Molecular Approaches for Characterization of Aflatoxin Producing Aspergillus flavus Isolates from Poultry Feed

Saba Sana1, Aftab Ahmad Anjum1*, Tahir Yaqub1, Muhammad Nasir2, Muhammad Asad Ali1 and Mateen Abbas3

1Department of Microbiology; 2Department of Food Science and Human Nutrition; 3Quality Operations Laboratory, University of Veterinary and Animal Sciences Lahore Pakistan

*Corresponding author: aftab.anjum@uvas.edu.pk

ABSTRACT

Aflatoxins are secondary toxic metabolites produced by aspergilli. Aspergillus flavus is one of the major aflatoxins producing specie. Present study was conducted to enumerate mycoflora of poultry feed and aflatoxin production potential of A. flavus. Home mixed and commercial poultry feed (n=20, each) were processed for determination of fungal load and isolation of mycoflora. Isolates were identified by culture and microscopic characters. Thin layer (TL) and high-performance liquid chromatography (HPLC) were used for screening, identification and quantification of aflatoxins produced by A. flavus respectively. A. flavus were confirmed by specie specific polymerase chain reaction (PCR). Isolation frequency of different genera, Aspergillus species and toxigenic A. flavus was calculated. The fungal count in home mixed feed was 2x10^6 to 1.6x10^7 CFU/g whereas, in commercial poultry feed from 2x10^3 to 6x10^4 CFU/g. Aspergillus was the most prevalent genus in home mixed and commercial feed followed by Mucor. Among aspergilli, the highest percentage was of flavus (95%) followed by A. niger (75%), A. fumigatus (15%) and A. terreus (5%). A total of 32.61 percent (223/685) aflatoxin producing A. flavus from commercial and 16.67 percent (23/140) from home mixed feed were detected by TLC. These aflatoxins (AFs) were identified as AFB₁ and AFB₂ and AFG₁ by HPLC. Amplicon (500 bps) of A. flavus was observed on 2 percent agarose gel. It was concluded that poultry feed may be a source of transmission of disease producing fungi and aflatoxins to poultry birds and human beings.

INTRODUCTION

Filamentous fungi of genus Aspergillus, Penicillium, Fusarium Claviceps, Alternaria and Stachybotrys are producers of low molecular weight secondary metabolites; toxic to human, animals and birds. These metabolites are termed as Mycotoxins. Among several mycotoxins the most important ones are Ochratoxins, Zearealenone, Deoxynivalenol, Fumonisin, T-2 toxin and Aflatoxins (Fokunang et al., 2006). Aflatoxins are difuranocoumarin derivatives of polyketide pathway, generally activates in A. flavus and A. parasiticus under certain physico-chemical conditions (Silva et al., 2004). A. flavus are present in air, soil and thus commonly isolated from feed material. Under improper storage of feed, A. flavus produces aflatoxins up to toxic level (Ghaemmaghami et al., 2016). Aflatoxins are classified as AFB₁, AFB₂, AFG₁ and AFG₂ based upon natural ability to fluoresce under Ultra Violet (UV) light and chromatographic mobility by thin layer chromatography. Among four; AFB₁ is the most potent carcinogen and frequently produced toxin (Naseem et al., 2018). The contamination of feed with toxin producer fungi causes economic losses not only to farmer but also to consumer (Morrison et al., 2017).

Ingestion of feed contaminated with aflatoxins develops aflatoxicosis in poultry. Aflatoxicosis is an intoxication which may occur either by high exposure over shorter time called as acute aflatoxicosis or low exposure over longer time known as chronic aflatoxicosis (Gong et al., 2016). Aflatoxins reduced weight gain, disturbances in muscle arrangement and bone deformities in poultry (Morrison et al., 2017). Aflatoxins enter human food chain through poultry meat and cause public health problems.
The toxic actions of mycotoxins in human are characterized by carcinogenicity, mutagenicity, teratogenicity, and estrogenic properties resulting in hepatocellular carcinoma, impaired child growth, allergic response and immune suppression (Greco et al., 2014; Gong et al., 2016).

