

Towards Managing and Controlling Aflatoxin Producers within *Aspergillus* Species in Infested Rice Grains Collected from Local Market in Kenya

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ABSTRACT

Rice grain can be attacked by a range of pathogens, including *Aspergillus* species, which can cause accumulation of aflatoxins that represent a serious threat to the consumers. Aflatoxins are naturally occurring toxic metabolites synthesized by certain species of *Aspergillus*. This study was designed to analyze the prevalence of *Aspergillus* species and aflatoxin-producing *Aspergillus* using polymerase chain reaction (PCR) in rice grains being sold in the local markets. A total of 98 samples were randomly collected and primarily analyzed to observe the moisture content and fungal growth. Subsequently, *Aspergillus* species were isolated, characterized using ITS primers and screened for aflatoxigenic fungal targeting specific genes (nor-1 and ver-1) involved in aflatoxin biosynthetic pathway using PCR assay. It was observed that all tested samples were found contaminated with the highest prevalence of *Aspergillus* species and aflatoxigenic fungal, 55.4% and 36.4% for nor-1 and ver-1 respectively. Occurrence of high contamination level of *Aspergillus* species indicates the possible production of aflatoxins in rice grains. A total of 74 genomic DNA extracted from the isolates in this study, 55.4% of isolates were confirmed the aflatoxin producers by targeted genes. This research provides the baseline studies for the occurrence of Mycotoxigenic fungal species in rice grains being sold in local markets.

Keywords: Rice grains; *Aspergillus* species; Prevalence; ITS; Nor-1; Ver-1; Aflatoxigenic

INTRODUCTION

Rice is a cereal consumed by great part of the human population throughout the world in many forms of products, such as white rice, parboiled rice, meal rice and rice bran [1]. After harvesting, it is generally dried and under inappropriate storage conditions such as storage dumpy/misty, lack of controlling airborne, insects, temperature and humidity during storage, rice is considered a viable substrate for growth of fungi [2,3]. During processing, rice kernels can be contaminated with fungi especially those producing mycotoxins such as aflatoxins given the conditions are favorable [4]. Additionally, delayed drying process and excess moisture (above 13%) [5] can promote the growth of fungi. Several strains of *Aspergillus* section Flavi are common in plants and its processed derivatives, with some producing diverse mycotoxins, such as aflatoxins, 3-nitropropionic acid, tenuazonic acid and cyclopiazonic acid [6]. The production of aflatoxins is associated with spore production by species of *Aspergillus* [7]. They are produced as

secondary metabolites and 4 types i.e., aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂) are the most important with reference to carcinogenic effect, mutagenic, teratogenic, and immunosuppressive capabilities [8]. They are classified in group I as first class carcinogens, mutagens and immunosuppressive agents by International Agency for Research on Cancer [9]. About 5.2 million cancer deaths occur each year, 55% of which occur in developing countries [10]. The removal of AF_s is difficult due to their stability and thermal resistance in dried products [11]. In fact, AF_s are resistant to food processing and thus they may remain throughout the food chain [12]. Therefore, AF_s are potential threats to human health, either by consumption of direct contaminated food products or by carry over aflatoxins and their metabolites in milk and meat [13]. World health authorities warn that low doses with long-term dietary exposure to aflatoxins are a major risk to lead hepatocellular carcinoma [14]. In this context, the aim of this study was to characterize the nature of *Aspergillus* species contaminating rice grains on one hand and the aflatoxigenic genes profile of

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Aspergillus species isolated by using the phylogenetic analysis of Internally Transcribed Spacer (ITS) regions on the other hand.

MATERIALS AND METHODS

Samples

Samples comprising local and imported rice grains were collected randomly (1 kg) from local retail markets and millers in Mwea and Thika (Kenya) and labelled appropriately. A total of 98 samples (local rice produced in Mwea and imported rice originating from Biryani, India, Pakistan, Thailand) were taken according to the alternative sampling plan for official control of mycotoxins in food [15]. To give a representative sample which was then put in sealed bags and transported to the Molecular Biology and Biotechnology Laboratory in PAUSTI.

Moisture content analysis

To better understand the factors that may have led to aflatoxins contamination of the samples, each sample of rice grains collected was analyzed for its moisture content using Grainier meter (Model Japonica).

