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Isolation, Extraction and Identification of Aflatoxin Producing *Aspergillus* Fungi by HPLC Analysis and ITS Sequencing

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Abstract

Aflatoxins are naturally occurring mycotoxins that contaminate food and agro commodities, leading to acute and chronic health conditions in human and animals. Due to high toxicity, stability and diverse structures, aflatoxins in food chain is an increasing global food safety concern. This study sought to isolate and identify aflatoxigenic fungi, as well as detect the presence of Aflatoxin in food and feed samples. Grains and kernels from various agricultural produces were collected from regions in and around Mysore. Fungal cultures obtained from the samples were subjected to morphological, microscopical examinations; followed by chemical and chromatographic studies. The HPLC analysis indicated 129/220 fungal isolates produced aflatoxins and showed a population distribution of 43% of *A. flavus*, 29% of *A. parasiticus*, 7% of *A. fumigatus*, 7% of *A. oryzae*, 5% of *A. ochraceus*, 4% of *A. versicolor* and 3% of *A. tamarii* and 2% of *A. niger*.

Keywords: Aflatoxins; ITS sequencing; HPLC; TLC; *Aspergillus*

Introduction

Globalization of the food industry has led to the vast spread of food commodities and also illnesses related it, which are mostly caused by fungi with *Aspergillus flavus* being an important postharvest fungus in grain stores. Along with their secondary metabolites termed as "Mycotoxins", fungal infestation has raised concern on public health due to their toxic properties [1]. Among them, Aflatoxin (AF) contamination in food has gained importance worldwide due to their hazardous impact on both human and animal populations as carcinogens, mutagens and teratogens [2]. Aflatoxins are a group of difuranocoumarin derivatives produced through the polyketide pathway by filamentous fungi of the genus *Aspergillus*. Mycotoxins produced by members of *Aspergillus* are commonly known as "Aflatoxins". The main producers include species of *A. flavus*, *A. fumigates*, *A. parasiticus* and *A. nomius* that are predominant in tropical and subtropical regions [3]. There are about 20 different types of AFs identified as byproducts of *Aspergillus* contamination in food commodities such as cereals, nuts, fruits, oilseeds, and dried fruits both in field and storage [4]. The most predominant AFs in food, agriculture, and dairy products that are designated as Aflatoxin B1, B2, G1, G2, M1, and M2 [5]. Among these, Aflatoxins B1 (AFB1) is classified as a group 1A carcinogen by International Agency for Research on Cancer (IARC) due to its substantial liver damage and hepatocellular carcinoma apart from plentiful of deleterious health conditions [6].

Activities during crop cultivation (preharvest, harvest and postharvest), and other factors such as temperature, humidity and pest damage affect the level of infection of grains by mycotoxigenic fungi. Aflatoxin contaminated food and feed incur huge losses to farming communities as the harvest is made unwholesome for consumption. Aflatoxin contamination of 88% with concentration of 5 µg/kg and 14 g/kg (AFB1), 58 g/kg (AFG1) was reported in stored maize in Benin respectively [7]. Agbetiameh et al. [8], reported that 35% of 509 (326 maize and 183 groundnut) samples had detectable aflatoxin [8]. A study conducted in Doha, Qatar revealed aflatoxin contamination in spices and nut samples as 371.6 ng/g and 534.15 ng/g, respectively [9]. Al-Jaal et al. [9] highlighted the limitations on studies of aflatoxin contamination of food and feed globally and in the Gulf Cooperation Council countries (GCC). In recent years, numerous studies have revealed high levels of aflatoxins and fungal contamination in rice in many countries [10]. However, mycotoxin-producing fungi are less commonly reported for rice than for many other cereal crops [11].

In order to protect the consumers from the aflatoxin effects, many countries have set stringent

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regulations in monitoring and surveillance of aflatoxin levels in for human food as well as animal feed. The Food Safety and Standards Authority of India (FSSAI) limit AF concentration to 15 ppb to 30 ppb (parts per billion). According to the U.S. Food and Drug Administration (FDA) (2002), the concentration of AFs in the range of 4 ppb to 30 ppb is allowed in human food, depending on the importing countries. In the United States, the maximum limit of aflatoxin in the human food – except for milk is 20 ppb, while in the European Union it is 2 ppb to 4 ppb [12].

According to Sowley [1], the prevalence of mycotoxins, especially aflatoxins and its associated health problems is a silent threat in developing countries where most of the people are oblivious of the dangers associated with the consumption of contaminated produce. Therefore, monitoring and management of mycotoxins in food and feed is an important prerequisite in food industry. The objective of the study was to isolate and identify aflatoxigenic fungi and detect aflatoxin in various food samples from farms, stores and markets in and around Mysore, Karnataka, India.

