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RESEARCH ARTICLE

Isolation of Aflatoxigenic *Aspergillus flavus* from Animal-Feed and Exploration of the Genetic Basis of Aflatoxin Biosynthesis

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Prevalence of aflatoxigenic fungi in the animal feed produces aflatoxins in the feed posing serious health complications. Aflatoxins are toxic, carcinogenic and mutagenic for livestock and human. In the current study, animal feed was evaluated for the presence of aflatoxigenic filamentous fungi and the isolates were subjected to identification using microbiological, biochemical and molecular biology methods. Based on the DNA sequences of Internally Transcribed Spacer (ITS) regions, the isolates were recognized as *Aspergillus unguis*, *A. niger* and *A. flavus*. Phylogenetic relationship was deduced by constructing a phylogenetic tree using Neighbor-Joining method. Biochemical characterization revealed that isolated *Aspergillus flavus* is aflatoxigenic. The genes responsible for the aflatoxins biosynthesis in *A. flavus* were amplified using polymerase chain reaction. The genome of *A. flavus* was shown to harbor the aflatoxin synthesizing genes namely *aflO*, *aflR*, *aflS*, *aflP*, *aflD*, *aflM* and *aflQ*. The amplicons were sequenced and submitted to GenBank at NCBI. It was observed that *A. flavus* was the only aflatoxigenic species responsible for feed contamination in collected samples and all the seven targeted aflatoxin biosynthesis genes were found in the studied aflatoxigenic species. The sequencing of ITS regions has shown to be a reliable tool for the identification of atoxigenic and toxigenic fungal species. These findings on gene cluster variability may be useful for better understanding of genetic control as well as toxicological risks of aflatoxins.

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INTRODUCTION

Aflatoxins are the chemical compounds produced by *Aspergillus* species commonly present in a variety of agricultural and livestock products. Mycotoxins contamination of poultry and livestock feeds is the second most important problem after the high feed prices (Naseem *et al*., 2018). Contamination of food by these toxic metabolites has been a great challenge in the food industry. Among aflatoxin producing species, *Aspergillus flavus* is most commonly responsible for feed spoilage (Saleemi *et al*., 2017). The taxonomic study and identification of the toxigenic *A. flavus* is essential and always need an expert approach (Miranda *et al*., 2019) to develop an effective control strategy. Molecular identification based on ITS region sequencing has been widely used. The ITS regions are hyper-variable among various species, so these sequences are useful to study phylogenetics of fungal species. Use of molecular approaches in identification of microbial species has fulfilled many gaps that were left by biochemical and morphological characterization. The sequence analysis of variable DNA regions like ITS-1 / ITS-2 has made molecular characterization more rationalized for species like *A. flavus* (Jogee *et al.*, 2017).

The capability of fungal species to produce aflatoxins is strain specific, which occurs in the conidia, fungal hyphae, and sclerotia. The aflatoxin biosynthesis gene pathway consists of around 25 genes assembled in a 70 kb DNA region (Yu *et al.*, 2004), among which *aflR*, *aflS*, *aflQ*, *aflP*, *aflD*, *aflM*, and *aflO* are the dominant genes. Different *Aspergillus* species share closely matching sequences and conserved gene order within the cluster. During the recent past, PCR detection of aflatoxin

biosynthesis gene expression or existence has been used as an analytical tool for the characterization of toxigenic fungi in particular feed ingredients (Miranda *et al*., 2019). Sequence variability and omissions in numerous regions of the aflatoxin biosynthetic pathway have been used to conclude the polyphyletic assemblage of toxigenic *A. flavus* species (Okoth *et al*., 2018).

Currently in Pakistan, little scientific reports are published about molecular characterization of indigenous toxigenic *A. flavus* and no significant published work is available on aflatoxin biosynthesis gene profiling in locally isolated toxigenic *A. flavus.* Though individual work related to morphological and biochemical identification of *Aspergillus* sp. is available (Hossain *et al*., 2018), yet polyphasic approach of identification is rarely reported. In the current study, apart from molecular identification of *A. flavus* based on ITS-region, the isolated strain was also evaluated for the presence of two regulatory (*afl*R and *afl*S) and five structural (*afl*D, *afl*M, *afl*O, *afl*P, and *afl*Q) genes of aflatoxin biosynthesis within genetic cluster. Our study will lead to establish a robust technique for the screening of feed samples and to develop a molecular-based control strategy to ensure food/feed safety.

