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Molecular probing of Aflatoxigenic fungi in rice grains collected from local markets of Lahore, Pakistan

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Abstract

Background: Aflatoxigenic *Aspergillus* strains have emerged as a serious threat to food safety and quality assurance. The objective of this study was to identify the aflatoxigenic *Aspergillus* sp. by targeting the amplification of aflatoxigenic genes *i.e., aflR, nor1, omt1, ver1*, in different fugal strains isolated from the rice grains being marketed in local markets of Lahore city, Pakistan.

Methods: Total eleven (11) *Aspergillus* strains were isolated from rice grains and aflatoxigenic genes *i.e., aflR, nor1, omt1, ver1* were amplified to differentiate between aflatoxin producing and non-producing strains.

Results: Four (04) out of total eleven (11) strains showed the presence of aflatoxins producing genes, indicating the possible contamination of aflatoxins in rice grains being sold in local markets of Lahore.

Conclusion: This research provides the basis for the quantification of aflatoxins; a significant threat to the quality of foodstuffs and consumers. The situation demands the attention of rice growers, processors as well as government officials to tackle the problem to assure the safety of rice eaters.

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Introduction

Rice (*Oryza sativa* L.) is being widely consumed in the world and it is ranked as a second (2nd) commonly used food item in Pakistan [1]. In Pakistan, its annual production was 7442 thousand tonnes during 2017-18. Contamination of aflatoxins occurs during the pre-harvest and post-harvest stages of rice grains. Production of aflatoxins in rice grains may be triggered by high humidity levels and delayed drying processes. Rice has been reported to contain high aflatoxin levels (up to 28.30 µg / Kg) as a cereal commodity [1]. Moreover, compromised storage conditions can also be helpful for the enhanced production of aflatoxins. Even aflatoxins may contaminate boiled rice in the husk (commonly known as parboiled rice) and white rice. According to a study, the most susceptible rice for aflatoxigenic fungi, is the parboiled rice, if it is not dried properly after boiling [2].

A variety of fungal strains have been reported as aflatoxins producers. These may include the strains of *Aspergillus flavus*, *Aspergillus niger, Aspergillus parasiticus,* and rarely by *Aspergillus nomius*, producing aflatoxins as secondary metabolites [3]. Initially, aflatoxins were reported from the *Aspergillus flavus,* so named aflatoxin that is derived from a combination of four (04) alphabets i.e., AFLA among which A represents genus Aspergillus and FLA means *flavus*, while toxins mean poison. Aflatoxins have been of most significance than all other mycotoxins due to their post consumption effects. Initially they were screened in the 1960s and recognized as the cause of hepatic necrosis [4].

These toxins have been proved major contributors to cause severe toxicity leading to carcinogenic properties in humans as well as their adverse effects on a variety of life including animals, birds, and fish, which are of foremost important with respect to the safety of public health. Aflatoxin is the most dangerous type of mycotoxins especially in developing countries targeting to the population of 4.5 billion. Various studies have exposed that aflatoxins are cause of many types of toxicosis including carcinogenic, teratogenic, hepatotoxic, genotoxic, immunosuppressive and mutagenic effects [5,18-21]. Aflatoxicosis results with decreased immunity, decreased food efficiency, abnormality in the liver, and carcinogenesis [6].

Aflatoxin production is mainly related to the moisture content of grains, which is highly influenced by the humidity of the area. Aflatoxins production can be predicted by average relative humidity. The toxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* when the moisture content exceeds 10% (7% without ventilation), and the temperature is between 24 to 35°C [7]. Rice represents an ideal substrate for mold growth. However, mycotoxin contamination is less commonly reported for rice than many other major cereal crops.