Methods

Chemicals and reagents

Mycotoxins (Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), Ochratoxin A (OTA), Fumonisin B1 (FB1), Patulin, Citrinin, Zearalonone (ZEA), Deoxynivalenol (DON) and T-2 toxin), Potassium Chloride (KCl), Sodium Chloride (NaCl), hemin, TMB (3,3′,5,5′–Tetramethylbenzidine) substrate reagent set, Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), Coconut Cream Agar (CCA), chloroform, acetone, methanol acetonitrile and glacial acetic acid required for present study were procured from Sigma-Aldrich (St. Louis, USA) unless mentioned otherwise. All the solutions were prepared with ultra-high purity water. All other solvents and reagents used in the experiments were of analytical grade. Ultrapure water (Mill-Q, Millipore, India) was used throughout this study. Standards of mycotoxins were prepared according to manufactures protocol and diluted from stock solutions to working concentration (1 mg/mL) in absolute methanol (HPLC grade).

Collection of samples suspected for fungal contamination

As it is known, food and feed are considered as the major reservoirs of aflatoxin producers; grains and kernels from various agricultural produces namely, wheat, paddy, maize, groundnut, sorghum, and spices *viz.*, chili, and pepper were collected from regions in and around Mysore, India. All the samples were weighed to a sample size of 250 gm, respectively and stored at 4°C for the isolation, identification and molecular detection of *Aspergillus* species and further analysis of the extracted aflatoxins. The schematic representation adapted in the present work for isolation of aflatoxigenic fungi is depicted in the Figure 1.

Physical examination

Isolation of aflatoxin producers from the fungal isolates: The isolation of fungal strains from the collected samples was performed according to Abbas et al. [13]. Initially, the grains and kernels were surface sterilized by rinsing 3 to 4 times with distilled water followed by thorough washing in 1% Sodium hypochlorite + 0.1% Tween 20 for 10 min to 15 min by rapid shaking. This was followed by final washing of 3 to 5 times in sterile distilled water for 5 min each. Disinfected samples were allowed to air dry on blotting paper. Grains and kernels of 4 to 6 numbers were added to 1% Potato Dextrose Broth (PDB) and incubated at 25°C for 48 h to 72 h. After incubation,

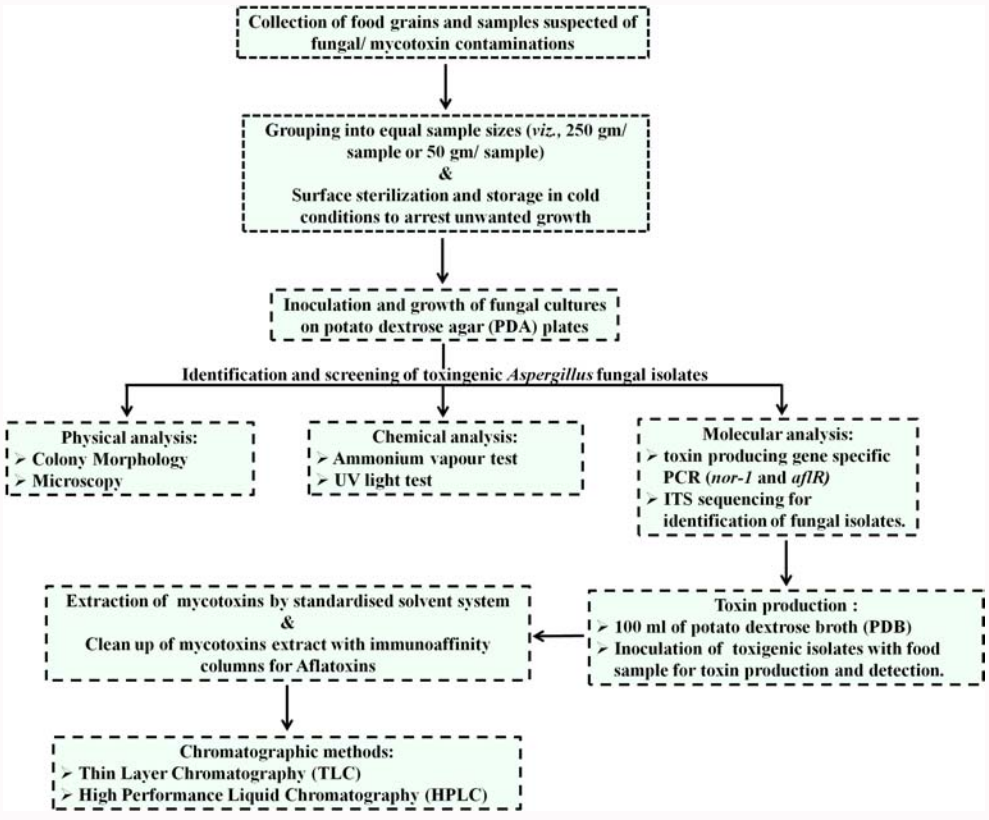


Figure 1: Schematic representation of isolation and identification of aflatoxin-producing *Aspergillus* species.