As poultry feed may be carrier of toxigenic fungi therefore regular monitoring of feed for presence of toxigenic fungi is important to ensure food safety and stop its transmission to human food chain. A study was conducted in which poultry feed was assessed for mycological quality and toxin producing A. flavus were characterized among isolated species.

MATERIALS AND METHODS

Poultry feed samples were processed to determine mycological quality of feed. Among isolated fungi, A. flavus isolates were evaluated for Aflatoxin production. Aflatoxins were characterized and production potential of A. flavus isolates was determined.

Poultry feed samples: Poultry feed samples (n=40) were collected from poultry feed market in and around Lahore (1Kilo gram, each) in sterile polythene bags and transported to Mycology Laboratory, Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan (Greco et al., 2014).

Fungal load: Fungal load was determined using spread plate technique. Ten percent stock suspension of poultry feed was prepared by adding 25 g of poultry feed in 225 mL peptone water (0.1%) with constant shaking. Ten-fold serial dilutions (10⁻² to 10⁻⁴) were prepared from this suspension. One mL from each dilution was spread on Sabouraud’s dextrose agar (SDA) plates followed by incubation at 25°C±3°C for a period of 3 days (Ghaemmaghami et al., 2016). The colony forming units (CFU) were counted. The isolation frequency of different genera and Aspergillus specie and aflatoxin producing A. flavus was calculated (Greco et al., 2014). Mycological quality of poultry feed was determined based on provided criterion (Gimeno et al., 2002).

Mycoflora identification: Fungi were purified on SDA by single spot technique. Purified cultures were identified based on macroscopic and microscopic features. Macroscopic features recorded were colony color from obverse and reverse side of purified growth of fungi on SDA plate, colony texture and production of diffusible pigments. Macroscopic features; types and arrangements of spores and types of hyphae; were observed by agar drop slide culture. Lacto phenol cotton blue stained slide cultures were observed under microscope at 100 and 400X magnification (Gilman, 1957).

Screening for aflatoxin producing Aspergillus flavus: Toxigenic potential of A. flavus was determined by Thin layer Chromatography. Autoclaved, homogenized 12.5 g fungal culture (45 days old) was mixed with extractant (Chloroform 45mL; Methanol 5mL; NaCl 5mL and Distilled H₂O 5mL). Mixture was placed at 37°C for 30 minutes with constant stirring followed by filtration through whatman filter paper. The filtrate was evaporated, crystals obtained were crushed and dissolved in one mL chloroform. The crude extracts were passed through membrane filter (pore size 0.2µm) and 20µL spotted on silica gel coated thin layer chromatographic aluminum sheet along with aflatoxin standards. It was placed in chromatographic tank using Chloroform and acetone as mobile phase (95:5). Chromatograms were observed in UV lamp at 365 nm wavelength.

Characterization of aflatoxins by high performance liquid chromatography: Aflatoxins produced by A. flavus were identified and quantified by HPLC following the procedure described by Alvarado-Hernández et al. (2016) against reference aflatoxin standards. HPLC was performed using C8 column with injection volume of 100µL, acetonitrile; methanol and water (20:20:60 v/v) as mobile phase at a flow rate of 0.8 mL per min.

Confirmation of Aspergillus flavus: Identified toxin producing A. flavus were confirmed by Polymerase chain reaction (PCR) using specie specific primers (FLA1 5′-GTAGGGTTCCTAGCGAGCC-3′) and FLA2 (5′-GGAAAAAGATTGATTGCGTTC-3′). DNA was extracted using Plant DNA extraction Kit. Specific PCR was carried out using method of González-Salgado et al. (2008). A reaction volume of 25µL was prepared and amplified by providing one cycle of initial denaturation at 95°C for 5 minutes and 26 cycles at 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds followed by 1 cycle at 72°C for 5 minutes (González-Salgado et al., 2008).

Statistical analysis: For statistical analysis, Log₁₀ of fungal counts were calculated and mean Log₁₀ were compared using Statistical Package for social sciences (SPSS version 20) with five percent significance level (α=0.05). Isolation frequency of fungal genera, Aspergillus species and toxigenic A. flavus was calculated.