MATERIALS AND METHODS

Sample collection, isolation and identification of fungi: Feed samples were collected from various animal feed stocks located in the vicinity of Faisalabad, Pakistan during humid season of June-August and were transported aseptically to lab in plastic bags. Samples were immediately enriched in Buffered Peptone Water (BPW) solution on a horizontal shaker for 30 minutes at room temperature. *Aspergillus* spp. was identified and isolated on Aspergillus differentiation media (AFPA; M1127, HiMedia) as described earlier (Sekar *et al.*, 2008; Abriba *et al*., 2013; Hossain *et al*., 2018).

Genomic DNA extraction: Genomic DNA from all isolated fungal species was extracted by the modified method of Cenis (1992) as described previously (Saleemi *et al.*, 2012) and was analyzed on 1% (w/v) agarose gel electrophoresis.

PCR amplification of ITS region: The isolated genomic DNA was used as a template to amplify ITS (Internal transcribed spacer) region by polymerase chain reaction using ITS1 (forward) and ITS4 (reverse) primers (Saleemi *et al*., 2012; Okoth *et al*., 2018). A 25µL reaction mixture containing; 2.5µL (100ng) template DNA, 0.5µL dNTP mix (10mM), 2.5µL *Pfu* buffer with $MgCl_2$ (10X), 0.5µL of each Forward & Reverse Primers (10 pmol/µL), 0.5µL *Pfu* polymerase (2.5U/µL) and 18µL double distilled deionized H_2O was prepared. For PCR profile set up, *Pfu* polymerase was used with initial denaturation at 94°C for 5 min (1 cycle), 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1.5 min, followed by final extension at 72°C for 10 min (1 cycle).

Sequencing of amplified PCR products and phylogenetic analysis: Amplified PCR products were purified using FavorPrep PCR Clean-Up Mini Kit (FAVORGEN Biotech Corp., Taiwan) according to the instructions of the manufacturer. DNA was quantified by Nanodrop spectrometer as described earlier (Haque *et al.*, 2003). Three purified ITS region amplicons were sent for sequencing to Macrogen (Korea) and compared to ITS nucleotide sequences (Kwak *et al.*, 2013) in the GenBank database by BLASTN at NCBI (http://www.ncbi.nlm.nih.gov). Sequences were assembled in contigs by DNA Dragon Software (Version 1.6.0, SequentiX-Digital DNA Processing, Germany) and were submitted to the GenBank. Multiple sequence alignment of closely related type strains ITS sequences of validly named *Aspergillus* species retrieved from NCBI along with the sequences of current study was done using ClustalW and a phylogenetic tree was constructed by the Neighbour-Joining method using MEGA software (Version 7.0) with 1000 bootstrap value (Costa *et al.*, 2011; Okoth *et al*., 2018).

Screening of aflatoxin production on APA media: Aflatoxin production of identified isolates using APA (Aflatoxin Producing Ability) media was screened according to the method described earlier (Fente *et al*., 2001, Sekar *et al*., 2008). The fungi were plated on APA media and incubated at 28°C for 7 days in the dark. Diffusible zone of aflatoxin was detected by visualizing the plates under long wave UV light at 365nm and characteristics "Blue fluorescence" was observed (Paula *et al*., 2007).

Table 1: Primers used for aflatoxin biosynthesis gene profiling

Primer name	Primer sequence	Length (bp)	T_m (°C)
AflR-F	5′-AAGCTCCGGGATAGCTGTA-3′	19	54.9
AflR-R	5′-AGGCCACTAAACCCGAGTA-3′	19	54.9
AflS-F	5′-TGAATCCGTACCCTTTGAGG-3′	20	55.4
AflS-R	5′-GGAATGGGATGGAGATGAGA-3′	20	55.4
AflD-F	5′-CACTTAGCCATCACGGTCA-3′	19	54.9
AflD-R	5′-GAGTTGAGATCCATCCGTG-3′	19	54.9
AflM-F	5′-AAGTTAATGGCGGAGACG-3′	18	52.1
AflM-R	5′-TCTACCTGCTCATCGGTGA-3′	19	54.9
AflO-F	5′-TCCAGAACAGACGATGTGG-3′	19	54.9
AflO-R	5′-CGTTGGCTAGAGTTTGAGG-3′	19	54.9
AflP-F	5′-AGCCCCGAAGACCATAAAC-3′	19	54.9
AflP-R	5′-CCGAATGTCATGCTCCATC-3′	19	54.9
AflQ-F	5′-TCGTCCTTCCATCCTCTTG-3′	19	54.9
AflQ-R	5′-ATGTGAGTAGCATCGGCATTC-3′	21	55.9