Table 1: List of standard fungal cultures utilized in the present work.

S. No	Name of the fungal strain	Source
1	*Aspergillus flavus*	NCIM 152
2	*Aspergillus parasiticus*	MTCC 2797
3	DFR11	DFRL, Mysore, India
4	DFR12	
5	DFR13	

the grains suspected of fungal growth were transferred onto petri-dishes containing 1% Potato Dextrose Agar (PDA) and incubated at 25°C for 2 to 4 days. The resultant fungal colonies on PDA petri plates were further observed and purified. Obtained fungal isolates were subjected to screening and identification by morphology, microscopy, molecular and chromatographic methods as discussed in the following sections.

The single spore cultures of *Aspergillus* isolates were subcultured on PDA slants, overlayed with 5% glycerol and maintained in 4°C. The reference strains and *Aspergillus* isolates available from the in-house repository were also utilized as positive controls in the present study and are listed in the Table 1.

Microscopical analysis of *Aspergillus* species: Obtained fungal colonies were examined for their phenotypic morphologies as per Diba et al. [14]. The fungal isolates with characteristic morphologies similar to *Aspergillus* species were selected and further studied by microscopy using lactophenol cotton blue stain. The slides were then viewed under the light microscope (NIS-Elements imaging software, Nikon, India) and examined for characteristic features of *Aspergillus* species.

Chemical analysis

Ammonium vapor test: Ammonium vapor test was performed on 3-day old fungal cultures as per Fani et al. [15]. The cultures grown on Coconut Cream Agar medium (CCA) according to Degola et al. [16], containing fungal colonies were placed upside down, and a drop (approximately 200 µl) of 25% ammonia solution (v/v) was added onto the lid of each fungal petri-dish and color development was carefully observed. Pink color pigmentation upon contact with ammonium vapors served as an indication for the aflatoxin production and these isolates were selected for further analysis.

Ultraviolet (UV) light test: For Ultraviolet (UV) light test, the fungal colonies were grown on CCA plates at 28°C for 5 to 7 days and tested for characteristic blue fluorescence on the reverse side of each petri-dish under long-wavelength (365 nm) UV light.

Molecular analysis

Fungal DNA extraction: Genomic DNA extraction from fungal samples was carried out using phenol: Chloroform: Isoamyl alcohol method. A loopful of mycelium or spore suspension from purified fungal isolates was inoculated into 50 ml of sterile Potato Dextrose Broth (PDB) (Himedia, Mumbai, India). The inoculated PDB flasks were incubated at room temperature for 48 h to 72 h with mild agitation of 100 rpm. The mycelium growth was harvested from the inoculated flasks by filtration through Whatman filter paper No. 3 and snap-frozen with liquid nitrogen. The frozen mycelium was pulverized and ground to powdered form using a sterile mortar, pestle and stored at −20°C until further use. A small amount of mycelial powder was collected into a sterile 1.5 ml eppendorf tube and crushed with micropestle and 500 µl of lysis buffer (pH 8) (100 mM Tris-HCl, 50 mM EDTA, 150 mM NaCl and 10% SDS) was added and kept in a water bath for 5 min at 60°C. After boil lysis, the suspension was allowed to cool and 10 µl of 100 µg/ml proteinase K was added and incubated at 56°C for 1 h. After protein lysis, equal volumes of phenol (approximately 500 µl) was added and centrifuged at 12000 rpm for 5 min at 4°C conditions. The supernatant was collected in a fresh eppendorf tube and 200 µl of chloroform: Isoamyl alcohol (24:1, v/v), 200 µl of phenol was added and mixed thoroughly by inverting the tubes. Tubes were then centrifuged at 12000 rpm for 5 min at 4°C. The supernatant was collected in a fresh tube and phenol, chloroform; isoamyl alcohol step was repeated 3 times. Collected supernatants were pooled together and equal volumes of chloroform were added and subjected to centrifugation at 12,000 rpm for 5 min at 4°C. Supernatants thus collected were treated with 500 µl of ice-cold ethanol or isopropanol and incubated at −80°C for 30 min. After DNA precipitation the tubes were subjected to centrifugation at 12,000 rpm for 10 min at 4°C. The DNA pellets were washed two times with 70% ethanol (v/v) and dried in a vacuum chamber. Resultant DNA was resuspended in 50 µl of Tris-EDTA and stored at −20°C until further use.