Isolation and screening of aflatoxigenic fungal strains is not an easy task due to the higher similarity index among different strains. Normally, fungal strains are isolated on the basis of phenotypic characters including colony morphology and microscopic examinations. Though molecular methods are available for the identification of fungal strains, and these are more rapid, but the importance of phenotypic strain can't be denied. The most common method of identification of fungal microflora is the amplification of internally transcribed spacer (ITS) regions mainly the ITS1 and ITS4 and many variable regions at the 5th end of rRNA (28s) gene. While checking the potential to produce aflatoxins, a variety of genes have been identified and cloned. Those aflatoxigenic regulatory genes may include the locus *aflR* from *Aspergillus flavus* and *Aspergillus parasiticus*. While many other structural genes responsible for the productions of aflatoxins i.e., *pksA*, *nor*-1, *ver*-1, *uvm*-8, and *omtA* have been reported. By using the polyphasic approaches, we can identify the fungal strains responsible for the production of aflatoxins.

The current study was designed to check the presence of aflatoxigenic fungal strains on the basis of aflatoxigenic genes, in the rice grains being sold in local markets of Lahore city, Punjab, Pakistan. This study will help to understand the food safety status of rice, enabling the issue to be tackled by rice farmers, processors, and government officials.

Methods

Rice Sampling and Moisture Content (%age) Determination

Rice samples were collected from five (05) different markets in Lahore city according to previously reported method [1]. Approximately one (1) Kg rice sample was purchased randomly from different retail shops in each market. Samples were sealed in sterilized zip bags and transported immediately to lab and stored at 4 ℃ in a refrigerator until further process. Samples from each market were processed following the quadrant method to make the representative samples; coded as *i.e.,* R1, R2, R3, R4, R5 collected from Akbari, Ichra, Iqbal Town, Garden Town, and Mazang markets, respectively. Moisture contents (%age) of each sample (triplicated) were determined by the oven drying method in hot air oven by heating at 105˚C till constant weight obtained [1].

Isolation and Identification of Fungal Strains

Two approaches were used for media inoculation. Grains were directly plated on the potato dextrose agar (PDA) plates in the first method, while the suspension of ground rice grains was prepared in peptone water (0.5 %) in the second method and the 100 µL suspension was spread on the PDA. Plates were incubated at 26˚C up to 5 d, until the growth of fungal strains. Obtained colonies were identified on the basis of morphological characters to the genus level. While head seriation and conidia morphology were also done through microscopy [8,9]. Purified cultures were obtained by re-culturing colonies 3 to 4 times. Purified cultures were stored in slants at 4˚C till further use.

Total Genomic DNA Extraction

Fungal spores from each sample were inoculated on potato dextrose broth and incubated at 25˚C for 4 to 5 d

in 250 mL conical flasks on a rotary shaker (150 rpm). Filtration assembly was used to harvest mycelia and rinsed with normal saline (0.85% NaCl) solution. Harvested mycelia were stored in 1.5 mL tubes, 1 g aliquots of each sample at -80˚C. Total genomic DNA was then extracted using the readymade kit provided by Sigma Aldrich (Product No. E5038, Saint Louis, USA). The quality of DNA was checked by 0.84% agarose gel electrophoresis.

Polymerase Chain Reaction (PCR) for ITS region

Universal primers of internally transcribed spacer regions i.e., ITS 1 (5' TCCGTAGGTGAACCTGCGG '3) and ITS 4 (5' TCCTCCGCTTATTGATATGC '3) were used for amplification to analyze the fungal strains. PCR master mixture (Genei Red dye PCR master mix, purchased from Merk Millipore, Darmstadt, Germany) containing *Taq* DNA polymerase was used. PCR conditions in G-Storm Thermo Cycler GS4822 were optimized as 94°C for 5 min, denaturation at 94°C for 45 s, annealing at 57°C for 30 s, elongation at 72°C for 1 min, and a final extension at 72°C for 10 min. The total number of cycles was 35 withhold at 4°C and the total volume of the reaction mixture was 50 µL. Amplified PCR product was checked by using gel electrophoresis. After amplification, purification of PCR products for sequencing analyses was done by using Thermo Scientific GeneJET Gel Extraction Kit (K0691; Waltham, Massachusetts, United States).