Aflatoxin biosynthesis gene profiling: By using specific gene primers (Table 1), aflatoxin biosynthesis genes (*aflR*, *aflS*, *aflQ*, *aflP*, *aflD*, *aflM*, and *aflO*) of the aflatoxigenic fungus were amplified as described earlier (Gallo *et al.*, 2012; Fakruddin *et al.*, 2015). For targeted seven aflatoxigenic genes, PCR mixture as described in section-2.3 was prepared. The PCR profile was the same except that annealing temperature was according to the primer set being used (*aflR* and *aflS* at 54ºC; *aflD* and *aflP* at 50ºC; *aflM* 46ºC; *aflO* and *aflQ* at 45ºC) and extension time was as per amplicon size and *Pfu* polymerase synthesis rate. The amplified products were purified, analyzed, sequenced and assembled as described earlier in section-2.4. The sequences were submitted to GenBank to obtain accession numbers.

Aflatoxin analysis using HPLC: Extraction and purification of aflatoxins in sample (autoclaved corn grains inoculated with aflatoxigenic *A. flavus* in aseptic

conditions and incubated in dark for 7 days at 28°C) were carried out according to Iqbal *et al*., 2013 with some modifications. Briefly, 25g sample (contaminated corn grains) was grinded and extracted with 100 mL of acetonitrile: water (86:14; v/v) by horizontal shaking for 1 h at 100 rpm. Extract was then filtered through Whatman No. 1 filter paper. After filtration, 10 mL filtrate with the addition of 70 mL acetic acid was passed through an immunoaffinity column (AflaTest, VICAM, USA) at a flow rate of 1.0 mL/min. Aflatoxins were eluted from the column by applying 1.5 mL of methanol and the extract was collected in a glass vial. Then, aliquot was evaporated to dryness by N_2 stream at 40°C in a glass centrifuge tube. Pre-column derivatization was done with the addition of 100 mL TFA to the extract residue or aflatoxins standards to derivatize AFB1 and AFG1. The samples were left in dark at room temperature for 20 min and then 400 mL mixture of acetonitrile:water (1:9, v/v) was added into a tube. A fraction was subjected to HPLC analysis. The flow rate of isocratic mobile phase acetonitrile:methanol: water (20:20:60, v/v/v) was maintained at the rate of 1.0 mL/min. The samples were analyzed on reverse-phase HPLC (Shimadzu, Kyoto, Japan) with a Discovery HS C18 column (Sigma-Aldrich, USA) equipped with a fluorescence detector (RF-530). Excitation and emission wavelengths were 360 and 440 nm, respectively (Gallo *et al.*, 2012; Iqbal *et al*., 2013; Hossain *et al*., 2018).

RESULTS

Screening, isolation and identification of fungal isolates: The fungi were isolated from animal feed samples. Based on morphological and culture characteristics (growth pattern, reverse plate color, conidia appearance, and sporulation color) on Aspergillus Differentiation Media, three distinguished isolates were obtained (Fig. 1). The colonial growth of isolate-1 showed white to greenish color and eventually, it became dark green to brown. Isolate-2 showed characteristic pale yellow to white hyphae, turning to black / dark brown originating from the center of the culture, while the isolate-3 showed characteristic velvety to the woolly culture of yellow to green color (especially at center), with a white border surrounding the yellow to the greenish surface. On the reverse side of the AFPA culture plate, it also showed characteristics bright orange color (Fig. 2). Based on the observed characteristics, the isolates were recognized to be *Aspergillus unguis*, *A. niger*, and *A. flavus*, respectively.

Molecular characterization of the fungal isolates: The isolates were further processed for molecular characterization by sequencing their ITS regions. Genomic DNA of three isolates (Fig. 3) was used as a template for PCR amplification of ITS-region using forward (ITS1) and reverse (ITS4) primers. All amplicons were sent for sequencing to Macrogen (Korea). The BLASTN analysis of sequences thus obtained identified the isolates as *Aspergillus unguis, A. niger and A. flavus*, respectively. The sequences were submitted to GenBank at NCBI and accession numbers MH174087 (*A. unguis*), MH174088 (*A. niger*) and MH179066 (*A. flavus***)** were obtained.

Phylogenetic analysis: An evolutionary tree based on ITS region sequences of isolates and their close relatives was constructed. Multiple sequence alignment of closely related type strain sequences of validly named species retrieved from NCBI was done using ClustalW, and a "Phylogenetic Tree" was reconstructed by the "Neighbor-Joining Method" using MEGA (Version 7.0) software (Costa *et al.,* 2011; Okoth *et al*., 2018). The confidence level of each branch and stability of the relationship was tested by bootstrap analysis, by performing 1000 re-sampling for the tree topology of the neighbor-joining data. The optimal tree with the sum of branch length = 0.82474709 was drawn. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There was a total of 711 positions in the final dataset. The evolutionary tree showed that isolate-1 closely relates to *A. unguis*, while isolate-2 and 3 are related to *A. niger* and *A. flavus*, respectively (Fig. 4).