Isolation and enumeration of fungal species

Isolation of microflora from the grains was done following Ulster method [16]. The samples were analyzed by the direct plating technique. For each sample, 20 particles (rice grains suspected are contaminated by fungi) were placed aseptically on a layer of moistened filter paper in the Petri dishes. The Petri dishes were incubated in an upright position at 25°C for 5-7 days in darkness and ventilated for 12 hours in the 3rd day. After incubation, the plates were examined and the number of contaminated particles were counted and reported as percentages. Fungi growing on different seeds were isolated from emerging colonies on modified rose Bengal chloramphenicol agar (mRBA) [17]. Pure cultures were carried out for subsequent studies using two (2) culture media: malt extract agar (MEA) and potato dextrose agar (PDA) according to Pitt et al. [18] and Varga et al. [19] for seven days.

Phenotypic characterization

Phenotypic characterization was done on the basis of mycelium growth pattern, color and properties of fruiting bodies of the fungi. Growth pattern of fungal colonies, colony size (diameter) and color (reverse and coarse), exudates and colony margins were the macroscopic features used in identification. Size of head, vesicle shape, phalides, matulae, conidiophores and conidia (conidial diameter, wall, shape, and surface and conidia attachment with conidiophore) were done for microscopic identification. These features were finally compared with the synoptic keys for identification of the isolated fungi [20].

Percentage occurrence of each species isolated was also calculated

$$\% \text{ Occurrence of species} = \left(\frac{\text{No. of isolates of a species}}{\text{Total no. of isolates} \times 100} \right)$$

Molecular characterization of the isolates

The genomic DNA was extracted from seven days' old fungi cultures grown on MEA and PDA media and the fungal mass from the pure cultures was scraped-out from the plates. 50-100 mg of fungal mycelium was scraped and placed in a 2 ml tube and vortexed vigorously for 30 minutes with glass beads to crush the

mycelial wall and to release the DNA. A volume of 500 µl of Lysis Buffer (100 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 10 mM B-mercaptoethanol and 1% Sodium dodecyl sulfate), 5 µl of RNase A and 1 µl of Proteinase K were added. Tubes were then incubated at 65°C for 45 minutes into buffer.

Thereafter, 270 µl of Sodium/Potassium acetate (3 M) was added and samples were well mixed and centrifuged at 13000 rpm for 10 minutes. After centrifugation supernatant (700 µl) was transferred to a fresh tube, and an equal volume of Chloroform: Isoamylalcohol (24:1) was added and mixed well, samples were stood on bench for 5 mn followed by centrifugation at 13,000 rpm for 10 min. The supernatant (700 µl) was transferred into new tubes, 80 µl of Sodium/Potassium acetate (3 M) and 587 of ice cold Isopropanol were added then mixed well by inverting the tubes then incubate at -20°C overnight. After that, samples were centrifuged at 13,000 rpm for 30 min, then supernatant was discarded carefully. The DNA pellets were washed with 1 ml of 70% ethanol and centrifuged at 13,000 rpm for 10 min. The DNA pellets were air dried and dissolved in 50 µL of TE buffer and stored at -20°C until use.

Separation of the isolated genomic DNA was done using agarose gel electrophoresis followed by Sybr Green visualization using Lambda (λ) as DNA size marker. The concentration and purity of genomic DNA were measured using Nano-drop Spectrophotometer (Model PCR Max Lambda), purity of all extracted DNA was done by taking their absorbance at 260 nm and 280 nm.

Diagnostic PCR using Aspergillus universal primers

We used Aspergillus universal primer pairs to amplify the ITS1 and ITS4 region of different isolated Aspergillus strains for characterization and primer sequences already available in literature [21] to amplify 2 different genes involved in Aflatoxin biosynthetic pathway Norsolorinic Acid (NOR): aflD (nor-1), and Versicolorin: aflM (ver-1) fragments of aflatoxigenic fungal genomic DNA. The sequences of primers are listed in Table 1.

PCR assay was performed in 20 µL of a reaction mixture that contained 1 µL of extracted genomic DNA, 4 µL of master mix, 0.5 µL of each forward and reverse primer. The final volume was made up to 20 µL with nuclease free water. Amplification was performed in a Proflex PCR System (Model 4483636) with the following conditions (Table 2).