Primer	Sequence	Size (bp)
aflR-F	5' TAT CTC CCC CCG GGC ATC TCC CGG '3	1032
aflR-R	5' CCG TCA GAC AGC CAC TGG ACA CGG '3	
ver1-F	5' ATG TCG GAT AAT CAC CGT TTA GAT GGC '3	537
ver1-R	5' CGA AAA GCG CCA CCA TCC ACC CCA ATG '3	
omt1-F	5' GTG GAC GGA CCT AGT CCG ACA TCA C '3	797
omt1-R	5' GTC GGC GCC ACG CAC TGG GTT GGG G '3	
nor1-F	5' ACC GCT ACG CCG GCA CTC TCG GCA C '3	400
nor1-R	5' TTG GCC GCC AGC TTC GAC ACT CCG '3	

Table 1: Sequences of specific primers for amplification of aflatoxigenic genes.

Sequencing and Phylogenetic Analysis

Purified PCR products along with ITS-1 and ITS-4 primers, were sent to commercial labs for sequencing analyses. After getting the sequences of both (reverse and forward) primers of each strain, sequences were merged by using the SeqMan tool in DNA Star software and blasted on NCBI to find similar sequences. Sequences with a high similarity index were included to identify our query sequence. Phylogenetic relationship was found by using the maximum likelihood method with the help of MEGA6 [10].

Amplification of aflatoxigenic genes

Extracted DNA from all the eleven strains was amplified with the specific primers to check the potential of aflatoxigenic genes. Multiplex PCR amplification was carried in 25 µL reaction mixture for each sample containing 0.5 µL of each primer (20 n moles) for the detection of aflatoxin pathway genes i.e., *aflR*, *ver-1*, *omt-1,* and *nor-1* (Table 1) [11] and 10 ng of the total genomic DNA sample. PCR conditions were optimized as 95˚C for 6 min followed by 6 cycles of 45 s at 95˚C, 45 s at 58.2˚C and 80 s at 72˚C, then 30 cycles of 60 s at 95˚C, 60 s at 56˚C, 60 s at 72˚C. While the final extension of 10 min, were given at 72˚C. PCR products were visualized by 1% agarose gel electrophoresis.

Results

This study was conducted to check the presence of aflatoxigenic fungal strains present in rice grains available in local markets of Lahore, Pakistan. Initially, moisture contents of rice samples were analyzed (Fig. 1) to see the interaction between moisture and fungal growth. Maximum moisture contents were observed in parboiled rice grains (R2) as 14.67% and minimum moisture contents were 8.65% in polished rice (R3). While in R1, R4 and R5, the mean of moisture contents was 12.32%, 9.54%, and 13.63% respectively. Total of eleven (11) fungal strains were purified on the basis of phenotypic characters and subjected to a series of molecular analyses to check the presence of aflatoxigenic genes.

Molecular analyses were carried by DNA extraction of fungal strains and checked by using 0.84% agarose gel electrophoresis. DNA samples were subjected to a (PCR) by using internally transcribed spacer regions (ITS-1 and ITS-4). The results are shown in Fig. 2. DNA samples from all the eleven (11) strains showed the amplification of approximately 600 base pairs (bp) [12]. PCR products were purified and sent to commercial labs for Sanger sequencing by using both of the primers (reverse and forward). After joining the sequences, consensus sequences were BLAST on NCBI and revealed that all of the strains belong to *Aspergillus flavus* species.

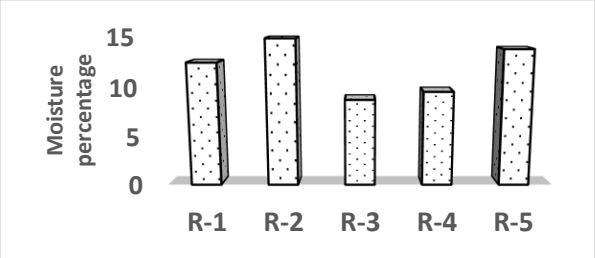

Figure 1: Percentage of moisture contents in rice grain samples collected from local markets of Lahore.