Screening of aflatoxigenic potential of fungal isolates using APA media: The isolates were grown separately on APA media for the screening of aflatoxigenic potential**.** Petri plates were inoculated aseptically in duplicate and then incubated at 28°C for 7 days in the dark. After the incubation period, all incubated plates were observed for aflatoxin zone formation by visualizing the culture plates under UV light (365 nm) for blue fluorescence (Paula *et al.,* 2007). Out of all Petri plates, the plates inoculated with "isolate-3" showed a zone of aflatoxin formation (Fig. 5). The control, as well as plates inoculated with isolate-1 and 2, did not show any blue fluorescence on APA media. Thus, characteristic orange color of colonies (reverse side culture on AFPA) and visible zone of aflatoxin production as a peculiar "Blue fluorescence" on APA confirmed the isolate-3 as aflatoxigenic *A. flavus*.

Aflatoxin biosynthesis gene profiling: The identified aflatoxigenic *A. flavus* (named as *A. flavus* strain GCUF-BNB01) was selected for aflatoxin biosynthesis gene profiling and the strain was characterized using specific primer sets at optimized annealing temperatures. PCR products were observed on 1.5% agarose gel along with DNA ladder and size of each amplified gene was determined i.e. *aflO* (790bp), *aflR* (1079bp), *aflS* (684bp), *aflP* (870bp), *aflD* (852bp), *aflM* (470bp) and *aflQ* (757bp), respectively (Fig. 6). The results showed that all the targeted genes were found in *A. flavus* GCUF-BNB01. Amplified gene fragments were extracted from agarose gel and were sent for sequencing to Macrogen (Korea) after quantification. The BLASTN analysis of sequences thus obtained confirmed aflatoxin biosynthesis genes of *Aspergillus flavus* i.e. *aflO*, *aflR*, *aflS*, *aflP*, *aflD*, *aflM* and *aflQ*. The sequences were submitted to GenBank at NCBI and accession numbers were obtained as *aflO* (MH280089), *aflR* (MH280087), *aflS* (MH280086), *aflP* (MH280088), *aflD* (MH280091), *aflM* (MH280092) and *aflQ* (MH280090).

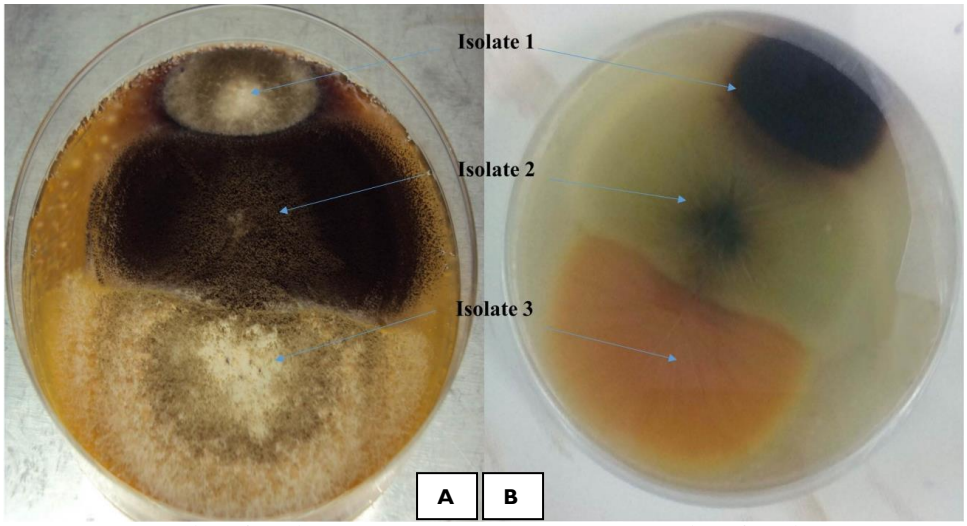

Fig. 1: Growth of *Aspergillus* isolates on Aspergillus Differentiation Media (AFPA).
A: Front side of AFPA petri plate showing various isolates after 5 days of incubation at 28°C.
B: Reverse side of AFPA petri plate showing characteristic colors of various isolates after 5 days of incubation at 28°C.