Screening of aflatoxin positive fungal isolates by gene-specific PCR: Gene-specific PCR targeting two genes of the metabolic pathway encoding toxin chemotypes *viz.*, nor-1 and aflR for aflatoxin production in fungal isolates were conducted [17]. The primers for the targeted genes are mentioned in the Table 2. Each PCR reaction consisted of 20 µl of reaction volume with 1x PCR buffer, 1.5 mM of MgCl2, 80 µM of each dNTPs, 1.0 U of Taq polymerase, 10 ng to 00 ng of template DNA and 6 µM each of forward and reverse primers. The reaction was carried out for 35 cycles in Eppendorf master thermal cycler (Hamburg, Germany) with denaturation at 94°C for 1 min, annealing of 56°C, 58°C for *nor-1 gene* and *aflR gene*, respectively for 1 min, extension at 72°C for 1 min. The DNA was initially denatured for 5 min at 94°C and finally extended for 8 min at 72°C before termination of the amplification. PCR amplicons thus obtained were resolved on 1% agarose gel by electrophoresis technique and visualized under UV transilluminator.

Identification of aflatoxin positive fungal isolates by Internal Transcribed Spacer (ITS) sequencing: The species-level identification of plausible aflatoxin positive fungal isolates was carried out by the Internal Transcribed Spacer (ITS) sequencing. The amplification of the internal transcribed spacer (ITS1-5.8S-ITS2) region in ribosomal DNA (rDNA) of fungal isolates by PCR was performed and sequencing of the amplicons for identification of the isolates was carried out.

PCR amplification of Internal Transcribed Spacer (ITS) region: Genomic DNA of the aflatoxin positive fungal isolates was used as a template for the PCR amplification of their ITS regions. Universal primers namely ITS1 and ITS4 were utilized as forward and reverse primers (Table 2) for the amplification [18]. Each PCR reaction of consisted of the 2.5 µl of 1x PCR buffer, 2.0 µl of 2.5 mM dNTPs, 1.5 µl of each primers ITS1 and ITS4 (10 pmol/ml), 1.0 µl of 50 mM MgCl2, 0.2 µl Taq DNA polymerase (5 U/ml), 1.0 µl of 10 ng/ml DNA and 15.3 µl of distilled water. The PCR reaction was carried out in Eppendorf master thermal cycler (Hamburg, Germany) and the program was set with an initial denaturation of DNA for 4 min at 95°C followed by 35 cycles. Each amplification cycle consisted of three steps: Denaturation for 60 sec at 95°C, annealing for 45 sec at 56°C and elongation for 2 min at 72°C. Final elongation of 72°C for 8 min

Table 2: Primer sequences used in the present work.

Gene	Primer	Sequence (5'-3')	Size (bp)	References
nor-1	*nor1 F*	TTGGCCGCCAGCTTCGACA	367	In this study
	nor1 R	TCGCTGAAGGCAGTCGGAT		
aflR	*aflR Fwd*	GCACCTGTCTTCCCTAACA	400	Priyanka et al. [17].
	aflR Rev	ACGAACATGCTCAGCAAGTA		
ITS	ITS 1	TCCGTAGGTGAACCTGCGG	550-600	White et al. [18].
	ITS4	TCCTCCGCTTATTGATATG		

was used at the termination of the program. The resultant amplified products were analyzed by gel electrophoresis on 1% agarose gel in 1x TBE buffer. The resolved gel was then visualized under UV light and the desired amplicons of 550 bps were excised and gel purified by GenElute Gel Extraction kit (Sigma, Bangalore, India).

Sequencing of the internal transcribed spacer (ITS) region: The gel purified PCR products encoding ITS regions were sequenced according to Sanger et al. [19]. The sequencing reaction was maintained at a volume of 10 µl, containing 2.0 µl of ITS4 primer, 4.0 µl of a premix of Dynamic ET terminator (Amersham Biosciences) and 4.0 µl of the purified ITS - PCR product (100 ng/ml). The PCR tubes were then placed in the Mastercycler thermocycler gradient (Eppendorf) and set to perform 35 cycles with the conditions of initial denaturation for 2 min at 95°C, followed by denaturation for 1 min at 95°C, annealing for 1 min at 55°C, elongation for 1 min at 60°C and final elongation for 5 min at 60°C. Analysis of the resultant sequences was performed by ABI 3500 Genetic Analyzer (Applied Biosystems, USA). The obtained sequences were compared with those available in the GenBank of the National Center for Biotechnology and Information (NCBI), using the BLAST search tool [20].

