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ISOLATION AND IDENTIFICATION OF *Aspergillus flavus* FROM POULTRY FEED SAMPLES USING COMBINED TRADITIONAL-MOLECULAR APPROACH AND EXPRESSION OF CYP64A1 AT mRNA LEVEL

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The research work was aimed for isolation and identification of *Aspergillus flavus* from poultry feed samples using conventional and molecular techniques. Out of 23 samples, 8 (34.78%) were found positive for *A. flavus*. The molecular techniques comprise comparison of ITS1-5.8S-ITS2 region. In addition, the ability of the isolates to express aflatoxin synthase (CYP64A1) gene in synthetic liquid medium was also analyzed. Based on cultural characteristics, unique nature of the ITS 1 and 2 genes and expression of CYP64A1 at mRNA level, *A. flavus* was accurately identified from the feed samples. The isolated *A. flavus* would be a potential candidate for further studies and application.
Keywords: *Aspergillus flavus*, aflatoxins, CYP64A1, feed sample, internal transcribed spacer

INTRODUCTION

Aspergillus flavus is an important fungal species which may occurs in foods and feeds producing a number of toxins including aflatoxins being the most relevant with food safety. The aflatoxinogenic fungi can contaminate several food commodities including cereals (Pittet, 1988; Sultana *et al*., 2013), peanuts (Jelinek *et al*., 1989), spices (Bartine and Tantaoui-Elaraki, 1997) and figs (Färber *et al*., 1997). The starchy foods and feeds are especially susceptible of colonization by *Aspergillus* species where they may produce aflatoxins along several stages of the food chain: either at pre-harvest, processing, transportation or storage (Ellis *et al*., 1991). The level of infestation by mold-spore and the identification of the species are important indicators of the quality of the raw material and predict the potential risk for the presence of mycotoxins (Shapira *et al*., 1996; Muhammad *et al*., 2012; Khan *et al*., 2013).

Aflatoxin producing fungi are an important group of food-borne fungi. This group includes *A. flavus*, *A. parasiticus* and *A. nomius*. On the other hand, *A. oryzae*, an industrially useful fungus, is recognized as safe since its biosynthesis pathway of aflatoxin is proved to be silent (Nazir *et al*., 2010; Kiyota *et al*., 2011). Cytochrome P450 64A1 (CYP64A1) is a gene homolog involved in the aflatoxin biosynthesis pathway in *A. flavus*, *A. parasiticus* and *A. nomius* (Prieto and Woloshuk, 1997). Though *A. flavus* can be differentiated from other aspergilli by morphology and/or molecular studies, it is very difficult to distinguish from *A. oryzae* as morphological characteristics of these two fungi are similar. Moreover, discrimination of these two fungi using PCR method is virtually impossible as DNA relatedness between them was found to be almost 100% (Kurtzman *et al*., 1986; Kurtzman *et al*., 1987; Saleemi *et al*., 2012).

The traditional methods for identification and detection of the fungi are time-consuming, laborious, and require facilities and mycological expertise (Edwards *et al*., 2002). Also, these methods have low degree of sensitivity and do not allow the specification of mycotoxigenic fungal species (Zhao *et al*., 2001). The highly variable regions, internal transcribed spacer (ITS) from the rDNA units, are widely used for phylogenesis and diagnostics of closely related fungi such as *Aspergillus* (Henry *et al*., 2000; Parenicová *et al*., 2001; Varga *et al*., 2004; González-Salgado *et al*., 2005; Patino *et al*., 2005; González-Salgado *et al*., 2008) or *Fusarium* (González-Jaén *et al*., 2004). ITS sequence, however, is not unique for discriminating *A. flavus* from *A. oryzae*. To overcome the problem, expression of CYP64A1 at mRNA level can be assessed. Here, we applied a combined traditional and molecular approach and expression of CYP64A1 in synthetic medium at mRNA level for accurate identification of *A. flavus*, which allowed us to investigate the prevalence of *A. flavus* in feed samples.