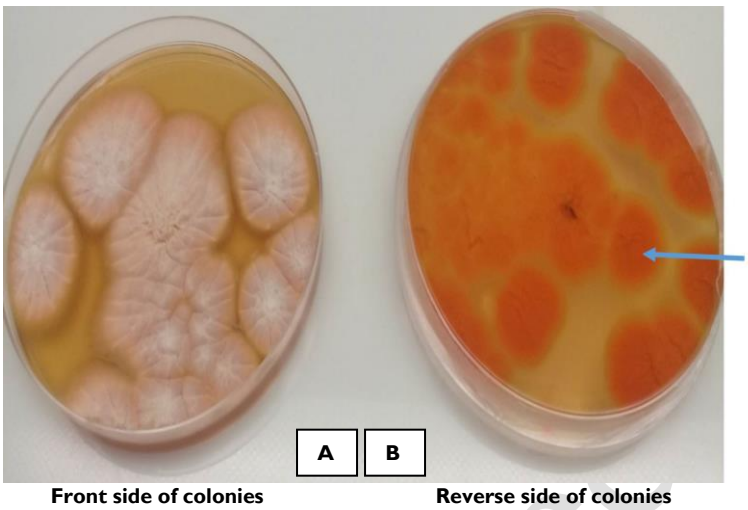

Fig. 2: Growth of *Aspergillus flavus* on Aspergillus Differentiation Media (AFPA).
A: Front side of colonies of *A. flavus* (Isolate-3) showing good luxuriant growth on AFPA after 2-3 days of incubation at 28°C.
B: *A. flavus* (Isolate-3) showing characteristics bright orange color on reverse side of colonies on AFPA after 2-3 days of incubation at 28°C.

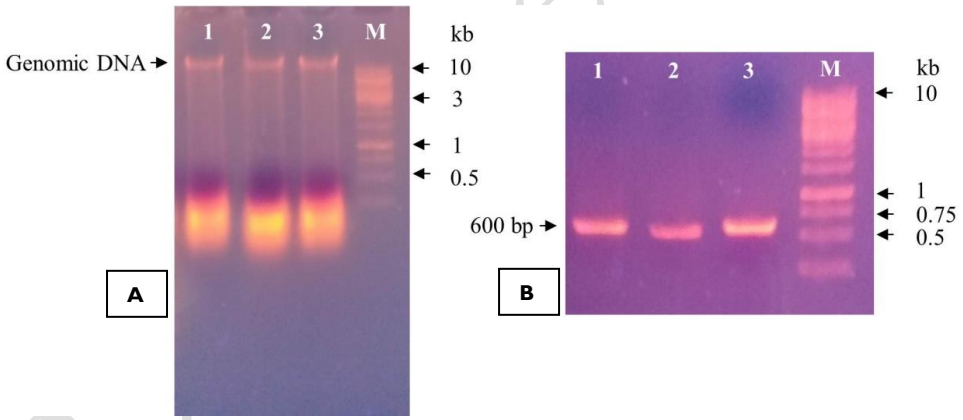

Fig. 3: Different steps in molecular characterization of ITS region.
A: Isolation of genomic DNA from isolates.
M: 1 kb DNA ladder,
1: Isolate-1 (*Aspergillus unguis*),
2: Isolate-2 (*Aspergillus niger*),
3: Isolate-3 (*Aspergillus flavus*).
B: PCR amplification of ITS regions of isolates.
M: 1 kb DNA ladder,
1: Isolate-1 (*Aspergillus unguis*),
2: Isolate-2 (*Aspergillus niger*),
3: Isolate-3 (*Aspergillus flavus*).

Aflatoxin analysis using HPLC: The extracts (in duplicate) of the sample were analyzed on HPLC as described in Materials and Methods. For quantitation, a mix of aflatoxin standards (Sigma-Aldrich, USA) containing 3 mg of each toxin (AFB1, AFG1, AFB2 and AFG2) in 1mL of benzene:acetonitrile (98:2 v/v) was also applied on HPLC column. The peak at the similar retention time and five-times higher than the baseline noise indicated the presence of a particular aflatoxin in the sample. The HPLC analysis of the extract indicated the presence of 0.15 ng AFB1 per g sample with non-detectable amounts of AFB2, AFG1 and AFG2.

