	**7**

Table 4. Aflatoxin amplicon patterns and PCR/ELISA results according to *Fungal* spp.

***Fungal* spp. No. and % of PCR +VE**	**PCR positive amplicon profiles**								**PCR -Ve**	**Total PCR +ve & PCR -ve**	**PCR/ ELISA +VE Results**
	nor-1,ver1 AflR	***nor-1, omt-A***	***nor-1,omt-A AflR***	***nor-1***	***nor-1, ver-1, omt-A***	***nor-1, AflR***	***nor-1, ver-1, omt-A, AflR***	***omt-A***			
A. parasiticus ***n=15(75%)***	3	10	1	1	0	0	0	0	*n=5*	***n=20***	12(60.0%)
A. flavus ***n=39(95.1)***	13	5	12	3	2	2	1	1	*n=2*	***n=41***	24(58.5%)
Total ***n=54***	16	15	13	4	2	2	1	1	*n=7*	***n=61***	

3.2.3 Aflatoxin amplicon profiles and variation of PCR/ELISA results according to fungal species

Among all isolates that were positively amplified in this study (*n=54),* the distribution according to fungal spp. was such that while 15 out of 20 isolates of *A. parasiticus* (75%) were PCR positive, prevalence was 39 out of 41 (95.1%) for *A. flavus* isolates. Contrastingly, among all the PCR negative isolates (*n=7)*, majority (*n=5)* were from *A. parasiticus* (Table 4). Further, the agro-ecological pattern of these PCR negatives according to fungal spp. demonstrated that none of the *A. flavus* isolates from Kitui/Kibwezi were among these isolates. Instead, the only negative amplification result (1 out 27) was from an *A. parasiticus* isolate. This implies that all the 20 *A. flavus* isolates (100%) from Kitui/Kibwezi had at least one of the four aflatoxin genes under assay in this study in their DNA. Contrastingly, *A. flavus* isolates from both Uasin-Gishu and Perkerra maize samples each had at least one PCR negative amplification results (Table 3).

Among the 54 PCR positive isolates, the variation of amplicon pattern according to fungal spp. revealed that *A. parasiticus* had a comparatively narrower spectrum of 4 amplicon patterns including (*nor-1,ver-1,AflR), (nor-1,omt-A,AflR), (nor-1,omt-A),* and *(nor-1).* On the other hand, *A. flavus* had a wider spectrum of the 8 gene clusters among them, the 4 present in *A. parasiticus* besides 4 additional namely: *(nor-1, ver-1, omt-A), (nor-1,AflR), (nor-1, ver-1, omt-A, AflR)* and *(nor-1)* as illustrated in (Table 4).

The results of PCR amplification assay were complemented and validated by establishing the presence of aflatoxins in the maize grain samples from which the isolates were obtained. The prevalence of combinations of these two variables was expressed as either a positive or negative designate and analyzed according to fungal spp. The distribution of positive results for these two variables was such that 12 PCR positive *A. parasiticus* isolates out of 20 (60.0%) were obtained from maize samples that had natural aflatoxin contamination. Contrastingly, prevalence for *A. flavus* isolates was 24 PCR positive isolates out of 41 (58.5%) were from Aflatoxin positive maize samples (Table 4).

3.2.4 Correlation between PCR and aflatoxin ELISA results according to agro-ecological origin of fungal isolates

The results on the prevalence of various combinations of PCR amplification and ELISA results as dependent study variables were similarly expressed as either a positive or negative designate according to the agro-ecological origin of both the fungal isolates and maize samples' (Table 5).

Among the 27 fungal isolates from Kitui/Kibwezi region, the 22 isolates (81.5%) indicating PCR positive amplification results were similarly all obtained from aflatoxin positive maize samples as established by ELISA tests. Interestingly, the only isolate that tested PCR positive for all the four genes coding for aflatoxin biosynthesis including *nor-1, ver-1, omt-A* and *AflR* was coincidentally obtained from Kitui/Kibwezi maize samples and was aflatoxin positive as well (6.7 ppb). Among Perkerra samples *(n=19*), the prevalence for positive results for combination of both variables was 10 (52.6%). Contrastingly, results among Uasin-Gishu maize samples indicated the lowest prevalence whereby, out of a total of 15 isolates that were PCR tested, only four PCR positive isolates (26.7%) were obtained from aflatoxin positive maize samples (Table 5).

