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Molecular Identification of *Aspergillus* Strains and Quick Detection of Aflatoxin from Selected Common Spices in Tanzania

Gladness Elibariki Temu[1*]

[1]Department of Molecular Biology and Biotechnology, College of Natural and Applied Sciences, University of Dar es Salaam, P.O.Box 35179, Dar es Salaam, Tanzania.

Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Twenty three *Aspergillus* species isolated from nine commonly used spices in Tanzania were characterized to determine presence of potential aflatoxin producers. PCR of one regulatory (aflR) and three structural (aflD, aflM and aflO) aflatoxin biosynthetic pathway genes followed by nucleotide sequence analysis of 5.8S ITS rDNA region identified the potential aflatoxin producing strains. Four *Aspergillus* strains had all four genes which were missing in two strains while the other strains had 1 to 3 genes. Among the four strains having all four genes, three were identified as *A. flavus* and one *A. parasiticus*. Red chill was contaminated with three potential aflatoxin producer strains: *A. flavus*, *A. parasiticus* and *A. tamarii*. *A. flavus* was identified from red chill, black pepper and ginger. Using lateral flow immune-chromatographic assay, red chill tested positive with detectable ≥ 4 ppb of total aflatoxins. These results demonstrated that *A. flavus* is the most contaminant strain in the spices tested and thus may have risk implications based on their potential to produce aflatoxin. Further, both PCR of genes involved in aflatoxin production pathway and quick detection of total aflatoxin can be used to assess the quality of spices and predict its safety to consumers.

Keywords: Aflatoxin; Aspergillus; diversity; spices; Tanzania.

**Corresponding author: E-mail: gladness@amu.udsm.ac.tz, gladye2000@yahoo.com;*

1. INTRODUCTION

Aspergillus section *Flavi* is a group of fungi known to produce the most dangerous toxic secondary metabolites in agricultural products. Members of A. section *Flavi* consists mainly of *A. flavus, A. parasiticus, A .nomius and A. psedotamarii* [1,2]. Among the secondary metabolites produced by these fungi, aflatoxin is the most acute toxic metabolite which causes serious health problems to humans and animals. Four types of principal aflatoxin known to date are aflatoxin B1, B2, G1 and G2, which are named based on the innate fluorescent properties when illuminated under ultraviolet light. Among the four, aflatoxin B1 is the most toxic and frequently encountered in a variety of food crops and products such as, meat, corns, maize, nuts, grains and spices [3]. Aflatoxins are known to cause tremendous harmful effects to humans and animals including liver cancer, mutagenic and teratogenic effects to human beings [4,5].

Ability to produce aflatoxin is highly conserved in some species such as *A. parasiticus* but highly variable for other *Aspergillus* species. *A. parasiticus* is reported to have more than 95% aflatoxin producing strains while toxigenicity of *A. flavus* varies greatly depending on the strain, substrate and geographic origin [6,2]. Identification of *Aspergillus* species using conventional methods remains a big challenge to researchers [7]. For instance, *A. flavus* and *A. sojae* are considered nearly impossible to discriminate precisely since their DNA relatedness was found to be very high [8]. Therefore, even molecular discrimination of *Aspergillus* species is also complex with variable challenges depending on the method of choice. The most widely used molecular method is the rDNA region including the internal transcribed spacer regions and the D1-D2 domains [9,10]. Multiplex PCR, using several primer pairs targeting aflatoxin biosynthetic pathway, has also been used for species differentiation using both regulatory and structural genes such as *aflR*, *aflS, aflM, aflD and aflO* [11]. Real time PCR and digestion of nuclear DNA using SmaI enzyme was reported to distinguish species in the section *Flavi* [9]. Consequently, methods of detection of aflatoxins in foods and food products are complicated and require sophisticated machines which sometimes are unaffordable in most developing countries. Several methods are currently used to allow detection of these toxic metabolites from various food sources. One-step lateral flow immune-chromatographic assay with a cut-off level of total aflatoxin based on an inhibition immunoassay format is a quick and simple method of aflatoxin detection. Despite being qualitative, sample preparation (extraction) and result interpretation are rapid and simple, thus serving as useful assay of total aflatoxin from various food stuffs.

