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RESEARCH ARTICLE

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

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A B S T R A C T

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 $2x10^2$ to $1.6x10^4$CFU/g whereas, in commercial poultry feed from $2x10^1$ to $6x10^3$ 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 AFB_1 and AFB_2 and AFG_1 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.

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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, Zearalenone, 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 AFB_1, AFB_2, AFG_1 and AFG_2 based upon natural ability to fluoresce under Ultra Violet (UV) light and chromatographic mobility by thin layer chromatography. Among four; AFB_1 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 *at 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 *at 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^{-1} 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 25ºC±3ºC 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 H_2O 5mL). Mixture was placed at 37ºC 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 she*et al*ong 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 aflatoxins 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′-GGAAAAAGATTGATTTGCGTTC-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, Log_{10} of fungal counts were calculated and mean Log_{10} were compared using Statistical Package for social sciences (SPSS version 20) with five percent significance level ($\alpha<0.05$). 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 $1.6x10^4$ (Log_{10} 4.21) and $6x10^3$ CFU/g (Log_{10} 3.77) in home mixed and commercial feed, respectively. Similarly, the lowest CFU/g were $2x10^2$CFU/g (Log_{10} 2.30) in home mixed and $2x10^1$ (Log_{10} 1.30) in commercial feed. Mean Log_{10} 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 3.10^4 CFU/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

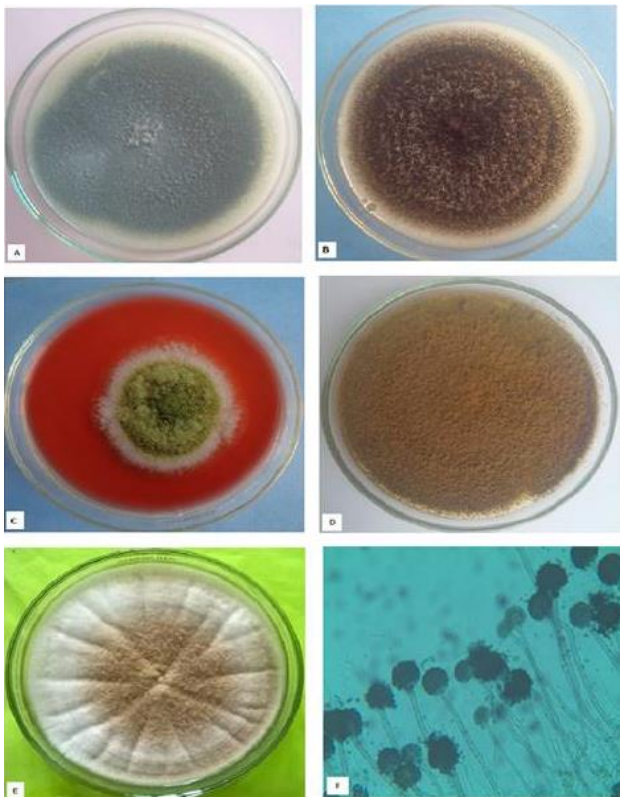

Fig. 1a: Macroscopic and microscopic identification of *Aspergillus* species (A): *Aspergillus fumigatus*: (B) *Aspergillus niger*: (C) *Aspergillus flavus*: (D) *Aspergillus parasiticusn:* (E) *Aspergillus terreus*: (F) Microscopic view of *Aspergillus.*

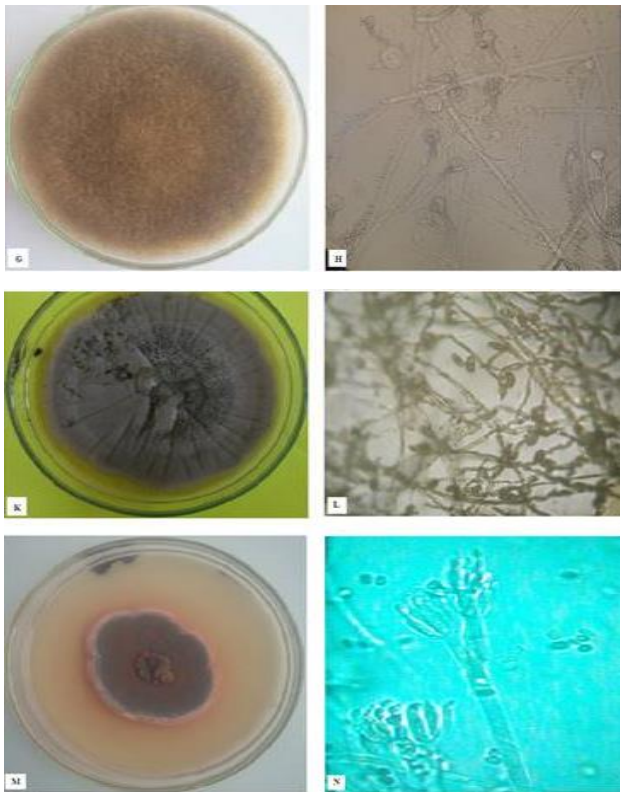

Fig. 1b: Macroscopic and microscopic identification of fungal species; G) *Mucor spp.*: (H) Microscopic view of *Mucor spp.*: (I) *Fusarium spp.*: (J) Microscopic view of *Fusarium spp.* (K) *Phaeoid fungi*: (L) Microscopic view of *Phaeoid fungi*: (M) *Penicillium spp.* (N) Microscopic view of *Penicillium spp.*