Table 5. Relationship between quadruplex PCR amplification results for aflatoxin biosynthesis genes and aflatoxin status of maize samples

Region	Isolates positive for PCR assay				Isolates negative for PCR assay				PCR +VE: at >10 ppb		Max toxin level (ppb)
	Aflatoxin Presence		Aflatoxin Absence		Aflatoxin Presence		Aflatoxin Absence				
	A.F[a]	R.F[b]	A.F[a]	R.F[b]	A.F[a]	R.F[b]	A.F[a]	R.F[b]	A.F[a]	R.F[b]	
U-Gishu (*n=15)*	4	26.7%	8	53.2%	1	6.7%	2	13.3%	1	6.7%	13.3
Perkerra*(n=19)*	10	52.6%	6	31.6%	2	10.5%	1	5.3%	7	36.8%	100
Kitui/Kibwzi (*n=27)*	22	81.5%	4	14.8%	1	3.7%	0	0%	10	37.0%	102
TOTAL *(n=61)*											

A.F[a]: Absolute Frequency R.F[b]: Relative Frequency

Conversely, the regional prevalence for negative results for these two study variables was relatively high for isolates and maize samples from Uasin-Gishu at 13.3% representation, whereas it was 0% for Kitui/Kibwezi (Table 5). This demonstrated that when a given maize sample and the corresponding fungal isolate from Kitui/Kibwezi agro-ecological region was considered, the results were positive for at least one of these two dependent variables.

The prevalence of aflatoxins in the maize samples of isolates' origin at levels of >10 ppb, the statutory safety limit set for total aflatoxins in foods in Kenya by KEBS (2009), in relation to the prevalence of PCR positive isolates, similarly demonstrated a regional variation. Among the isolates that were positive for PCR amplification and whose sources of maize samples were positive for aflatoxins tests, the only one sample from Uasin-Gishu (6.7%) had aflatoxin contamination of 13.3 ppb. Contrastingly, the difference in the frequency for Perkerra and Kitui/Kibwezi was not significant where each had 7 and 10 samples, representing 36.8% and 37.0%, respectively, (χ^2*=16.764, P=0.05*).

4. DISCUSSION

4.1 Incidence of Aflatoxins in Relation to Maize Variety

The mean aflatoxin load as established from this study was significantly lower among certified seed compared to indigenous maize. The use of improved maize varieties over conventional indigenous varieties in the control of aflatoxin contamination has widely been documented. The use of uncertified seed varieties has previously been established as a factor contributing to the prevalent fungal and aflatoxin contamination of maize and maize products in Eastern Province of Kenya compared to the North Rift regions [21]. Previous work showed that flint maize genotypes were more resistant to preharvest aflatoxin accumulation than dent genotypes [70].

The current study, however, established that H614 was the most contaminated variety among the 7 from Uasin-Gishu, its popularity notwithstanding. Findings by Mutinga [28] in Western Kenya also established that, despite its flint textured endosperm it was the most contaminated variety. Its vulnerability to insects' damage has been found to account for its high mycotoxin accumulation [71].

On the other hand, use of old stock of previous crops harvest as seeds had been found to contribute to prevalence of aflatoxins in the local *Kinyanya* maize variety of Eastern Province [29].

Elsewhere in Africa, in a study to establish farming practices contributing the low aflatoxin levels in maize, improved maize varieties commonly cultivated in Northern part of Benin were found to have comparatively lower aflatoxin levels relative to the local varieties of Southern region [72]. In another study on the effect of maize hybrids with varying ear size on mycotoxin contamination levels, the lowest levels of aflatoxin and fumonisin contamination in harvested grain were associated with the flexible ear trait compared to fixed ear trait [73]. These observations are in agreement with the results of the current study whereby Uasin-Gishu maize varieties including H613, H629 and H6213 whose ears droop at physiological maturity had comparatively lower aflatoxin contamination relative to the upright indigenous *Kinyanya* and *Kipkaa* varieties.