DISCUSSION

Fungal contamination in animal feed is a widespread problem especially when farming community does not follow the standard operating procedures. The problem becomes even worse in warm and humid regions. Aflatoxin-producing *Aspergillus* species like *A. flavus* is reported to cause huge losses in terms of contamination of agriculture and feed ingredients (Sohrabi and Taghizadeh, 2018). When dairy animals eat aflatoxin contaminated feed, they secrete AFM1 in milk, which is a carcinogenic aflatoxin (Giovati *et al.,* 2015). Because, animal feed is

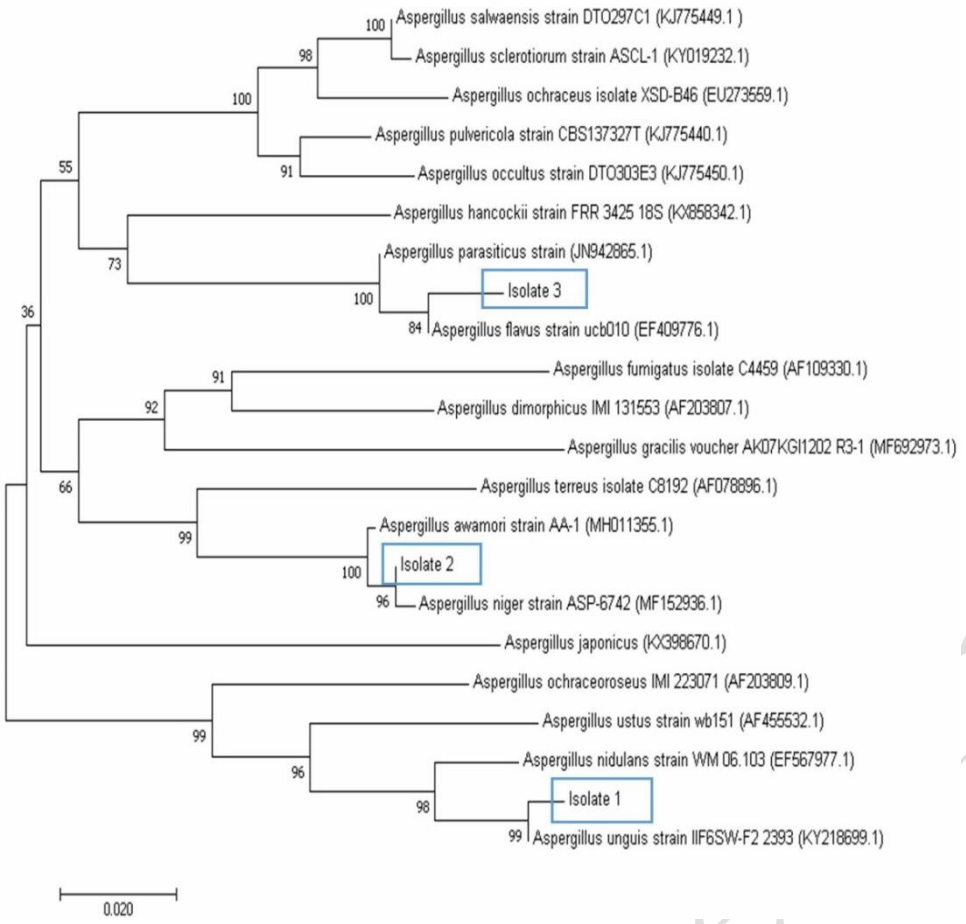

Fig. 4: Phylogenetic tree reconstruction by using MEGA 7 software among ITS sequences retrieved from GenBank-NCBI and sequenced fungal isolates. The confidence level of each branch and stability of the relationship was tested by bootstrap analysis, by performing 1000 re-sampling for the tree topology of the Neighbour-Joining data. Isolates obtained in current study are shown within boxes.