Chromatographic analysis

Extraction and cleanup of mycotoxins: Depending on the matrices of the test samples, the toxin extraction procedure was performed with slight modifications. The samples size of 50 gm for solid samples (food) and 100 ml for samples in liquid state was used in the extraction procedure. Non fatty test samples such as wheat, maize, and sorghum were mixed with 100 ml of methanol: water (55:45, v/v) and for samples with fatty matrices and spices, like groundnuts, chili, pepper, and turmeric, 2 gm of NaCl and 50 ml of hexane were additionally added to the above mentioned solvent system. The samples were then blended in a blending jar at high speed for 5 min to 10 min and filtered through Whatman No.4 filter paper. The filtrate was mixed with equal volumes of chloroform and shaken vigorously for 1 min to 2 min and left undisturbed for separation. The lower phase was collected and evaporated to near dryness under a gentle stream of liquid nitrogen. The sample was then redissolved in 10 ml of methanol: Water (80:20, v/v). Further clean-up of the extracted toxin was preceded with specific immunoaffinity columns (LCTech, Germany) following the manufacturer's instructions. Elutions were collected in 2 ml methanol and stored at – 20°C until further use. Standard aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), Citrinin (CIT), Deoxynivalenol (DON), Fumonisin B1 (FB1), Ochratoxin A (OTA), Patulin (PAT), T2 toxin (T2) and Zearalenone (ZEA) (Sigma-Aldrich, USA) were prepared according to the supplier's instructions.

Thin Layer Chromatography (TLC): Toxin extracts and aflatoxin standards (AFB1, B2, G1, and G2) of 10 µl each were spotted on thin-layer chromatography Silica gel 60 F254 plates (Merck, Darmstadt, Germany) in dark conditions. The plates were developed in the mobile phase consisting of chloroform: acetone solution (85:15, v/v) for 60 min and air-dried for 3 min to 5 min in dark conditions. The plates were then visualized under long wavelength Ultraviolet (UV) light (365 nm) for the presence of fluorescent spots *viz.*, blue color (AFB1 and AFB2) and green color (AFG1 and AFG2). The Retardation Factor (Rf) values were calculated and compared to the Rf values of the aflatoxin standards. The solvent phase (chloroform: Acetone) and extracts from non-toxigenic isolates were used as the negative control in the experiment.

High-Performance Liquid Chromatography (HPLC): Quantitative and qualitative analysis of the aflatoxin extracted from fungal isolates and suspected food samples was carried out using HPLC analysis as per Priyanka et al. [21], with minor modifications. Toxin extracts and aflatoxin standards of 10 µl each were injected onto the RP-C18 column (Jasco, UK) with dimensions of 3 µm × 250 mm × 46 mm. The analysis was performed on a JASCO HPLC system (JASCO, UK) with a fluorescence detector at wavelength settings of emission at 365 nm and excitation at 455 nm with a flow rate of 0.8 ml/min and mobile phase consisting of water: Acetonitrile: Glacial acetic acid (52:47:1, v/v/v). For other mycotoxins (OTA, DON, and ZEA) emission wavelengths were set at 365, 336 and 262 nm at a flow rate of 1.2 ml/min. Fumonisin B1 (FB1) was analyzed using a UV detector with an excitation wavelength of 455 nm and a flow rate of 0.8 ml/min. The solvent system consisted of methanol: Acetonitrile: Acetic acid (50:40:10 v/v), methanol: Water (70:30 v/v), methanol: Water (80:20 v/v), acetonitrile: Water (40:60) for OTA, FB1, DON, and ZEA respectively.

Result and Discussion

Isolation of *Aspergillus* species from diverse sources

A total of 220 fungal cultures were isolated from the collected food and agricultural samples suspected for fungal infestation. These isolates were screened for the presence of aflatoxin producers by phenotypic characters, chromatography and molecular methods. The Figure 2 depicts the morphological and biochemical tests performed on the fungal isolates in the present study. As a preliminary step in screening, macro and micro-morphological features were observed and 196 isolates were screened with characteristics morphologies of *Aspergillus* strains. The presumptive *Aspergillus* isolates were then screened for aflatoxin production by ammonium vapors test and UV light test (Figure 2). A total of 188 isolates developed pink color pigmentation upon contact with ammonium vapors and characteristic blue fluorescence under long wavelength (365 nm) of UV light. For further confirmation and identification; tests such as molecular methods (gene-specific PCR, ITS sequencing) and chromatography (TLC and HPLC) were performed for the fungal isolates.