RESULTS

Mycological quality of poultry feed: The highest fungal counts recorded were 1.6x10⁴ (Log₁₀ 4.21) and 6x10³ CFU/g (Log₁₀ 3.77) in home mixed and commercial feed, respectively. Similarly, the lowest CFU/g were 2x10⁵CFU/g (Log₁₀ 2.30) in home mixed and 2x10¹ (Log₁₀ 1.30) in commercial feed. Mean Log₁₀ of home mixed (3.24±0.69) and commercial feed (2.97±0.54) differed non-significantly with p value 0.178 (>0.05) as shown in (Table 1). All feed samples from both categories were of good quality showing less than 3.10⁴CFU/g.

Isolation frequency of fungal genera and species: Different fungal genera and Aspergillus species were identified on basis of macroscopic and microscopic features (Fig. 1a and 1b). The highest frequency of genus Aspergillus was recorded in home mixed and commercial feed. Two genera isolated from home mixed feed were Aspergillus and Mucor, whereas, from commercial feed Aspergillus, Mucor, Penicillium and Cladosporium recovered. Aspergillus was present in 20/20 (100%) home mixed and 19/20 (95%) commercial feed samples and
Macrocotic and microscopic identification of  Aspergillus species (A): Aspergillus fumigatus; (B) Aspergillus niger; (C) Aspergillus flavus; (D) Aspergillus parasiticus; (E) Aspergillus terreus: (F) Microscopic view of Aspergillus.

Characterization of Aflatoxin producing Aspergillus flavus: A total of 685 and 140 A. flavus were isolated from home mixed and commercial feed respectively. These isolates were characterized for aflatoxin production and confirmed as A. flavus by Polymerase chain reaction. Thin layer chromatography was performed for screening of Aflatoxin producing A. flavus (Fig. 2). It was revealed that 16.67 percent isolated A. flavus from home mixed feed and 32.61 percent from commercial poultry feed respectively showed bands with blue or green fluorescence under UV light were considered positive for aflatoxin production. The type of aflatoxins giving fluorescence were identified by HPLC.

Aflatoxins were identified and quantified by HPLC. Among toxigenic isolates of home mixed A. flavus 6.25 percent produced both AFB1; and AFB2; 6.25 were AFB1 and 10.42 percent of these were AFG1 producers. Among toxigenic A. flavus isolated from commercial feed 17.4% were producers of two Aflatoxins (AFB1; and AFB2); 10.9 percent produced only one aflatoxin (AFB1) and 2.2 percent produced three toxins (AFB1; AFB2 and AFG1). The AFB1 was quantified in range of 0.22ng to 11622.24 ng by HPLC. While AFB2 and AFG1 ranged 0.14 to 9016 and 0.33 to 40.12ng respectively. HPLC chromatogram of standard and sample are in (Fig. 3). The results obtained from HPLC were correlated with TLC results.

Table 1: Fungal load of home mixed and commercial poultry feed (n=40)