4.2 Multiplex PCR Method Molecular Characterization of Fungal Isolates

4.2.1 Variation of PCR banding patterns and specific aflatoxin genes in fungal isolates

The results on molecular characterization of aflatoxin producing fungal isolates through application of multiplex quadruplex PCR clearly demonstrated that the isolates obtained from maize samples in this study contained the four critical genes that control aflatoxin biosynthesis. The four critical genes include *nor-1, ver-1, omt-A* and *aflR,* whose molecular weights are 400 bp, 537 bp, 797 bp and 1032 bp [49,68]. The finding of an isolate with a quadruplex DNA banding pattern thereby indicating presence of all the four genes controlling aflatoxin synthesis, this amplification using the multiplex type of PCR is a great contribution as a diagnostic technique in supplementing the methods routinely used for molecular characterization of aflatoxin producing fungi besides application in microbiological food quality tests in Kenya, notwithstanding having been only one out of the 61 isolates tested. Similarly, the fact that these fungal isolates were obtained from maize grain food samples is of great relevance owing its strategic importance as a staple diet in the country [1,3].

In the recent few studies on molecular characterization of aflatoxigenic fungi contaminating maize in Kenya, the single gene *AflR* was used in the identification of distinct deadly strains *of A. flavus* [26]. Similarly, two genes including *aflD (=nor-1)* and *AflQ (=ord1=ordA*) were used to compare the distribution of *Aspergillus* and their toxicity in maize obtained from two agro-ecological zones in Kenya, namely Nandi and Makueni counties [25]. Unlike the previous molecular techniques used in Kenya, the multiplex PCR technique that amplifies structural genes such as (*nor-1, ver-1, omt-A*) and regulatory gene such as *aflR* of the aflatoxin biosynthesis pathway was successfully applied in the current study.

The finding that triplicate banding of varying amplicon patterns, particularly (*nor-1, ver-1, aflR*) was the most prevalent corroborates other similar studies on molecular detection of aflatoxin producing strains of *A. flavus* from Peanut [55], where the same amplicon was most the most prevalent. Similarly, in findings of a study aimed at establishing the frequencies of the aflatoxin structural genes in non-aflatoxigenic *A. flavus* strains isolated from food and feed commodities in Italy, PCR products with triplicate DNA banding had the greatest spectrum of amplicon patterns. However, in contrast to the current study, the amplicon pattern (*nor-1, ver-1, omt-A)* was the most frequent gene combination, occurring in 12.7% of the isolates while *(nor-1, omt-A, aflR)* and *(nor-1, ver-1, aflR)* occurred in 11.2% and 5.2% of the isolates, respectively [68].

The gene *nor-1 (aflD*) was the most prevalent in the current study. It is responsible for the conversion of norsolorinic acid (NOR) to averantin (AVN) in the middle of the aflatoxin biosynthetic pathway therefore molecular analysis of isolates usually involve this gene, because its expression indicates high correlation to aflatoxigenic ability of fungal isolates [51,74]. Prevalence of the gene *nor-1* in this study implies that most of the isolates of *A. flavus* and *A. parasiticus* strains were capable of producing aflatoxins. Prevalence of the *nor-1* gene was also established in a molecular study on aflatoxigenic fungi contaminating foods in Zimbabwe where it occurred in 88.2% of the fungal isolates [54]. Similarly, in a quality test of animal feeds in South Africa with respect to aflatoxin-producing fungi and establishing the correlation between prevalence of aflatoxin levels and an aflatoxin biosynthesis gene, only the gene *(nor-1)* was used for molecular assay by Iheanacho et al. [75]. However, in the study on aflatoxin structural genes in non-aflatoxigenic *A. flavus* strains in Italy by Criseo et al. [68] the gene *nor-1* was equally the most representative as is the case in the current study.

In contrasting findings of a multiplex PCR assay involving the genes (*aflR, nor-1, ver-1* and *omt-A*) in the detection of aflatoxigenic *A. flavus* isolates in Egyptian chili, *Omt-A* was the most prevalent gene occurring in 90% of the isolates. Interestingly, as established in the current study, *ver-1* had the lowest prevalence of 80% [57]. Further, the presence the two genes *omt-A* and *aflR* at equivalent prevalence in the current study indicates the aflatoxin production machinery. These two genes, *aflR* regulates the expression of *omt-A* gene within the aflatoxin biosynthetic pathway, while *omt-A* gene is critical for all the final formalities of aflatoxin biosynthesis [76]. The gene *aflR* was also the second most prevalent in multiplex molecular characterisation studies of isolates from spices in Tanzania [58].