the major source of aflatoxins, so it is necessary to develop reliable screening methods for toxigenic *A. flavus* and to develop it's biological control to minimize the risks. The current study used a polyphasic approach for the identification of toxigenic *A. flavus* comprising morphological and molecular characterization. The Aspergillus differentiation media (AFPA) was used for the determination of culture characteristics. The AFPA is a selective media that identifies various *Aspergillus* species and gives the distinct characteristic color of many *Aspergillus* species especially *A. flavus, A. parasiticus* and *A. niger*. Many researchers have previously identified *Aspergillus flavus* using AFPA (Abriba *et al*., 2013; Fakruddin *et al.,* 2015; Hossain *et al*., 2018). In the current study, one of the isolates was shown to be *A. niger* because it produced pale yellow to white hyphae, turning to dark brown to black on AFPA, which is in agreement to the previous results (Abriba *et al.,* 2013). Similarly, another isolate showed dark green to brown color hyphae which is the characteristic feature of *A. unguis* (Hamano *et al.,* 1992). Whereas, the *A. flavus* showed characteristic velvety to the woolly culture of yellow to green color (especially at center), with a white border surrounding the yellow to the greenish surface. On the reverse side of the AFPA culture plate, it also showed characteristics bright orange color. Though, AFPA was previously used for the identification of *Aspergillus flavus* (Hossain *et al*., 2018) yet the toxigenic potential is not usually determined by this media. While, to determine the toxigenic potential of *A. flavus*, APA media is used which shows a zone of toxin formation (in case of toxigenic fungus) under UV light (365nm). Sekar *et al.* (2008) had identified aflatoxigenic species (*Aspergillus flavus*) using the same APA media and confirmed it by TLC. In the current study, the identified isolates were grown on APA media to determine the toxicity of species, as described previously (Fakruddin *et al.,* 2015). Out of three identified species, only *A. flavus* was shown to be aflatoxigenic. Though culture characteristics and morphological identification of atoxigenic and toxigenic species provide precise results yet false positive results are common (Sudini *et al.,* 2015). Therefore, the toxigenic *A. flavus* was grown on corn grains and the sample was analyzed through HPLC to determine aflatoxin production which was shown to be positive for AFB1. Fente *et al*., 2001 and Rodrigues *et al*., 2009 have also reported a positive corelation between the observation of blue flourescence on culturing media and aflatoxin detection on HPLC. In a study Hossain *et al*., 2018 have reported 12.052 ppb to 68.674 ppb aflatoxin level in 75% of collected raw red chilli samples contaminated with *A. flavus*.

The taxonomic and ITS-based molecular characterization of the isolates was performed. ITS is a spacer DNA that is located between small subunit rRNA to large subunit rRNA. Sequencing-based on ITS regions is very common in the molecular phylogenetic system (Makhlouf *et al*., 2019) due to multiple reasons including ease in identification even in small quantities of DNA, smaller amplicon size and enhanced variability in closely related species. All these features make ITS based sequencing more reliable. Molsems and co-workers used molecular methods for the identification of *Aspergillus* from coffee beans (Moslem *et al.,* 2010). Likewise, Saleemi and co-scientists (2012) also described the

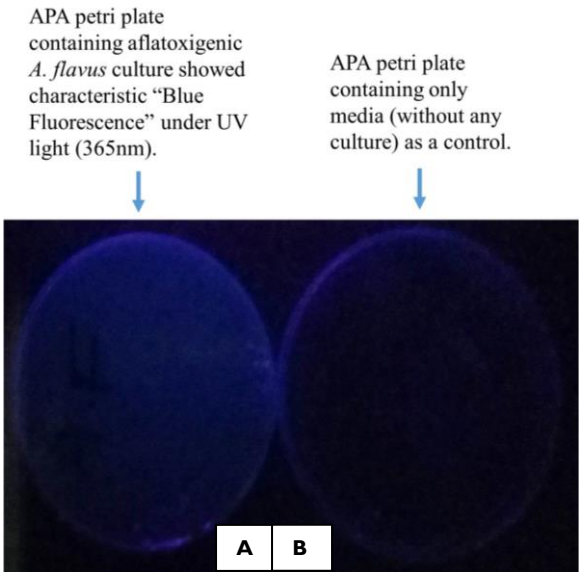

Fig. 5: Determination of aflatoxin production under UV light (365nm) on APA media. **A**: Characteristic "Blue flourescence" as shown by aflatoxigenic *A. flavus*. **B**: Control APA media plate (without fungal culture) did not exhibit "Blue flourescence".

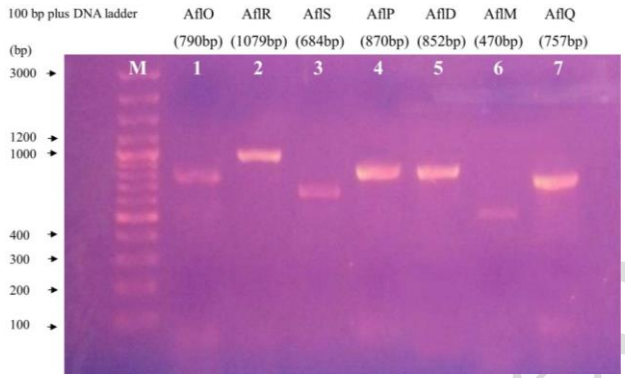

Fig. 6: Aflatoxin biosynthesis gene profiling of *Aspergillus flavus*, determined on 1.5% agarose gel. M: 100 bp plus DNA ladder; PCR amplicons of 1: *aflO* (790bp), 2: *aflR* (1079bp), 3: *aflS* (684bp), 4: *aflP* (870bp), 5: *aflD* (852bp), 6: *aflM* (470bp) and 7: *aflQ* (757bp) genes.

molecular identification of *Aspergillus* species based on ITS regions and suggested it as a best approach for identification of fungal species. Similarly, our findings will be helpful to analyze local biodiversity of aflatoxigenic strains and can contribute in the development of a robust molecular method to screen the food/feed samples for the presence of toxigenic fungi.