The amplified PCR products were resolved by gel electrophoresis in a 1.5% agarose (Sigma) gel stained in 10 µL of Trugel Fluorescent Dye. The DNA bands resolved on agarose gel were visualized in UV Documentation system with camera (Model UV Doc. HDS UITEC Cambridge) transilluminator. The sizes of the amplicon were estimated by comparing with a commercial 1 kb DNA ladder on agarose gel.

RESULTS

Moisture content

In present study mycoflora was isolated from rice grains to evaluate the contamination level in rice grains sold in the market. Samples collected were previously examined for moisture content and the prevalence of Aspergillus species. Fungal growth in food samples is usually influenced by various factors. Climatic conditions, especially temperature and higher moisture content of the food samples play an important role in this process. All samples were

Table 1: Oligonucleotide primer sets used for the study.

Set	Primer name	Sequences (5'-3')	Length of PCR Product (bp)
1	ITS1	F-TCCGTAGGTGAACCTGCGG	598
	ITS4	R-TCCTCCGCTTATTGATATGC	
2	aflD	F-ACCGCTACGCCGGCACTCTCGGCAC	400
		R-GTTGGCCGCCAGCTTCGACACTCCG	
3	aflM	F-GCCGCAGGCCGCGGAGAAAGTGGT	536
		R-GGGGATATACTCCCGCGACACAGCC	

Abbreviations: F-Forward; R-Reverse

Table 2: PCR parameters of the oligonucleotide primer sets used for the study.

S.No	PCR fragment	Initial denaturation	Denaturation	Annealing	Elongation	Final elongation	Number of cycles
1	ITS	94°C 5 mn	95°C 60 s	52°C 60 s	72°C 60 s	72°C 10 min	35
2	aflD	94°C 5 mn	94°C 60 s	64°C 60 s	72°C 60 s	72°C 10 min	33
3	aflM	95°C 5 mn	95°C 60 s	65°C 60 s	72°C 2 mn	72°C 10 min	33

initially analyzed for moisture content to check for the relationship correlation ($Y=4.211 \times -54.39$) between moisture level in rice grains and fungal growth. 4 groups (group 1, group 2, group 3 and group 4) of samples were clustered according to their percentage 14-14.5; 15-15.5; 15.6-16.5; and 16.6-17.9 respectively as shown in Figure 1.

Samples with high moisture contents for a length of time offer best microclimate for the growth of mycotoxigenic fungi and mycotoxins production. A positive correlation between moisture content and isolates was observed in this study: $R^2=0.2926$, with the increase of moisture content the number of isolates was increased, indicating a higher level of fungal growth and contamination of rice grains.

Isolation of *Aspergillus* species

The results of isolates for different samples showed the presence of *Aspergillus* in all samples. From the observations made in infested rice grains the fungal growth was seen in all the inoculated plates except negative control.

Morphological analysis

All the samples were positive for *Aspergillus* with a total of eight (8) fungal strains including. *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus nomius*, *Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus versicolor*, and *Aspergillus* spp being observed. These strains were purified based on their distinct morphology on malt extract agar (MEA) and potato dextrose agar (PDA) plates and differentiated based on their macroscopic and microscopic characteristics. Macroscopic identification was based on colony and reverse color, diameter, exudates and texture. On the basis of macroscopic study, isolates can't be differentiated. Further microscopic study involved the arrangement, color, diameter, shape, size, wall characters, cellular contents, conidial heads, conidiophore, sterigmata, conidia and conidial arrangements was observed in microscope [22]. As the macroscopy and microscopy characteristics of fungal isolates that are presented in Figure 2.

Morphological analysis revealed that *Aspergillus flavus* (37.5%) was the most represented strain followed by *Aspergillus parasiticus* (27.9%), *Aspergillus fumigatus* (10.8%), *Aspergillus Nomius* (6.2%), *Aspergillus oryzae* (5%), *Aspergillus Clavatus* (3.1%), *Aspergillus Versicolor* (2.7%), and *Aspergillus* spp (6.5%). The