MATERIALS AND METHODS

A total of 23 feed samples were collected from commercial poultry farm according to the procedure mentioned by Klich and Pitt (1988). The feeds were treated as ready-to-serve. Ten grams of each sample was blended with 90 ml of autoclaved distilled water and an aliquot inoculated in

triplicate on to potato dextrose agar (PDA). The PDA plates were incubated at 30ºC for 7-10 days. Colonies representative of *A. flavus* were sub-cultured again on to PDA (Samson *et al*., 2004). Primary identification was made based on cultural, morphological and microscopic characteristics (Klich and Pitt, 1988; Singh *et al*., 1991; Pitt and Hocking, 1997). The plates were incubated at 30ºC in dark for 10 days. Besides, the fungal isolate was also cultured on PDA slants, incubated at 30ºC for 10 days and stored at 4ºC in cold room for future studies.

Extraction of DNA from fungi was performed following the needle inoculation of 50 ml of Potato Dextrose (PD) broth (Difco Laboratories, Becton, Dickinson and Company, Sparks, MD 21152, USA) with conidia from a 7-day culture in PDA and incubation under shaking condition (120 rpm) for 72 h at 30°C. The hyphae were recovered on a 0.45-mm-pore-size filter and washed with distilled water. Aliquots of the fungal hyphae were stored frozen at -80°C until use. The DNA was extracted following the method described previously (Chow and Kafer, 1993) with some modifications. In brief, prior to lysis, about 1 gm of hyphae was thawed and grinded in liquid nitrogen using pestle and mortar, which was then transferred to microcentrifuge tube (1.5 ml) and suspended in 660 µl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol). After vortexing, the microcentrifuge tube containing the grinded powder and buffer was incubated at 65°C in water bath for 1 h. Following lysis, DNA was extracted using phenol-chloroform. The DNA was eluted with 300 µl distilled water and 1 µl of RNase (100 mg/ml) was added and incubated at 65°C for 15 min. The purified DNA was stored at -20°C until used.

The oligonucleotide primers described previously (González-Salgado *et al*., 2008) were used for amplification of ITS regions. The ITS region specific primers were: FLA1 (5´-GTAGGGTTCCTAGCGAGCC-3´) and FLA2 (5´-GGAAAAAGATTGATTTGCGTTC-3´). For assessing the expression of CYP64A1 at mRNA level, the fungal cells were grown with shaking (120 rpm) at 30°C for 5 days under aerobic conditions. Then, total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN). The RNA was treated with DNase I (Takara), and first-strand cDNAs were synthesized with QuantiTect Reverse Transcription Kit (QIAGEN) according to the instructions of the manufacturer. For PCR amplification of CYP64A1, gene specific primer set (CYP64A1-F, 5´-GTACTATCGTCACTTGCCTTCCAC-3´ and CYP64A1-R, 5´-GCAATACAGCGAATATGTATGTCTA-3´) was used as reported by Nazir *et al*. (2010). All the primers were purchased from the Cosmo Genetech Co, Ltd., South Korea. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized on a UV-transilluminator. The PCR assay was performed in a total reaction volume of 50 µl consisting of 5 µl of 10x PCR buffer (100 mM Tris-HCl [pH 9.0], 15 mM MgCl2, 500 mM KCl, 1.0% Triton X-100); 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP) mix and 10 pmol of each primer. The PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Using the FLA1 and FLA2 primers, the purified PCR products were directly sequenced by automatic DNA sequencer (ABI 3730XL; Applied Biosystem) using BigDye® Terminator v3.1Cycle Sequencing Kit following the instructions of the manufacturer. Sequence comparisons of referenced strains and isolated *Aspergillus* (Fig. 1) were made using ClustalX and MEGA (version 5.2) softwares. Sequences from referenced isolates were aligned to complete or partial ITS sequences available in GenBank. Comparison of sequences from referenced isolates, feed sample isolate, and GenBank sequences was performed using a non-gapped, advanced BLASTn search. The sequence of ITSI-5.8S-ITS2 region of the isolate isolated from feed sample was deposited in GenBank (accession no. KC495618). The ITS sequences of related fungal strains and the accession numbers (in parenthesis) obtained from GenBank database were used for analysis are as follows: *A. flavus* ATCC 200026 (JX535495), *A. oryzae* QM-M004/12 (KC341712), *A. parasiticus* CS21 (JF412787), *A. nomius* AsFL-07 (JX235353) and *A. fumigatus* ATCC 36607 (HQ026746).