In Tanzania, spices are widely consumed in various foods and drink preparations, making them an ingredient that a person can hardly miss on a daily basis. However, spices, such as red chill, ginger, black pepper, turmeric and others can be contaminated with aflatoxins during harvesting, storage and transportation [12]. Due to high temperatures and humidity exhibited in Dar es Salaam city almost throughout the year, coupled with poor management in harvesting, transportation, processing and packaging, spices may become heavily contaminated with aflatoxigenic molds. Studies on molecular diversity of *Aspergillus* populations contaminating agricultural products in Tanzania are very rare. In this study, characterization and identification of *Aspergillus* strains isolated from selected spices were done based on sequence analysis of 5.8S ITS rDNA region and PCR of four genes known to be involved in the aflatoxin biosynthetic pathway. Furthermore, a simple and quick determination of total aflatoxin from selected spices detected with potential aflatoxin-producer strains was conducted to predict their safety.

2. MATERIALS AND METHODS

2.1 Sample Collection

Nine different types of spices were bought randomly from different local markets in Dar es Salaam city, Tanzania and were packed well in plastic bags or paper envelopes and brought to Microbiology Laboratory, University of Dar es Salaam. Each type of spice was bought as seeds, barks or powder and from different common markets. Total number of samples collected and analyzed was 50.

2.2 Culturing and Isolation of Molds

Before culturing, 10 g of each sample was weighed and dried at 100℃ in a hot oven for 24 hours and the moisture content was determined. Filamentous fungi (molds) from all samples were isolated by using agar plate method whereby 4 g of each sample was transferred into 20 ml of Maximum Recovery Diluent (HiMedia®) and 100

or 200 µl from the mixture was used to inoculate petri dishes containing 20 ml of malt extract agar (Difco ™) supplemented with 25 mg/l chloramphenicol to suppress bacterial growth. All plates were incubated at 30ºC for 3 to 7 days and fungal colonies emerged were examined visually and microscopically and counted as colony forming units (CFU)/g.

2.3 DNA Extraction

DNA was extracted from fungal mycelia harvested from cultures grown overnight in malt extract agar broth. The mycelia were transferred into an Eppendorf tube and 300 µl extraction buffer (1M Tris –base, 5M NaCl, 0.5 M EDTA and 10% cetyltrimethylammonium bromide (CTAB) was added. The samples were ground using a plastic pestle until homogeneous mixture was formed. The total volume was kept at 500 µl and then the mixture was incubated at 65℃ for 10 minutes followed by centrifugation at 14000 rpm in a micro-centrifuge for 5 minutes. Equal volume of chloroform was added to the supernatant to precipitate proteins and all debris, vortexed for 10 seconds followed by centrifugation at 14000 rpm for 5 minutes. To precipitate DNA, a 2 X volume of pre-chilled isopropanol was added to the supernatant, mixed gently and incubated at -20℃ overnight. DNA was collected as a pellet by centrifugation at 16400 rpm, for 10 minutes. The supernatant was then removed carefully and the DNA pellet was rinsed with 100 µl of ice-cold 75% ethanol. Centrifugation followed at 16400 rpm for 12 minutes and the supernatant was decanted. The rinsing step was repeated using 80% ethanol and the pellet was air dried and dissolved in 50 µl of sterile distilled water. To verify the DNA integrity, 2 µl of each DNA sample was run on 0.8% agarose (TopVision™, Fermentas LifeSciences) gel stained with ethidium bromide, using 5 x Tris Acetate EDTA (TAE) buffer at 200 V for 30 minutes and the gel image was viewed using ATTO gel documentation system (Japan). The quantity and quality of the DNA was further determined using a Nano- spectrophotometer (XNanoDrop® ND1000, Thermo Scientific) and then normalized to 50 ng/ µl for further studies.