| Sr. No. | Home mixed CFU/g | Log10 CFU/g | Mean Log10 CFU/g | S.D. | Commercial CFU/g | Log10 CFU/g | Mean Log10 CFU/g | S.D. |
|--------|------------------|------------|-----------------|------|------------------|------------|-----------------|------|
| 1      | 7.6 x 10^3       | 3.88       | 3.24±0.69       | 3.34 | 2.97±20.54*     |            |                 |      |
| 2      | 3.4 x 10^3       | 3.53       | 1.0 x 10^3      | 3.00 |                  |            |                 |      |
| 3      | 7.4 x 10^3       | 3.86       | 1.1 x 10^3      | 3.04 |                  |            |                 |      |
| 4      | 4.6 x 10^3       | 3.66       | 1.8 x 10^3      | 3.25 |                  |            |                 |      |
| 5      | 4.5 x 10^3       | 3.65       | 2.6 x 10^3      | 3.41 |                  |            |                 |      |
| 6      | 4.4 x 10^3       | 3.64       | 2.9 x 10^3      | 3.46 |                  |            |                 |      |
| 7      | 3.0 x 10^3       | 3.47       | 9.0 x 10^2      | 2.95 |                  |            |                 |      |
| 8      | 1.0 x 10^4       | 4.00       | 7.0 x 10^3      | 2.84 |                  |            |                 |      |
| 9      | 6.5 x 10^3       | 3.81       | 5.0 x 10^3      | 2.69 |                  |            |                 |      |
| 10     | 9.2 x 10^3       | 3.96       | 6.0 x 10^3      | 3.77 |                  |            |                 |      |
| 11     | 5.7 x 10^3       | 3.75       | 7.0 x 10^2      | 2.84 |                  |            |                 |      |
| 12     | 1.64 x 10^4      | 4.21       | 1.0 x 10^4      | 3.00 |                  |            |                 |      |
| 13     | 6.7 x 10^3       | 2.77       | 3.8 x 10^3      | 3.57 |                  |            |                 |      |
| 14     | 6.0 x 10^3       | 2.30       | 1.3 x 10^3      | 3.11 |                  |            |                 |      |
| 15     | 2.0 x 10^3       | 2.90       | 1.2 x 10^3      | 3.07 |                  |            |                 |      |
| 16     | 8.0 x 10^3       | 2.47       | 1.0 x 10^3      | 3.00 |                  |            |                 |      |
| 17     | 3.0 x 10^3       | 3.00       | 7.0 x 10^3      | 2.84 |                  |            |                 |      |
| 18     | 1.0 x 10^4       | 2.77       | 8.0 x 10^2      | 2.90 |                  |            |                 |      |
| 19     | 6.0 x 10^3       | 2.77       | 2.0 x 10^3      | 1.30 |                  |            |                 |      |
| 20     | 6.0 x 10^3       | 2.77       | 1.0 x 10^3      | 2.00 |                  |            |                 |      |

*Mucor* was found in 5/20(25%) home mixed and 9/20 (45%) commercial feed. *Penicillium* and *Cladosporium* present in 3/20 (15%) and 1/20 (5%) commercial feed samples and absent in home mixed feed. Among Aspergilli isolated from home mixed feed, *A. flavus* was found in 19/20 (90%) followed by *A. niger* 15/20(75%), *A. fumigatus* 3/20(15%) and *A. terreus* 1/20(5%). No home mixed feed sample was positive for *A. parasiticus*. Commercial feed showed growth of *A. flavus* in 15/20 (75%), *A. niger, 13/20* (65%), *A. fumigatus* 9/20(45%), *A. parasiticus* 2/20 (10%) and *A. terreus* 1/20 (5%). Commercial feed showed more diversity of fungi as compared to home mixed poultry feed (Table 2).
Confirmation of aflatoxin producing *Aspergillus flavus*: Amplified products were loaded on two percent agarose gel along with 100 base pair DNA ladder. Amplicon of 500 base pairs appeared on gel considered as desired product and confirmed as *A. flavus* (Fig. 4).

**DISCUSSION**

The objective of present study was to evaluate poultry feed for mycological quality and the risk associated with presence of aflatoxin producing of *A. flavus*. Moulds can be true pathogens, opportunistic pathogens and toxigenic. Samples are categorized as good, bad and acceptable based upon level of fungal contamination. So, characterization of fungi is important along with fungal count.

Presence of fungi in poultry feed is a public health issue. Moulds (filamentous fungi) not only affect the organoleptic properties of feed but some are toxin producers (Greco et al., 2014). In present study, mould counts in home mixed (1.64 x 10⁸) and commercial feed (6.0 x 10⁷ CFU/g) were slightly lower than counts in poultry feed (6.5x10⁷ CFU/g) in Iraq (Shareef, 2010), counts (42 x 10⁶ CFU/g) in Nigarian poultry feed (Kehinde et al., 2014), fungal load (0-14 x 10⁴ CFU/g) in Serbia’s poultry feed (Krnića et al., 2014), counts of fungi (8.1 x 10⁵ CFU/g) in Nigarian bird’s feed (Matthew et al., 2017) and higher than fungal counts (3x10² - 4x10⁴ CFU/g) in poultry feed in Quetta, Pakistan (Rashid et al., 2020). No doubt all poultry feed samples (n=40) of present study were categorized as good (Gimeno et al., 2002) in contrast to above mentioned studies. Similarly, Greco et al. (2014) reported contradictory results and observed 56 percent poultry feed (26/46) as good, 7 percent (3/46) under regular category and 37 percent (17/46) as bad. 