RESULTS AND DISCUSSION

Based on morphological studies and multiple alignments of ITS regions, the fungus was primarily identified as *A. flavus*. The primarily identified fungus was inoculated into synthetic culture media as described previously (Kirk *et al*., 1978). A total of 23 feed samples were tested of which 8 (34.78%) were primarily identified as positive for *A. flavus* based on colony pigmentation and morphology of the conidial head. After a 7-day culture, colonies on PDA at 30°C were olive to lime green with a cream reverse. The isolates were used for molecular studies and all the eight isolates were successfully validated for the expression of CYP64A1 at mRNA level.

The prevalence of *A. flavus* found in this study was in line with the findings of Bokhari (2007) and Jabeen *et al*. (2012). The prevalence recorded in this study was high although the feed samples were of ready-to-serve and the storage period was within expire date. The high prevalence of *A. flavus* is largely depends on long time storing in poor condition and unhygienic preparation, and its high adaptability to growth substrates in a wide range of environment and the production of spores (conidia) that remain viable even under extremely harsh conditions (Saleemullah *et al*., 2006). Most prevalent fungi in pre- and post-storage were *Aspergillus* (mostly *A. flavus*), *Fusarium* and *Penicillium* and their counts increased with increasing of storage period (Islam *et al*., 2005; Youssef *et al*., 2008; Azarakhsh *et al*., 2011).