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

Sr. No.	Home mixed			Commercial		
	CFU/g	Log10 (CFU/g)	Mean $Log_{10\pm}$ S.D.	CFU/g	Log10 (CFU/g)	Mean $Log_{10\pm}$ S.D.
1	7.6×10^3	3.88	3.24±0.69[a]	2.2×10^3	3.34	2.97±0.54[a]
2	3.4×10^3	3.53		1.0×10^3	3.00	
3	7.4×10^3	3.86		1.1×10^3	3.04	
4	4.6×10^3	3.66		1.8×10^3	3.25	
5	4.5×10^3	3.65		2.6×10^3	3.41	
6	4.4×10^3	3.64		2.9×10^3	3.46	
7	3.0×10^3	3.47		9.0×10^2	2.95	
8	1.0×10^4	4.00		7.0×10^2	2.84	
9	6.5×10^3	3.81		5.0×10^2	2.69	
10	9.2×10^3	3.96		6.0×10^3	3.77	
11	5.7×10^3	3.75		7.0×10^2	2.84	
12	1.64×10^4	4.21		1.0×10^3	3.00	
13	6.7×10^3	2.77		3.8×10^3	3.57	
14	6.0×10^2	2.30		1.3×10^3	3.11	
15	2.0×10^2	2.90		1.2×10^3	3.07	
16	8.0×10^2	2.47		1.0×10^3	3.00	
17	3.0×10^2	3.00		7.0×10^2	2.84	
18	1.0×10^3	2.77		8.0×10^2	2.90	
19	6.0×10^2	2.77		2.0×10^1	1.30	
20	6.0×10^2	2.77		1.0×10^2	2.00	

*Mean Log_{10} with same superscripts are statistically non-significant.

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).

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 AFB_1 and AFB_2, 6.25 were AFB_1 and 10.42 percent of these were AFG_1 producers. Among toxigenic *A. flavus* isolated from commercial feed 17.4% were producers of two Aflatoxins (AFB_1 and AFB_2), 10.9 percent produced only one aflatoxin (AFB_1) and 2.2 percent produced three toxins (AFB_1, AFB_2 and AFG_1). The AFB_1 was quantified in range of 0.22ng to 11622.24 ng by HPLC. While AFB_2 and AFG_1 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 2: Isolation frequency of fungal genera and species in poultry feed

Sr. #	Fungi	Macroscopic		Microscopic	Isolation frequency (%)	
		Obverse	Reverse		Home mixed	Commercial
1	*Aspergillus*	Initially white turning to different colours on maturation depending upon specie 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
	A. flavus	Yellowish green/cottony	Colourless		95	75
	A. fumigatus	Bluish green turning to slate grey in old culture/cottony to dusty	Colourless		15	45
	A. niger	Black on maturation/granular	Pale		75	65
	A. parasiticus	Dark green/cottony	Dull pale		0	10
	A. terreus	Cinnamon brown/cottony	Colourless		5	5
2	*Mucor*	Initially white turning to ash grey/fluffy	white	Coenocytic, hyaline hyphae, Sporangium containing sporangiospores (Spherical to slightly oval), no rhizoids	25	45
3	*Penecilliun*	Bluish green with white periphery/velvety	Diffusable red pigment production	Septate hyaline hyphae, bifurcation of conidophore bearing phialospores which were of spherical shape, No vesicle and foot cell	0	15
4	*Cladosporium*	Blackish green with folds, valvety/black		Daemataceous septate hyphae, shape of spores is oblong	0	5

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).

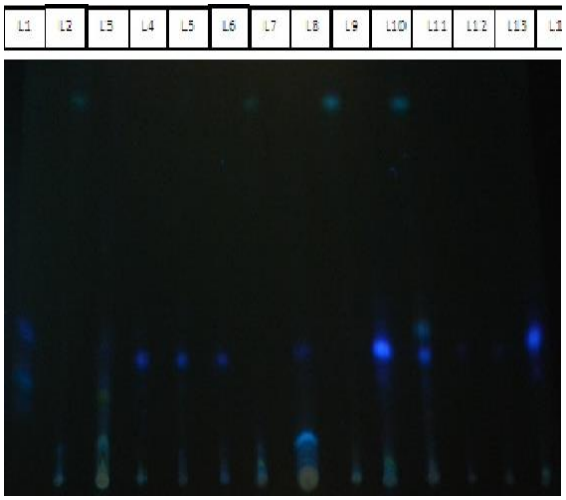

Fig. 2: Thin layer chromatogram of *Aspergillus species* mycotoxins. L1: Mycotoxin standard; L2,7,9,11: Ochratoxin; L3: G_1, G_2; L4,5,6,12,13: B_1; L8: B_1, G_1; L10,11,14: B_1, B_2.