*Mucor, Aspergillus, Fusarium* and *Penicillium* were recovered as predominante genera from poultry feed by Shareef (2010) somewhat related to present study. Labuda and Tančinová (2006) reported the isolation of *Penicillium* (89%), *Aspergillus* (69%), *Mucor* (50%) and *Cladosporium* (31%) from feed samples. Among aspergilli *A. flavus* was commonly isolated specie from poultry feed in accord with results of present study (Table 2). Several fungal species harbor poultry feed such as; *A. oryzae, Rhizopus oryzae* and *P. notatum* along with *A. flavus* and *A. flavus* as the most dominant species in feed (Kehinde et al., 2014), in agreement with present study.

**Table 2: Isolation frequency of fungal genera and species in poultry feed**

| Sr. # | Fungi          | Macroscopic Obverse | Reverse  | Microscopic | Isolation frequency (%) |
|-------|----------------|---------------------|----------|-------------|-------------------------|
| 1     | Aspergillus    | Initially white turning to different colours on maturation depending upon species with white periphery, cottony to granular/dusty texture | Pale to colourless | Septate hyaline hyphae, vesicle foot cell and Phialospores (Circular spores arranged in chains) | 100 | 95 |
| 2     | A. flavus      | Yellowish green/cottony | Colourless | Coenocytic, hyaline hyphae, Sporangium containing sporangiospores (Spherical to slightly oval), no rhizoids | 95 | 75 |
| 3     | A. fumigatus   | Blush green turning to slate grey in old culture/cottony to dusty | Colourless | Diffusible red pigment production | 15 | 45 |
| 4     | A. niger       | Black on maturation/granular | Pale | Daemateus septate hyphae, shape of spores is oblong | 75 | 65 |
| 5     | A. parastatus  | Dark green/cottony | Dull pale | 0 | 10 |
| 6     | A. terreus     | Cinnamon brown/cottony | Colourless | 5 | 5 |
| 7     | Mucor          | Initially white turning to ash grey/fluffy | White | 25 | 45 |
| 8     | Penicillium    | Blush green with white periphery/velvety | Diffusible red pigment production | 0 | 15 |
| 9     | Cladosporium   | Blackish green with folds, valvety/black | Coenocytic, hyaline hyphae, Sporangium containing sporangiospores (Spherical to slightly oval), no rhizoids | 0 | 5 |

**Fig. 2:** Thin layer chromatogram of Aspergillus species mycotoxins. L1: Mycotoxin standard; L2,7,9,11: Ochratoxin; L3: G1, G2; L4,5,6,12,13: B1, B2; L8: B1, G1; L10,11,14: B1, B2.

**Fig. 4:** Representative Polymerase chain reaction for Aflatoxin producing *Aspergillus flavus* L: DNA ladder, Lane 1: positive reaction for *A. flavus*. Lane 2-7 negative reaction.
In Nigeria, Aspergillus (48.3%), Penicillium (18.5%) and Fusarium (7.8%) were isolated from commercial feed samples. In self compound poultry feed the highest and lowest genera were Aspergillus (53.3%), Fusarium (6.7%) respectively. Matthew et al. (2017) declared 37.5 percent Rhizopus, 27.1 percent Mucor, 20.8 percent Aspergillus and 14.6 percent Penicillium frequency in feed. Greco et al. (2014) found 10.5 percent Aspergillus, 11.5 percent Cladosporium, 12.5 percent Eurotium, 16.7 percent Fusarium, 9.4 percent Mucor, 10.9 percent Penicillium, 0.5 percent Paeceilomyces and 6.5 percent frequency of A. flavus in poultry feed samples. Saleemi et al. (2010) observed Aspergillus (43.82%), Alternaria (1.12%), Fusarium (5.61%) and Penicillium (22.47%), in commercial feed and Aspergillus (46.66), Alternaria (10), Fusarium (10) and Penicillum (23.33%) in farm-mixed poultry feeds. Above mentioned data showed more diversity in fungal isolates present in poultry feed opposite to present findings. The differences or similarities in diversity might be due to climatic conditions prevailing in different regions (Shareef, 2010). But all studies strengthen the presence of opportunistic fungal species in poultry feed. A. flavus is common agricultural contaminants reported by Ismaiel and Papenbrook (2015) and Ibrahim et al. (2017) in accord to present study findings. Above mentioned and current data of poultry feed reveals A. flavus a frequent contaminant of poultry feed. The identification of A. flavus from other closely related species such as A. parasiticus and A. nomius is not an easy task. Other than mycotoxin profile, different selective media and molecular tools are used to differentiate these species. So, in present study different molecular approaches were used for characterization of aflatoxin producing A. flavus. A highly specific PCR was used for A. flavus confirmation. The primers used in this study were more efficient to amplify the variable portion of Internal Transcribed (ITS) regions of genus Aspergillus. This differentiate the A. flavus from other species of Aspergillus section Flavi (Gonzalez-Salgado et al., 2008).