4.2.2 PCR products according to agro-ecological origin of the fungal Isolates and prevalence of aflatoxin biosynthesis genes in fungal isolates in relation to aflatoxin levels in maize grain samples

The major finding regarding the agro-ecological variation of the PCR positive isolates in relation to aflatoxin test results strongly demonstrates that a good correlation between these dependent variables existed among Kitui/Kibwezi isolates and maize samples. Maize samples and fungal isolates from this region had the highest prevalence of positive results for PCR amplification and ELISA aflatoxin tests compared to the other two regions namely Perkerra irrigation scheme and Uasin-Gishu. These findings validate the comparatively wider gene spectrum of exhibited by PCR positive isolates from Kitui/Kibwezi region which had 6 different amplicon patterns whereas Uasin-Gishu and Perkerra isolates each had only 3 and 2 amplicons, respectively.

The relationship between increased expression of the determinate genes controlling aflatoxin biosynthesis, particularly both the structural *nor-1* and the regulatory *aflR* genes in *A. flavus* and aflatoxin production has recently been demonstrated to be enhanced by elevated temperature and water stress [41]. This could account for lower frequency of PCR positive

isolates from both the humid, cool Uasin-Gishu agro-ecological zone and the artificially modified humid agro-ecological conditions of Perkerra irrigation farming system in the current study. The observation that the only isolate that tested positive for all the four genes under assay in this study (*nor-1, ver-1, omt-A, AflR),* was from Kitui/Kibwezi which is considered a semi-arid region corroborates the relationship between these study variables and the ambient conditions.

In the findings of other similar comparative studies, factors including high levels of aflatoxigenic fungi in soils and the semi-arid climatic conditions of Kitui, Machakos and Makueni counties were found to correlate with the high levels of aflatoxins in maize in these regions compared to areas with humid and abundant rainfall including Uasin-Gishu, Nandi, Trans-Nzoia, Nyeri, Bungoma and Siaya counties [24,27]. The finding that ambient climatic conditions in Eastern Province and unsound farming practices may be favorable for proliferation of other types mycotoxigenic fungi besides aflatoxigenic fungi was confirmed by the incidence of *Fusarium* spp. and fumonisins in maize from this region [21,28,47].

Findings from the current study on the role of irrigation with respect to prevalence of aflatoxin biosynthetic genes in fungal isolates demonstrated that while *A. flavus* had a comparatively wider amplicon spectrum among isolates across the three agro-ecological zones than *A. parasiticus,* the difference in prevalence of PCR positive isolates for these two fungal species was not significant among isolates obtained from maize cultivated under irrigation farming system. In essence, irrigation reduces the soil temperatures and the water-stress thereby hindering proliferation of aflatoxin producing fungi and subsequent expression of aflatoxin genes, particularly in *A. flavus* [77].

The role of new climatic conditions in relation to fungal and aflatoxin contamination of food crops and particularly in agro-ecological zones previously considered to produce microbiologically safe maize is an emerging issue of serious public health concern not only in Kenya [25,26,28,46], but globally as well. Aflatoxin is now recognized an emerging food safety and security hazard particularly in Southern European countries including Italy, Croatia, Romania and Serbia countries where drought and warmer climatic conditions of autumn season have been associated with these outbreaks [78,79,80]. Recent findings in Kenya demonstrating that the difference in the prevalence of PCR positive isolates for the genes *nor-1* and *aflQ* genes was not significantly different agro-ecologically, having been 71% for Nandi county isolates and 62% for Makueni county isolates clearly attest to the role of global warming on changing geographical incidence of aflatoxin producing fungi in Kenya [25]. Similarly, the prevalence of these aflatoxin biosynthesis genes in *Aspergillus* spp. isolated from popular spices in Dar-es-Sallam city of neighboring Tanzania was attributed to the prevailing high temperatures and humidity exhibited almost throughout the year in this port city [58]. In this study, the four genes (*aflR, aflD, aflM and aflO* were also used under multiplex PCR whereby the major finding was that among the four strains containing all four genes, three were identified as *A. flavus* and one *A. parasiticus*