2.4 PCR of Aflatoxin Biosynthetic Pathway Cluster Genes

PCR targeting one regulatory (*aflR*) and three structural (*aflD, aflM* and *aflO*) genes involved in aflatoxin biosynthetic pathway was done in a GeneAmp® PCR System 9700 at different temperature regimes depending on the annealing temperature of each primer. The primers and primer sequences used were aflD (Nor-1) forward 5 –ACC GCT ACG CCG GCA CTC TCG GCAC-3 and reverse 5′-GTT GGC CGC CAG CTT CGA CAC TCC G-3; aflM (Ver-1) forward 5′-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3 and reverse 5 ′-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3′; aflO (Omt-1) forward 5′-GGC CCG GTT CCT TGG CTC CTA AGC-3 and reverse 5′-CGC CCC AGT GAG ACC CTT CCT CG-3′; and aflR forward 5′-TAT CTC CCC CCG GGC ATC TCC CGG-3′ and reverse 5′-CCG TCA GAC AGC CAC TGG ACA CGG-3′. The expected PCR products were 400, 895, 1232 and 1032 bp respectively.

Each PCR reaction mix contained 50 ng/µl of each gDNA sample, 1 X buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA pH 8.0), 0.25 mM dNTPs, 2.5 mM MgCl2; 0.1 µM of each of forward and reverse primers and 0.5 U Taq polymerase. PCR conditions were: Initial denaturation at 94℃ for 10 minutes followed by 35 cycles of denaturation at 94℃ for 1 minute, annealing at 55℃ or 60℃ (depending on the primer) for 1 minute, extension at 72℃ for 2 minutes and final extension at 72℃ for 5 minutes. PCR products were visualized on 1% agarose gel electrophoresis stained with ethidium bromide, submerged in 1 X TAE buffer and ran for 45 minutes at 200 V. Bench top UV trans-illuminator was used to view the gel and BioDoc-It ™ Imaging System documented the gel images.

2.5 Identification of Potential Aflatoxin Producers using 5.8S ITS rDNA Sequences

Total genomic DNA of selected *Aspergillus* strains showing all four or three aflatoxin biosynthetic genes were sent to Inqaba Biotec ™, South Africa. DNA nucleotide sequencing of 5.8S –ITS rRNA gene was done by using chain terminator method using ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) primers. Nucleotide sequence cleaning and analysis was done using CLC workbench 7.6.4 (QIAGEN©) software. Nucleotide basic alignment search tool (nBLAST) was used to compare the identity of *Aspergillus* strains in relation to those available in the National Center for Biotechnological Information (NCBI). Similarity sequences ≥ 99% of sample strains to accession strains were accepted as the maximum identity of the *Aspergillus* species isolated from the spices.

2.6 Lateral Flow Detection of Total Aflatoxin

A one-step lateral flow immunochromatographic assay (AgraStrip®, Romer Labs Singapore) with a cut off level of 4 ppb total aflatoxin was used to quickly detect presence of total aflatoxins (B1, B2, G1 and G2) in red chill, ginger and black pepper. Samples were extracted using 50% ethanol and assay performed following manufacturer's instruction and results recorded accordingly.

3. RESULTS

Twenty three *Aspergillus* species were isolated from all spices as identified using morphological and microscopic features. Description of spice samples used in this study and their moisture content and the mean total number of fungal colonies isolated from each are recorded in Table 1.

3.1 PCR Analysis of Aflatoxin Pathway Genes

Using four genes necessary for aflatoxin biosynthetic pathway, the *Aspergillus* species were clustered into five groups. Group I consisted of 4 strains with all four genes targeted in this study; group II consisted of 5 strains with three genes, group III had 5 strains with two genes; and group IV and V consisted of 7 and 2 strains having one and zero genes respectively (Table 2). Gel electrophoresis of selected potential aflatoxin-producing strains with all 4 genes is as shown in Fig. 1.

3.2 Nucleotide Sequence Analysis of 5.8S ITS rDNA

Nucleotide sequence analyses of the 5.8S ITS rDNA region of six selected species revealed the identity of each *Aspergillus* species where by four samples were 99% similar to *A. flavus*; one was 99% similar to *A. parasiticus* and one was 98% similar to *A. tamarii*. The control sample was found 99% similar to *A. flavus* isolate GGV-BT03 sourced from red chill (Table 3).

3.3 Total Aflatoxin Detection Using AgraStrip®

Only red chill was positive indicating total aflatoxin greater than or equal to 4ppb. The red chill tested positive by showing no visual line in the test zone of the strip while ginger and black pepper were negative showing total aflatoxin less than the cut off level as shown in Fig. 2.