In the study, an evolutionary tree based on ITS region sequences of isolates was constructed using the Neighbor-Joining method and the confidence level of each branch was verified by bootstrap analysis, by executing 1000 re-sampling (for the tree topology of the neighbor-joining data) which also confirmed the stability of relationship (Costa *et al.,* 2011). The analysis comprised of 22 ITS sequences and evolutionary distances were calculated by "the Maximum Composite Likelihood method". Any uncertain positions were ignored for each pair of sequence so that final data set included total 711 positions. The evolutionary tree showed that isolate-1 closely relates to *A. unguis*, while isolate-2 and 3 are related to *A. niger* and *A. flavus*, respectively. Similar phylogenetic relationships based on ITS and IGS regions of the nuclear rRNA gene complex of fungus have been reported (Krimitzas *et al.*, 2013). Significant efforts have also been reported by Chiba and co-workers (2014) in which they developed phylogenetic tree analysis of aflatoxigenic and other non-aflatoxigenic species of *Aspergillus* group based on ITS sequences. The study was based on morphological identification of strains followed by phylogenetic tree analysis for identification of aflatoxigenic and non-aflatoxigenic species. Chiba and co-workers concluded that findings of phylogenetic tree analy1sis of tested strains were in consistent to the results of morphological methods.

Moreover, set of seven primers specific to major regulatory and structural genes involved in aflatoxins synthesis pathway were used to analyze *A. flavus* strain GCUF-BNB01 biosynthesis pathway. All the targeted genes were found to be present within the isolated strain. This finding corelates with findings of another study by Davari and co-workers (2015) in which they had shown targeted quadruplet pattern of aflatoxin genes and found all four toxic genes in molecular characterization. Similarly, Gallo and co-workers (2012) studied seven aflatoxin synthesis genes in toxigenic strains of *A. flavus* and found a similar pattern of genetic pathway though some species were devoid of the complete set of genes. The reasons of variation may be the inclusion of false positive results. Similarly, the results of gene profiling pathway of the current study are in accordance with the study conducted by Fakruddin *et al.* (2015) with a difference that some of *A. flavus* strains exhibited major aflatoxigenic genes but no aflatoxins production. So, despite the presence of aflatoxin gene amplicons, these *A. flavus* isolates was found to be negative for aflatoxin production. In this regard, our study differs with their findings, as our studied strain was found to be involved in aflatoxins biosynthesis as confirmed by zone of aflatoxin production on APA media observed under UV light (365nm) and HPLC analysis. Fakruddin *et al.* (2015) have also reported that despite the absence of targeted aflatoxigenic genes, some strains showed aflatoxin production under lab conditions. Okoth *et al.* (2018) have also observed that aflatoxin production potential was not consistent with the presence of aflatoxigenic genes. However, this indistinctness was assumed to be observed due to the inability of primers to amplify targeted genes or presence of genetic mutations.

The study of fungal genetic pathway and variations at the molecular level are of high interest in biological control for reducing mycotoxin contamination risks. Biocontrol strategies for fungal toxins can be developed, as such procedures in agriculture crops are already in process. Further studies may be conducted to extend a complete classification system of atoxigenic and toxigenic fungal species based on aflatoxin biosynthesis gene profiling.

Conclusions: Aflatoxin biosynthesis genomics and cluster variability among *A. flavus* population provide a new avenue in understanding the mechanisms governing aflatoxin synthesis as well as associated toxicological risks. Regular monitoring of toxigenic mycoflora, reliable data and sufficient scientific information are pre-requisite to develop effective strategies to identify and control the

toxigenic fungi in food and feed. Therefore, the envisaged study used a polyphasic approach for the identification of toxigenic *Aspergillus* species involved in feed ingredient contamination and their aflatoxigenic gene profiling, to scrutinize possible genetic pathway of aflatoxin production. This will ultimately lead to prevent the human population from the health hazards of aflatoxins and the development of target-specific fungal growth inhibitors.

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Authors contribution: The study planning and data analysis were carried out by MRJ, MAM and TH. The study execution and initial manuscript drafting was carried out by MU. Proofreading and final revision were carried out by MRJ and AI. All authors have interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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