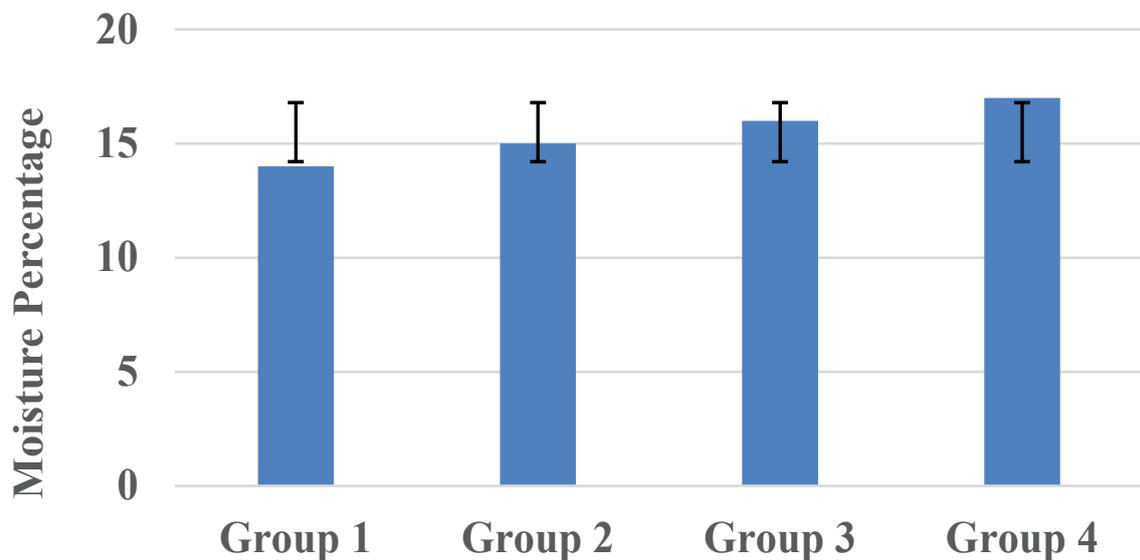
ability of fungi to change their morphology at different stages of growth and reproduction renders phenotypic characterization inefficient. Therefore, these isolates were subjected to molecular analysis through PCR amplification of their Internally Transcribed Spacer (ITS) regions and 2 genes profile of *Aspergillus* species involved in aflatoxin biosynthetic pathway Norsolorinic Acid (NOR): aflD (nor-1), and Versicolorin: aflM (ver-1) fragments of aflatoxigenic fungal genomic DNA Figure 3.

Molecular characterization

Seventy-four (74) genomic DNA isolated were selected from *Aspergillus* strains for molecular analysis. Quantity of extracted DNA from all the samples was between 369 µg/mL to 1998 µg/mL, which is an excellent quantity range. Genomic DNA isolated was amplified through PCR according to the described conditions. The PCR was conducted using the sets of three primers specifically ITS1-ITS4 for characterization of *Aspergillus* species, aflD and aflM genes profile targeted DNA for screening aflatoxigenic strains from *Aspergillus* isolates. The expected size of each primer pair produced a single DNA fragment is 598 bp for ITS, 400 and 536 bp for aflD and aflM respectively. From a total of 74 phenotypically characterized *Aspergillus* species isolates, all samples showed positive PCR results for ITS primer pair. Although ITS1-ITS4 is the most commonly used primer pair for the detection of *Aspergillus* species [23], it does not have sufficient discriminative power to differentiate between aflatoxigenic and non-aflatoxigenic (atoxigenic) species. Therefore, primers pair for specific genes profile involved in aflatoxin biosynthetic pathway aflD and aflM were amplified. Altogether, 74 samples were screened in which, 49 samples were found positive to targeted gene aflD, and 27 samples to targeted gene aflM. The amplified products were separated by Agarose gel electrophoresis as shown in Figure 4.

DISCUSSION

Moisture content is one of the determining factors for the growth of *Aspergillus* fungi. Here, high moisture content (14 to 17.9%) was recorded. This is way above of the standard levels of 12-13% for rice grains as reported by Magan et al. [24]. These results are in line with those reported by Hina et al. [25] who attributed the same to the processing, wet weather and transport conditions.



Representative Samples of Rice Grains (Groups)

Figure 1: Percentage of moisture content of rice samples.