Table 1. Description of spices used, moisture content and fungal colony count

S.N	Source / spice	Scientific name	Part used	Moisture content (%)	Mean cfu/g (10^2)
1	Turmeric	*Curcuma longa*	Powder	9.6	8.0
2	Caraway	*Carum carvi*	Powder	10.1	1.0
3	Red chill	*Capsicum annuum*	Powder	7.8	7.0
4	Ginger	*Zingiber offficinale*	Powder	11.0	7.3
5	Fenugreek	*Trigonella foenum-graecum*	Seeds/powder	10.9	2.3
6	Black pepper	*Piper nigrum*	Powder	8.0	12
7	Cinnamon	*Cinnamomum zeylanicum*	Barks	14.4	1.2
8	Coriander	*Coriandrum sativum*	Seeds	8.1	5.1
9	Cumin	*Cuminum cyminum*	Seeds	9.0	3.0

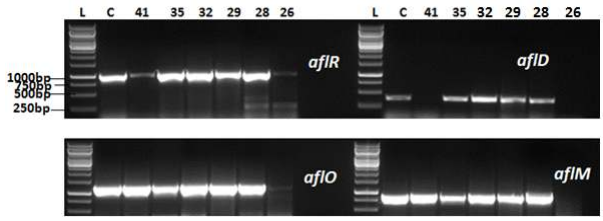

Fig. 1. Gel electrophoresis of selected *Aspergillus* strains. L: 1Kb ladder; C: +Ve control (35-28 positive samples with 4 genes)

Table 2. Characterization of *Aspergillus* sp using aflatoxin biosynthetic pathway cluster genes

Sample ID	Source/ spice	AflR	AflO	AflD	AflM	Total	Group
9B-4 (29)	Red chill	+	+	+	+	4	
9B-3 (28)	Red chill	+	+	+	+	4	**I**
12B-1 (32)	Ginger	+	+	+	+	4	
12B-4 (35)	Ginger	+	+	+	+	4	
9B-6	Red chill	+	+	-	+	3	
12B-2	Ginger	+	-	+	+	3	
12B-3	Ginger	+	-	+	+	3	**II**
6B-3	Turmeric	-	+	+	+	3	
Bp-3 (41)	Black pepper	+	+	-	+	3	
8B-6	Caraway	-	-	+	+	2	
13B-4	Fenugreek	-	-	+	+	2	
9B-1 (26)	Red chill	+	+	-	-	2	**III**
Ci-2	Cinnamon	-	+	+	-	2	
9B-2	Red chill	+	-	-	+	2	
Co-1	Coriander	-	-	+	-	1	
Co-3	Coriander	-	-	+	-	1	
Co-4	Coriander	-	-	+	-	1	
Cu-1	Cumin	-	-	+	-	1	
13B-1	Fenugreek	+	-	-	-	1	**IV**
13B-2	Fenugreek	+	-	-	-	1	
6B-1	Turmeric	-	+	-	-	1	
Bp-1	Black pepper	-	-	-	-	0	**V**
Ci-1	Cinnamon	-	-	-	-	0	

**+/- is presence/absence of the PCR product*

Table 3. Identification of selected *Aspergillus* species using nucleotide sequence of 5.8S ITS rDNA

Sample ID	Source	Similarity (%)	Accession	Name	Source
PB-3 (41)	Black pepper	99	KF221065	*A. flavus*	Spices
12B-4 (35)	Ginger	99	KF221065	*A. flavus*	Spices
12B-1 (32)	Ginger	99	KJ175417	*A. flavus*	Unknown
9B-4 (29)	Red chill	99	KJ175420	*A. flavus*	Unknown
9B-3 (28)	Red chill	99	AY373859	*A. parasiticus*	Unknown
9B-1 (26)	Red chill	98	KJ175440	*A. tamarii*	Unknown
Control (C)	IITA	99	KC907367	*A. flavus*	Red chill

Fig. 2. Qualitative detection of total aflatoxin using AgraStrip®

4. DISCUSSION

Twenty three *Aspergillus* species isolated from 50 samples of 9 commonly used spices were characterized in this study. Complete drying of spices may lead to a relative long shelf life but does not necessarily reduce the microbial contamination load. Cinnamon had the highest

moisture content (14.4%) followed by ginger (11%) while red chill had the lowest moisture content. Despite high moisture content observed in cinnamon the cfu/g recorded was lower ($1.2x10^2$) than the rest of the samples. The most contaminated spice was black pepper (12×10^2) followed by turmeric (8×10^2) while caraway was the least contaminated (10^2). However, other factors such as composition of substrate and presence of antimicrobial properties can affect mold infestation [12].