Identification of aflatoxigenic *Aspergillus* species

The suspected fungal isolates were subjected to genomic DNA

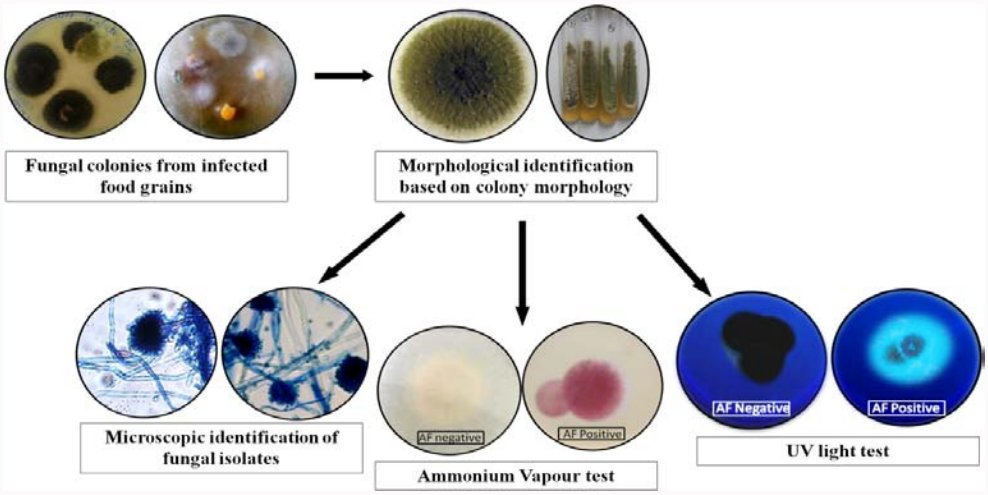


Figure 2: Morphological and biochemical tests performed on the fungal isolates.

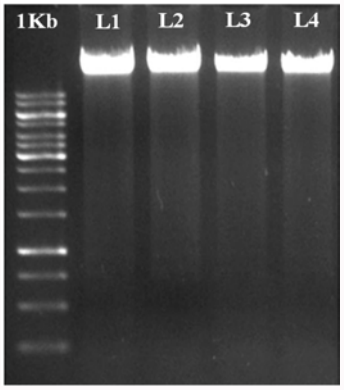


Figure 3: Fungal DNA bands against 1 Kb ladder.

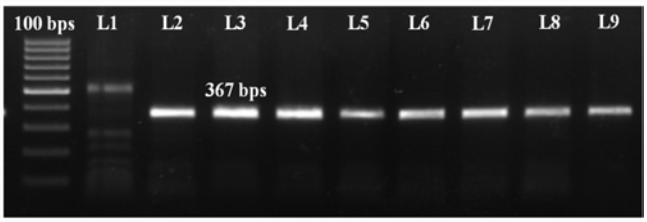


Figure 4: PCR using toxin specific *Nor-1* gene, showing bands of the desired amplicon size against 100 bps DNA ladder.

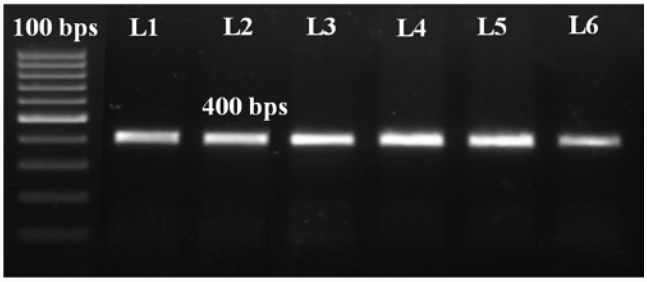


Figure 5: PCR using toxin specific *aflR* gene, showing bands of the desired amplicon size against 100 bps DNA ladder.

Table 3: List of aflatoxin producing isolates obtained in the present study.

Species name	ITS	TLC	HPLC
A. flavus	80	77	77
A. fumigatus	13	1	–
A. niger	4	1	–
A. oryzae	4	0	–
A. ochraceus	10	0	–
A. parasiticus	55	52	52
A. tamarii	6	0	–
A. versicolor	5	0	–
Total	177	131	129

extraction by phenol, chloroform, isoamyl alcohol method. Obtained DNA (Figure 3) were utilized in the molecular methods for further screening and characterization of the aflatoxin producing isolates.

Putative fungal isolates screened from the morphological and biochemical tests were examined for the presence of aflatoxin encoding genes namely *nor-1* and *aflR-1*. Gene-specific PCR amplification of *nor-1* and *aflR-1* was carried out with the respective primers mentioned in the Table 2. Amplicons of 367 bps and 400 bps were considered positive for the presence of *nor-1* (Figure 4) and *aflR-1* (Figure 5) genes respectively. A total of 178 isolates from 188 were found positive for the presence of one or both the genes (*nor-1*,170 isolates; *aflR*, 168 isolates).