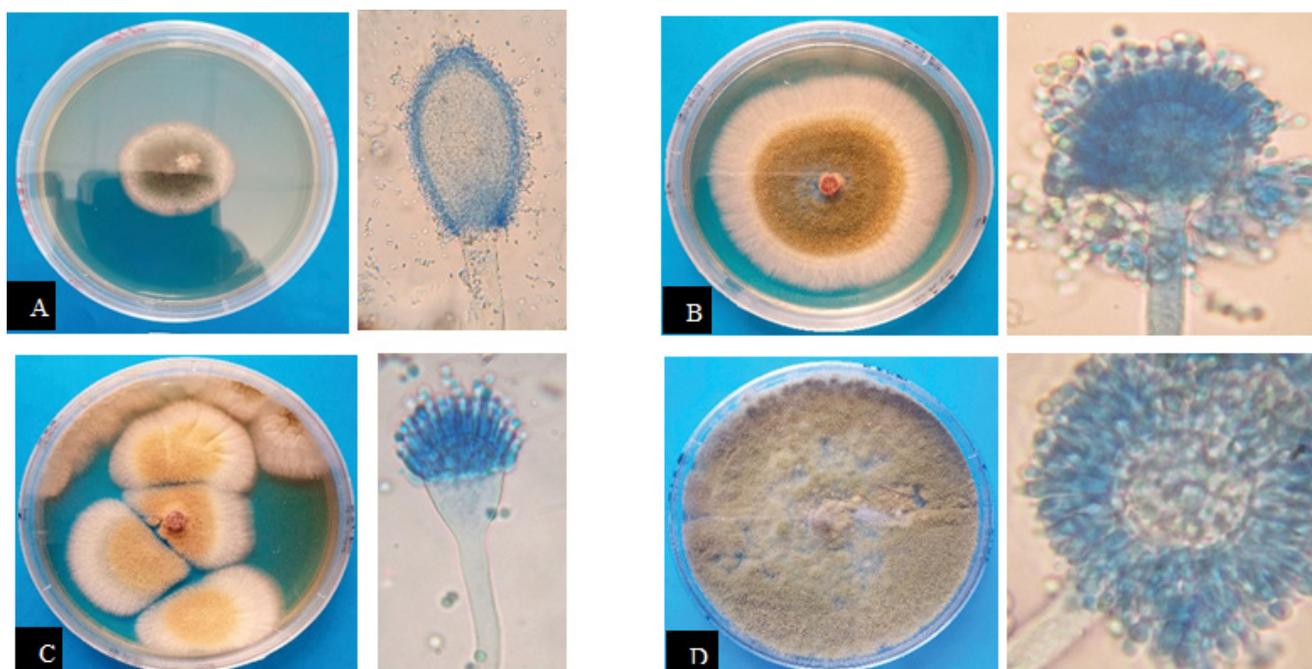


Figure 2: Left to right: 7 days old colonies on PDA and MEA; Top to bottom: A-*Aspergillus clavatus*; B-*Aspergillus flavus*; C- *Aspergillus fumigatus*; D-*Aspergillus parasiticus*.

The prevalence of *Aspergillus* species was analyzed in all samples under study (Figure 1). The investigation of occurrence fungal species showed that all the samples were contaminated. *Aspergillus flavus* was the most predominant followed by *Aspergillus parasiticus* as compared to other *Aspergillus* strains. These dominances could be attributed to the ability of *Aspergillus* to growth on substrates in a wide range of environment and the production of spores that remain viable even under extremely strict conditions. Our present data (Figure 3) are in agreement with that recorded by several investigators [25-27] who revealed the high prevalence of *Aspergillus* species in contamination of rice grains from local markets. In that case, it might be due to the rice grain processing, transport and storage conditions such as improper packaging, poor ventilation and increased humidity in the ware houses that favor

the growth of storage fungi i.e., *Aspergillus* species, instead of field fungi i.e., *Fusarium* species, more frequently found in field rather than on processed and stored grains [28]. The highest prevalence of *Aspergillus* species detected from different samples, which are the main contaminant of rice grains in present study. The presence of fungal growth indicates the anticipated contamination of mycotoxins; as high prevalence of *Aspergillus* species indicates the contamination of aflatoxins in rice grains as reported by Aiden et al. [29]. Due to the toxic and carcinogenic properties of aflatoxins, there is an urgent need to develop sensitive, rapid, and specific technique for the identification of aflatoxin producing from food samples. In our study the PCR reaction was targeted against ITS1-ITS4 for detection of *Aspergillus* species, aflD and aflM genes profile targeted DNA for screening aflatoxigenic strains from