4.2.3 Genetic variability of aspergilli aflatoxin gene cluster according to multiplex PCR results

Despite aflatoxigenic potential tests of the fungal isolates having not been undertaken in this study, the isolate *(A. flavus)* that had a quadruplet gene amplicon pattern thereby indicating positive results for all the four genes under assay similarly had natural aflatoxin contamination in the maize sample of its origin as established by ELISA. In other findings of a multiplex PCR using the four aflatoxin biosynthesis genes *aflR, nor-1, ver-1* and *omt-A* , all the 21 aflatoxigenic isolates and 1 non-aflatoxigenic isolate of *A. flavus* showed DNA fragments that correspond to the complete set of the targeted genes [57]. The presence of four targeted genes confirmed the abilities of isolates to produce aflatoxins as previously mentioned by other researchers [49,51,53,57,81]. Findings by [49,59] proved that the aflatoxigenic *A. flavus* isolates always show the complete gene set comprising *aflR, nor-1, ver-1* and *omtA*, whereas non-aflatoxigenic isolates lacking one, two, three or four PCR products indicated that the genes do not exist in these strains or that the primer binding sites changed. Studies by [78] on presence of seven aflatoxin biosynthesis genes in relation to their capability to produce aflatoxin B1 illustrated that isolates containing all seven amplicons produced AFB_1, whereas those lacking three, four or all seven PCR products were non-producers.

Results from the current study indicated that triplicate amplicon banding had the highest representation (50.8%) among the 61 isolates tested. However, majority (88.9%) of the isolates with triplex amplicon pattern from the cool humid ample rain-fed maize agro-ecological zone had maize samples of origin testing ELISA negative for aflatoxin assay. Similarly, [43] reported different results by multiplex-PCR and enzyme-linked immunosorbent assay (ELISA) whereby despite *norA, ver1, omtA* and *aflR* genes not having been detected in all tested strains, some of these samples were negative for aflatoxin tests. These results are in concurrence with the results of [56] which demonstrated complete set of genes among isolates which did not produce aflatoxins, notwithstanding. It was suggested by [81] that the lack of aflatoxin production could also be due to simple mutations, including the substitution of some bases. It was also suggested by [82] that a variety of different physiological conditions affect aflatoxin biosynthesis.

It is noteworthy to mention that the production of aflatoxins by aflatoxinogenic fungi requires certain natural environmental conditions along the maize value food chain but which may be different under the artificial culture media used for toxigenicity potential tests of isolates [41,48]. Therefore, food samples which are devoid of aflatoxins but are contaminated with fungal strains containing aflatoxin biosynthesis genes might be vulnerable to aflatoxins contamination when environmental conditions during storage are suitable for aflatoxins production. The application of Multiplex of PCR in the current study in the molecular characterization of fungal isolates with four genes *nor-1, ver-1, omt-A* and *aflR* complements the conventional aflatoxin quality tests using ELISA. This circumvents the constrain whereby though maize samples are contaminated by undetectable amounts of aflatoxins thereby implying aflatoxin free, the contaminating fungal spp. could be aflatoxin producing strain, notwithstanding [66,83].

5. CONCLUSION

The findings from this research demonstrated that the prevalence of biosynthetic genes in fungal isolates exhibited a pattern according to the aflatoxin levels in maize grain, type of maize variety and agro-ecological origin. Samples from certified seed varieties of Uasin-Gishu county had significantly lower aflatoxin contamination levels compared to both the indigenous maize varieties from Kitui/Kibwezi and also the certified seeds from irrigation farming system, the commercial variety H513 from Perkerra, notwithstanding. Similarly, the prevalence of the four biosynthesis genes varied according to the aflatoxin levels in maize samples of isolates natural origin whereby the regional amplicon spectrum and prevalence of PCR positives were comparatively higher among isolates from the semi-arid climatic zones of Kitui/Kibwezi and Perkerra (Baringo) than to those from the humid, abundant rainfall Uasin-Gishu County.

The results of this study are of immense significance with regards to Kenyas' food safety considering that despite the widely documented data indicating *A. flavus* as responsible for aflatoxin contaminant in Kenya's food supply system, the co-prevalence of aflatoxin biosynthesis genes among both *A. parasiticus* and *A. flavus* isolates from Perkerra irrigation scheme clearly indicates the serious potential of the threat posed by aflatoxin food poisoning in Kenya. It is on the background that irrigation farming system, particularly Galana Kalalu scheme is poised to become the country's' alternate food security strategy. The success in the application of the multiplex PCR in this study is a significant contribution as diagnostic tool that can be used in assessment of aflatoxin production potential of isolates obtained from food, feed, environmental and clinical matrices.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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