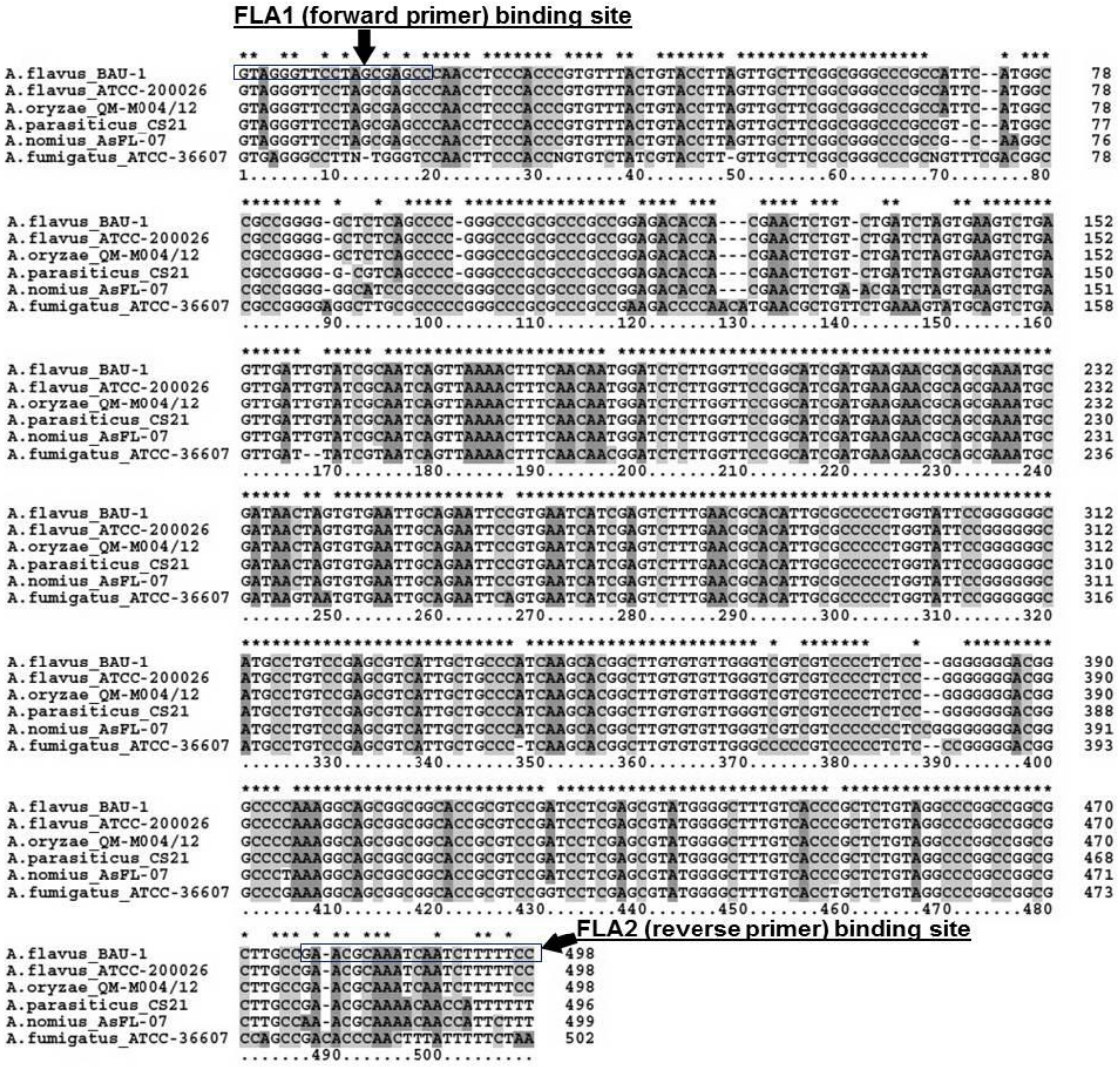

Figure 1. Nucleotide sequence alignment of *A. flavus* BAU-1 ITS1-5.8S-ITS2 regions (GenBank accession no. KC495618) with other intra- and interspecies fungal strains.

The FLA1 binds properly in the 5` end of the region except in *A. fumigatus*. However, the FLA2 binds with the 3` end region present in *A. flavus*. Thus, this set of primer differentiates *A. flavus* from other aflatoxin producing *Aspergilli* (González-Salgado *et al*., 2008). *****indicates the conserved bases among the nucleotides.

Because of high specificity and sensitivity, PCR-based methods are considered as a good alternative for rapid diagnosis, which have been used for the detection of *A. flavus* and *A. parasiticus* (Shapira *et al*., 1996; Sweeney *et al*., 2000; Chen *et al*., 2002; Mayer *et al*., 2003; Somashekar *et al*., 2004). However, none has yet been able to reliably differentiate *A. flavus* from other species of the *A. flavus* group. González-Salgado *et al*. (2008) developed a highly sensitive PCR-based detection method specific for *A. flavus*, which can discriminate the fungus from several other *Aspergilli* but the method is not applicable for differentiating the fungus from *A. oryzae*. We developed an alternate method for the first time that can be used for accurate identification of *A. flavus* discriminating *A. oryzae* using a combined traditional-molecular approach and expression of CYP64A1 at mRNA level. Amplified fragment length polymorphism (AFLP) can also be used to differentiate *A. flavus* from *A. oryzae* (Lee *et al*., 2004), however, the method needs more expertize in experiments and interpretation. Amplification of the ITS1-5.8S-ITS2 regions from the isolated *A. flavus* strains generated a PCR product size of 498-bp. After sequencing the regions, the nucleotide has been deposited in the GenBank (accession no. KC495618). Based on the multiple alignments, our isolated

A. flavus strain was found to be 100% similar to both *A. flavus* ATCC 200026 and *A. oryzae* QM-M004/12. On the other hand, considerable differences in gene similarities among some other species observed (Fig. 1); the sequenced region was 99, 96, and 90% similar to the corresponding region in *A. parasiticus* CS21, *A. nomius* AsFL-07 and *A. fumigatus* ATCC 36607, respectively.

For differentiating *A. flavus* from *A. oryzae*, a synthetic liquid culture medium under nitrogen-limited condition was used in which a series of cytochrome P450 genes of different filamentous fungi were expressed at mRNA level (Matsuzaki and Wariishi 2004; Nazir *et al*., 2010; Ide *et al*., 2012). Beside the nitrogen-limited synthetic medium, these authors used several other media such as Potato Dextrose broth, Yeast-extract Peptone Dextrose (YPD) medium, synthetic liquid culture medium under high-nitrogen condition etc., but CYP64A1 was not expressed. We used the same primers, culture condition and PCR reaction condition as reported by Nazir *et al*. (2010), and the gene was amplified (data not shown), which confirms that the fungal isolate was not *A. oryzae*. Thus, the isolated fungus was identified as *A. flavus*. After confirmatory identification of the fungus, the strain has been named as *Aspergillus flavus* BAU-1 and kept at the Yeungnam University, South Korea as a stock culture.

Conclusion**:** *A. flavus* was successfully isolated and identified from poultry feed sample in Bangladesh. This study describes a novel method for isolation and accurate identification of *A. flavus* using a combined traditional and molecular approach and expression of CYP64A1 gene at mRNA level in a synthetic liquid culture medium under nitrogen-limited condition. The findings observed here highlight a potential risk of poultry feed getting contaminated with potentially infective *A. flavus* in Bangladesh, thus making it for further analysis and continual monitoring and evaluation of feeds.

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