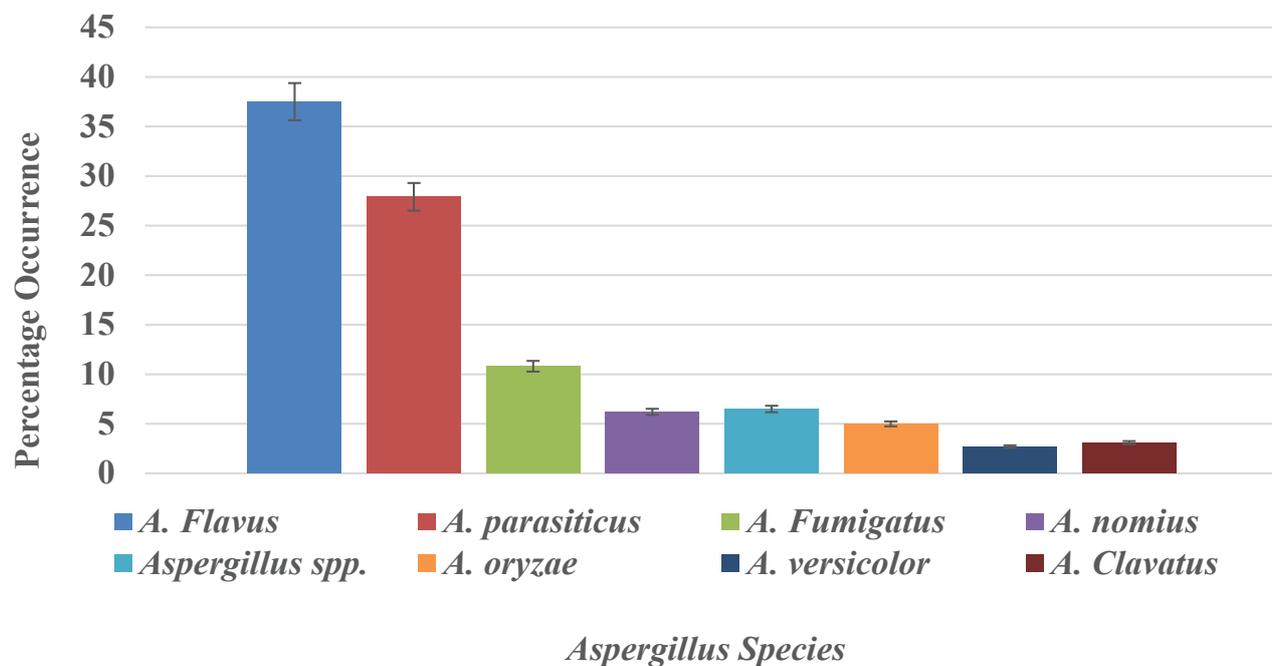


Figure 3: Occurrence of different *Aspergillus* species isolated from rice grains in percentage.