This study also showed that dried spices sold in various markets of Dar es Salaam are contaminated with *Aspergillus* strains that can be potentially aflatoxin producers. Based on the 4 genes involved in aflatoxin biosynthetic pathway, five groups of *Aspergillus* strains were identified in which 4 strains (17.4%) had all four genes while these genes were absent in only 2 (8.7%) strains. Genetic variation in aflatoxin production can arise from mutations in the aflatoxin biosynthetic gene cluster which may include gene loss, DNA inversion, recombination, partial deletions or other genomic rearrangements of the cluster [13]. However, a species may have all genes involved in the pathway but not necessarily produce aflatoxin till certain favorable conditions trigger its production. However, a species missing aflatoxin biosynthetic genes cannot produce aflatoxin. Presence of biosynthetic genes has been used as a diagnostic tool for aflatoxigenic fungi in certain food stuffs [9,11]. In this study 4 strains had all four genes which might indicate their potentiality in producing aflatoxins. Nucleotide sequence analysis of the 5.8S ITS rDNA identified four strains of *A. flavus*, one strain of *A. parasiticus* and one *A. tamari*i from red chill, ginger and pepper (Table 3). These *Aspergillus* species are well known for their potential to produce aflatoxins under favourable conditions. Based on the identification of these species, the samples (red chill, ginger and pepper) were analysed directly for presence/absence of detectable total aflatoxin. A simple and quick method of aflatoxin detection that was used in this study was able to determine total aflatoxin in red chill amounting to or greater than 4 ppb. The aflatoxin standards for cereals, dried fruits, and nuts intended for direct human consumption are more stringent in European Commission regulation [14] with limit of 4 ppb for total aflatoxins. According to EC, red chill was therefore found with maximum level of total aflatoxin. However for Tanzania, the limit of aflatoxins in most foods is 5 ppb -15 ppb depending on the type of food [15]. Red chill has been reported to contain relative higher levels of aflatoxins compared with other spices [16,17,12].

Due to limiting levels of aflatoxins in agricultural crops, there is a need for routine detection of aflatoxin levels in our food products because aflatoxins have significant economic implications for the agricultural industry worldwide. Since equipments for detection of aflatoxin in foods and food products are expensive and mostly unavailable in developing countries, lateral flow assay can be used to quickly assess spices and other food stuffs while further quantification methods can be employed when available.

Sometimes, presence of fungi in a substrate does not necessary relate to the presence or amount of aflatoxin. However, in this study red chill was contaminated with three potential aflatoxin producing strains namely *A. flavus, A. parasiticus* and *A. tamarii* (Table 3) and it tested positive for total aflatoxin. Both high incidence of *A. flavus* and high total aflatoxin in red chill was also observed by [5]. Presence of *A. parasiticus*, a strain that is known to have less than 5% non-aflatoxin producing might account for the total aflatoxin detected in red chill but not in ginger and black pepper. However, low concentration of aflatoxins in various spices may be attributed to the inhibitory effects of essential oils and other metabolites of the spice [12] and also sensitivity of the method used.

In Tanzania studies of mycotoxins and particularly aflatoxin are very limited in food crops and food products. Children who were consuming maize-based complementary foods in Northern Tanzania were at a risk of exposure to multiple mycotoxins [18]. Accordingly, [19] reported that 45% of maize samples collected from three agro-ecological zones in Tanzania were co-contaminated with aflatoxins and fumonisins thus posing health risks to consumers. Therefore, there is a need for more research on detection of *Aspergillus* populations, fungal contaminations and aflatoxin detections in other foods and agricultural products. This will help reducing risk of exposure of these deadly poisonous substances to both humans and animals.

5. CONCLUSION

Spices are prone to contamination by members of *Aspergillu*s section *Flavi* which are potential producers of aflatoxins. Without enforcement and monitoring of microbiological and aflatoxin

standards, spices as other crops may become over contaminated and hence compromise health status of consumers. Simple methods for detection of aflatoxins can be employed to enlighten on the quality and safety status of spices Tanzania.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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