Fungal isolates positive for aflatoxin encoding genes were subjected to sequencing for the identification of *Aspergillus* species. Sequencing of the internal transcribed spacer (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) was performed by amplification of the region by ITS1 and ITS4 primers (Table 3) with standardized PCR program. An amplicon of 550 bps (Figure 6) was gel purified and utilized for sequencing procedure. The BLAST analysis of resultant nucleotide sequences and comparison with those available at Gen Bank, NCBI, [20] revealed a total of 177 out of 220 fungal isolates as members of *Aspergillus* family. The percentage distribution of members of *Aspergillus* family recovered in the present study is depicted in (Figure 7) accounting for 43% of *A. flavus*, 29% of *A. parasiticus*, 7% of *A. fumigatus*, 7% of *A. oryzae*, 5% of *A. ochraceus*, 4% of *A. versicolor* and 3% of *A. tamarii* and 2% of *A. niger*.

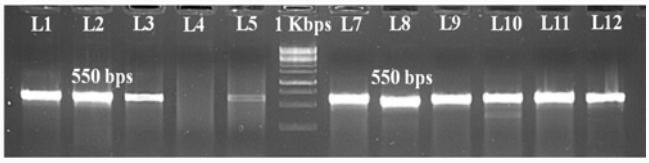


Figure 6: PCR using ITS primers, showing bands of the desired amplicon size against 1 Kb DNA ladder.

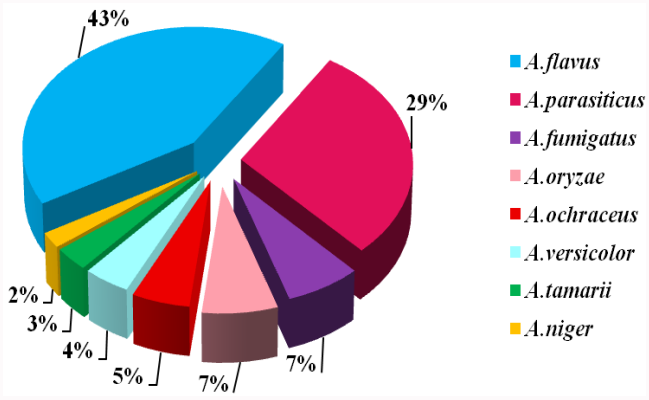


Figure 7: Percentage distribution of members of *Aspergillus* species obtained from ITS sequencing.

Chromatographic analysis of toxin extracts from *Aspergillus* species

Chromatographic analysis (TLC and HPLC) of the mycotoxin extracts from the *Aspergillus* isolates was performed as the confirmatory tests for the presence of aflatoxin. The toxin extracts from the isolates were spotted on silica gel plates and tested by TLC method. When visualized under UV light, the spots on the silica gel plates emitted blue and green fluorescence indicating the presence of Aflatoxin B1, B2, and Aflatoxin G1, G2 respectively (Figure 8). The Rf values of the test toxins were compared to that of the standard aflatoxin values and observations were drawn. A total of 131 *Aspergillus* isolates were able to produce extracts emitting blue fluorescence suggesting AFB production [22].

The toxin extracts found positive for the presence of blue fluorescence form TLC analysis were subjected to High Performance Liquid Chromatography (HPLC). A volume of 20 µl of the extracts was loaded into the HPLC system and analyzed. The Retention time (tR) and area of the peak were noted down and compared with values of the standard AFB1. The observations as depicted in Figure 9,

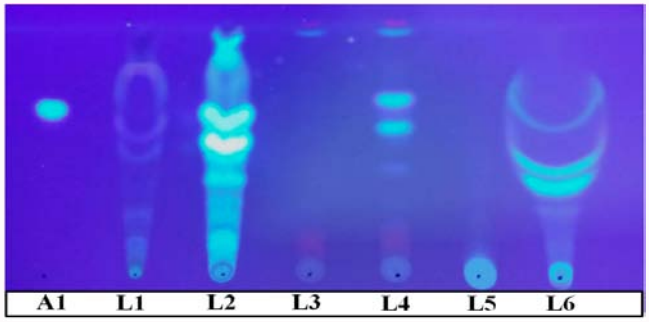
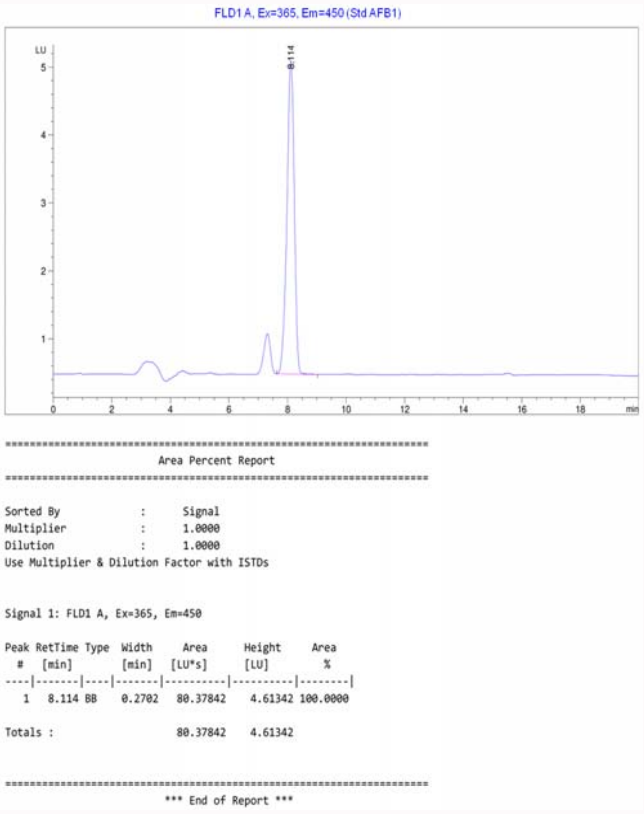