Fungal species have a specific mycotoxin profile. Fusarium species are zearealenone (ZEA), fumonisins (FUM), moniliformin (MON) and trichothecenes producers (Greco et al., 2015). Citrinin, ochratoxin A, patulin and penicillic acid were extracted from Penicillium species (Ismaiel and Papenbrook, 2015). A. ochraceous are well known for Ochratoxins production (Okiki and Ogbimi, 2017). Aflatoxins are mycotoxins mainly produced by A. parasiticus and by A. flavus. A. flavus produces Aflatoxins in poultry feed if storage conditions are inappropriate. Ingestion of contaminated feed results in mycotoxicosis. Several qualitative and quantitative methods are available for aflatoxins analysis. The commonly used technique is thin layer chromatography (TLC), others are High Performance liquid chromatography (HPLC), Enzyme linked immune sorbent assay (ELISA). Dafalla and Sulieman, (2015) used ELISA and Fluorometry for aflatoxin detection in poultry feed ad concluded fluorometry as more sensitive as compared to ELISA. Gutleb et al. (2015) determined aflatoxins and fumonisins using ELISA. Alshawabkeh et al. (2015) determined e 40% and 23.07% of feed samples positive for AFB1 by ELISA ad HPLC and concluded similar results in both techniques. Khayoon et al. (2010) applied HPLC for determination of Aflatoxins in animal feed. In current study TLC used for screening of A. flavus for aflatoxins whereas HPLC was used for identification and quantification of aflatoxins. Mostly studies were performed to detect mycotoxins directly from feed. However in a study, 83.33 percent A. flavus isolates were reported as toxicigenic. It was observed that six isolates produced four major aflatoxins (AFB1, AFB2, AFG1 and AFG2) whereas two produced three aflatoxins (AFB1, AFB2, and AFG1), one produced aflatoxin; B1, B2 and G2; and one was able to produce aflatoxins of category B1 and B2 (Saleemi et al., 2010) partially in accord with present findings. Different isolates of A. flavus produced AFB1 in highest quantity among four aflatoxins and quantified as 0.00157-1.9887ug lower than present study isolates. Fakrudin et al. (2015) recovered 15 A. flavus from grains and feed samples and screened 11 produced both AFB1 and AFB2. The AFB1 was quantified in range of 7-22ug by HPLC. Saleemi et al. (2012) isolated 33 percent aflatoxin producing A. flavus from maize and maize gluten in Pakistan and quantified AFB1 (635.50ng) in contrast to present study findings. All studies indicate that HPLC is widely used technique for identification and quantification of Aflatoxin.

Poultry feed is a good growth medium for fungal species. So, to avoid the contamination of poultry feed with fungi and aflatoxins, there is need to adopt strict hygienic and proper storage conditions to stop fungal growth and toxin production.

Conclusions: True pathogenic, opportunistic and aflatoxin producing A. flavus were present in poultry feed and may be a serious threat to poultry and human health.
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**Authors contribution:** AAA, TY and MN conceived and designed study. SS and MA executed the experiments. SS and AAA analyzed the data. SS, AAA and MAA prepared the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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