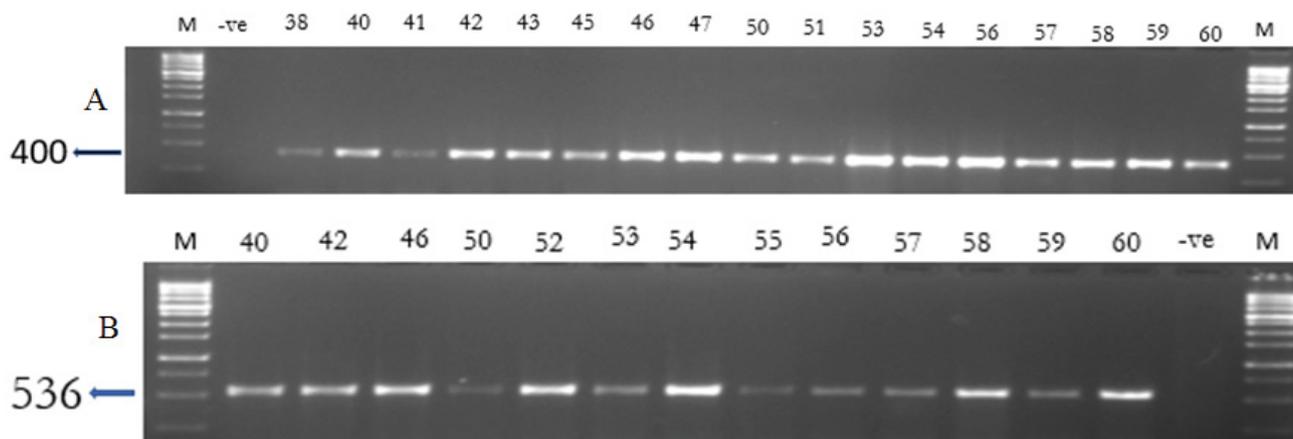


Figure 4: PCR amplicons of A: 400bp product of aflD; B: 536bp product of aflM markers; M: Molecular Weight Marker 1kb (Solis Biodyne); -ve: Negative Control. Numbers are the code of samples.

Aspergillus isolates. The primers ITS1 and IS4 were previously amplified successfully all the ITS region isolates selected using conventional PCR. In gel electrophoresis of PCR product and amplicon corresponding to 598 bp in size was seen only in positive sample, which clearly indicated that the primers (ITS1-ITS4) were specific for *Aspergillus* species. This result is in concordance with that recorded of many investigators [23,30] that ITS1-ITS4 is the most commonly used primer pair for the detection of *Aspergillus* species.

On one hand, these fungi deteriorate the quality of food products and on the other hand, they are able of producing harmful toxins such as aflatoxins. Biosynthetic pathway comprises of many enzymatic steps with aflatoxins as the end product. Several papers have reported use of PCR technology as rapid and sensitive method for detection and diagnosis of aflatoxin production [31] and to detect aflatoxigenic strain from non-aflatoxigenic strain [32,33] in food. During the current study, PCR was performed for the detection of aflatoxigenic genes i.e., aflD (nor-1), and aflM (ver-1), respectively by using specific designed primers. Cluster genes in aflatoxin biosynthesis pathway contain structural genes, nor-1

and ver-1 are the genes that play a key role in the production of aflatoxins. In our PCR analysis, screening of aflatoxigenic genes profile of isolates revealed that 41 out of 74 isolates were aflatoxin producers for aflD. In this study it was observed that aflD gene was being amplified efficiently as it was a structural gene required in the initial step of aflatoxin biosynthesis pathway. However, aflM primers were less efficient with 27 isolates amplified for this gene. These two primers were carefully selected to be highly specific for these two genes known to be essential for aflatoxin biosynthesis. Each primer pair yielded a single DNA fragment of the expected size of 400 and 536 bp for nor-1 and ver-1, respectively. A total of 74 genomic DNA isolated, only 33 showed negative PCR results for these genes targeted. This result indicates these isolates are non aflatoxigenic. Non aflatoxigenicity of these isolates may be because of a mutation or gene deletion in one or more genes belonging to the biosynthetic gene cluster [34]. Houshyarfard et al. reported that production of aflatoxin is bound to several factors [35]. Firstly, the presence of certain genes, Secondly, the genes should be intact. Meaning that, there should not be major deletions or insertions within the gene regions or regions flanking the gene. Otherwise,

deletions of several portions of the aflatoxin biosynthesis gene cluster have been reported to be the main cause for the lack of aflatoxin production. Keeping in view the above results, it is obvious that the contamination level of mycotoxigenic fungi in rice grains sold in the markets is very high. Our results are in accordance with other reports demonstrated that the significance of PCR based techniques to detect aflatoxigenic potential of *Aspergillus* strains [6,21,26,27,36,37].

CONCLUSION

On the basis of achieved results, we can say that prevalence of *Aspergillus* species in rice grains sold in the markets which is very high, and indicates the possible high level of aflatoxigenic strains in rice grains under study. In this study, 55.4% of isolates were able to confirm the aflatoxin production by amplifying the two targeted genes (*nor-1* and *ver-1*) as these genes are considered as indicators of aflatoxin production, so this percentage indicates the possible contamination of aflatoxins in rice grains. Molecular method is proved as rapid and accurate detection system to differentiate the aflatoxigenic and non-aflatoxigenic isolates. Further studies on sequencing and the quantification of aflatoxins in rice grains and their relationship with the level of contaminating *Aspergillus* species are in progress.

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