Figure 8: TLC image of aflatoxin producing isolates. Lane A1 contains the standard AFB1 against the lane L1-L6 with extracted toxins from fungal isolates.



Area Percent Report

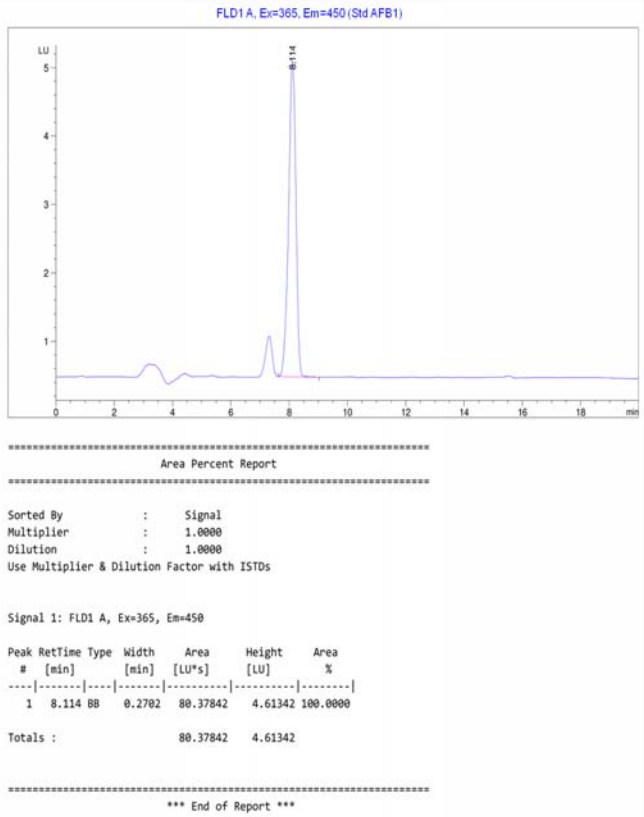
Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=365, Em=450

Peak #	RetTime [min]	Type	Width [min]	Area [LU*s]	Height [LU]	Area %
1	8.114	BB	0.2702	80.37842	4.61342	100.0000
Totals :				80.37842	4.61342	

*** End of Report ***

Figure 9: HPLC chromatogram with standard AFB1. Displaying peak at a retention time of 8.11 min.



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=365, Em=450

Peak #	RetTime [min]	Type	Width [min]	Area [LU*s]	Height [LU]	Area %
1	8.114	BB	0.2702	80.37842	4.61342	100.0000
Totals :				80.37842	4.61342	

*** End of Report ***

Figure 10: HPLC chromatogram with toxin extraction from corn sample isolates. Displaying peak at a retention time of 8.13 min.

showed similar Retention time (tR) intervals of 8.114 min as that of the standard AFB1 and the respective peak areas revealed the quantitative analysis of the aflatoxin present in the extracts. Among the tested 131 *Aspergillus* isolates, 77 of *A. flavus* and 52 of *A. prasiticus* were found to produce AFB1 whereas the remaining TLC positive *Aspergillus* members did not produce aflatoxins (Table 3).

Conclusion

A total of 220 fungal cultures were isolated from the collection and were screened morphologically and examined for microscopic features. Isolates were further screened by biochemical, molecular methods and identified by ITS sequencing. HPLC analysis revealed 129 isolates for aflatoxin production (77 of *A. flavus* and 52 of *A. prasiticus*). From our observation, it is recommended to carryout routine analysis of grains, food and feed in order to limit aflatoxin contamination in food chain. Management strategies should therefore be oriented to avoid fungal colonization during the crops and storage areas to avoid production of mycotoxins.

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