After the identification of fungal strains, DNA samples were further subjected to PCR to amplify the aflatoxigenic genes using a specific set of primers. Multiplex PCR was optimized for this purpose and after several repeated experiments, four (04) fungal strains out of a total of eleven (11) strains showed the amplification of the aflatoxigenic genes. PCR was performed using positive control i.e. known fungal strain containing the aflatoxigenic genes, and negative controls i.e. known fungal strain lacking the aflatoxigenic genes. Positive and negative control strains were obtained from the first fungal culture bank of Pakistan (FCBP) at the Institute of Agricultural Sciences, University of the Punjab, Pakistan.

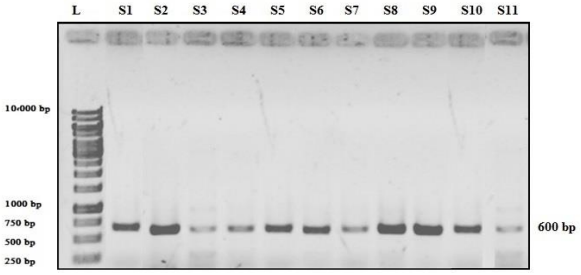

Figure 2: Amplification of strains with ITS (1 & 4) primers. All the eleven strains showing the position of PCR product near 600 bp, verifying the amplification for fungi.

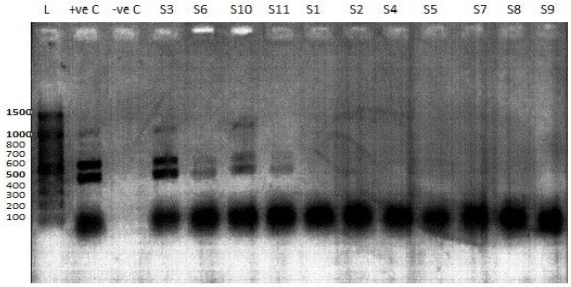

Figure 3: Amplification of aflatoxigenic genes (aflR1, omt1, ver1, and nor1), with positive and negative controls. Figure shows the amplification of strains 3 and 6 (S3 and S10) with all the four primers while strains 6 and 11 (S6 and S11) has amplified with nor1 and ver1.

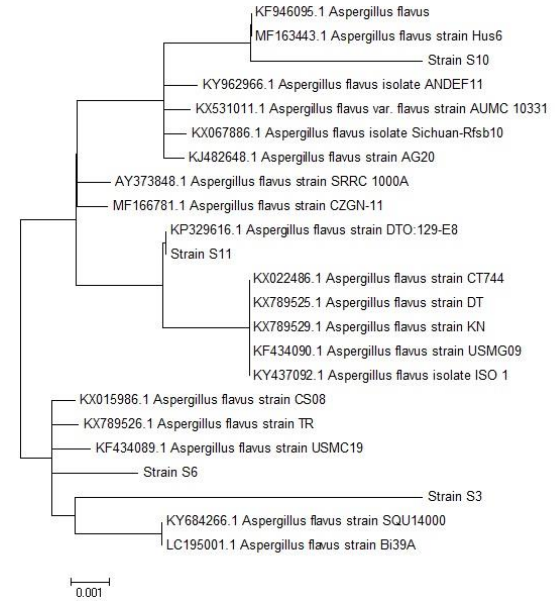

Figure 4: Phylogenetic dendogram of Aspergillus strains, which showed the aflatoxigenic genes, sequenced with ITS regions for identification by using sanger sequencing. Phylogenetic tree was constructed using the maximum likelihood method in MEGA 6 (software). The percentage of replicate trees in which the associated taxa clustered together in bootstrap test (1,000 replicates) are shown next to the nodes. Strains showed the amplification with aflatoxigenic genes are labelled as Strain S3, Strain S6, Strain S10, Strain S11.

Figure 3 shows the amplification of all aflatoxigenic genes in isolate S3 and S10 i.e., 1000 bp (*aflR*), 800 bp (*omt1*), 600 bp (*ver1*) and 400 bp (*nor1*). While S6 and S11 showed amplification of 600 bp (*ver1*) and 400 bp (*nor1*) in both of the isolates. All other strains didn't show the amplification of aflatoxigenic genes. The fungal strains showing the amplification of afltoxigenic genes were subjected to further analysis for homology and similarity-based upon their sequences obtained through the amplification of ITS regions (ITS-1 and ITS-4). The evolutionary history of the sequenced sample was inferred by using the Maximum Likelihood method based on the Tamura & Nei model [10]. Phylogenetic dendrogram placed all of the four (04) aflatoxigenic strains in the clade of *Aspergillus flavus* sp., (Fig. 4), though all of these were isolated from the rice grains procured from different markets.

Discussion

In the present study, Strain S3 was isolated from the Akbari market (R-1), Strain S6 from the Ichra market (R-2), Strain S10 and S11 from the Mazang market (R-5). Strain S3 showed the maximum homology with the strain of *A. flavus* (LC195001) reported from Egypt in 2016. Strain S6 showed the maximum homology with the strain of *A. flavus* (KF434089) reported from Malaysia in 2014. Strain S3 and S6 also showed close homology with each other. Strain S10 showed the maximum homology with the strain of *A. flavus* (MF163443) reported from Iraq in 2017. Strain S11 showed the maximum homology with the strain of *A. flavus* (KP329616) reported from the Netherlands in 2015.

There is a close interaction between the moisture percentage and presence of aflatoxigenic fungal strains, as strain S3 was isolated from R-1 having 12.32 % moisture contents, strain S6 was isolated from R-2 with 14.67% moisture contents, while strains S10 and S11 were isolated from R-5 having 13.63% moisture contents. High moisture contents play a vital role in the activation of aflatoxigenic genes which may effect the feeding species [6, 14]. These samples were collected from local markets, lacking the appropriate storage conditions i.e., improper ventilation, humidified environment, and lack of hygiene; to prevent the production of fungal strains, so this is another main reason for the prevalence of aflatoxigenic fungal strains [15]. As hepatic cancer and many other live disorders are being reported in Pakistan very frequently, but solid pieces of evidence are still unknown. By the findings of this study, it can be strongly assumed that a high level of aflatoxigenic fungal microbiota may be a major reason for these disorders because a large community of Pakistan is rice consumer in a variety of dishes as course meals. It is an alarming situation to the export of rice grains, as half of the rice production in Pakistan is being exported. Mycotoxins are global health concern but fungi is also a source of novel pharmacutical compounds [22, 23]. This study provides the baseline for the quantification and analyses of aflatoxins, which is becoming a major threat to food safety and quality. There is no research conducted at the molecular level in

Pakistan to identify the aflatoxin producing fungal strains by targeting the aflatoxigenic genes in rice borne mycoflora. There is a strong need for intensive research towards the quantification and decontamination strategies for aflatoxins in rice as well as other cereal grains i.e., wheat, barley, and oat.

During the current study, rice grains that are directly purchased by the consumers for consumption were studied for the contamination of aflatoxigenic fungi using molecular techniques. All aflatoxigenic genes were amplified from different isolates obtained from various rice samples collected from different markets of Lahore city. It was observed that rice grains being sold in open markets of Lahore are highly contaminated with aflatoxigenic fungi. So, it may be anticipated that these rice grains might be heavily contaminated with aflatoxins posing serious threat to the consumer's health. This study suggests the further research for the quantification of aflatoxins in rice grains available in local markets of Pakistan. In addition, extensive research on the decontamination strategies of aflatoxins in rice grains is strongly required.

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Author Contributions

Sohaib Afzaal, Muhammad Akhlaq and Usman Hameed conducted the experiments. Shinawar Waseem Ali planned the study and wrote the manuscript. Aftab Ahmad, Muhammad Arshad Javed and Muhammad Saleem Haider contributed in data analysis and manuscript preparation.

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