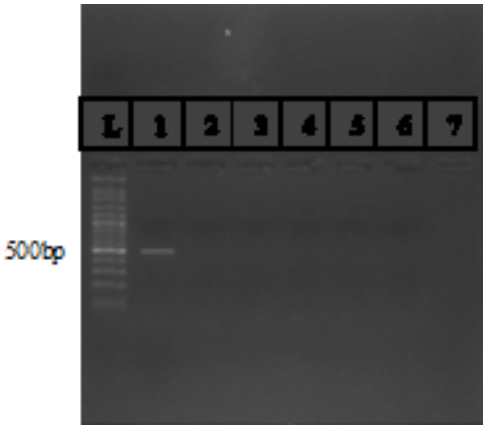

Fig. 4: Representative Polymerase chain reaction for Afaltoxin producing *Aspergillus flavus L: DNA ladder, Lane 1: positive reaction for A.flavus. Lane2-7 negative reaction.*

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 (1.64×10^4) and commercial feed (6.0×10^3 CFU/g) were slightly lower than counts in poultry feed (6.5×10^6 CFU/g) in Iraq (Shareef, 2010), counts (42×10^3 CFU/g) in Nigarian poultry feed (Kehinde *et al.,* 2014), fungal load ($0\text{-}14 \times 10^4$ CFU/g) in Serbia's poultry feed (Krnjaja *et al.,* 2014), counts of fungi (8.1×10^5 CFU/g) in Nigarian bird's feed (Matthew *et al.,* 2017) and higher than fungal counts (3×10^2- 4×10^4 CFU/g) in poultry feed in Quetta, Pakistan (Rashid *et al.,* 2017) and in Iran; mean fugal count $6.4 \times 10^4 \pm 1.12 \times 10^5$ CFU/g of poultry feed (Parviz *et al.,* 2014). 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 predominate 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. flams* and *A. flavus* as the most dominant species in feed (Kehinde *et al.,* 2014), in agreement with present study.

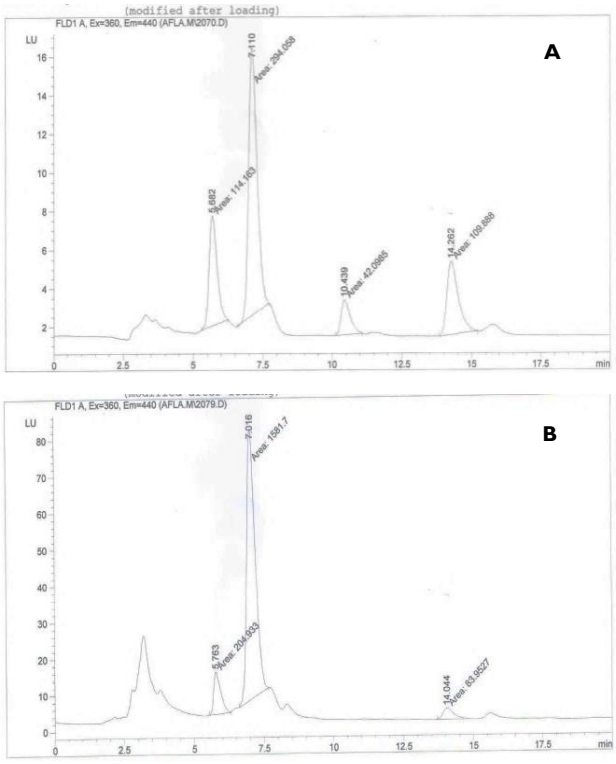

Fig. 3: Chromatogram for aflatoxins by high performance liquid chromatography; A: Standard; B: Sample.

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 *Penecilium* 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 *Penecilium*, 0.5 percent *Paceiliomyces* 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 *Penicillium* (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 Papenbrock (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* (González-Salgado *et al.*, 2008).

Fungal species have a specific mycotoxin profile. *Fusarium* species are zearalenone (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 Papenbrock, 2015). *A. ocharecous* 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 Fluorometery for aflatoxin detection in poultry feed ad concluded flurometery 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 AFB_1 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 toxigenic. 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 AFB_1 in highest quantity among four aflatoxins and quantified as 0.00157-1.9887ug lower than present study isolates. Fakruddin *et al.* (2015) recovered 15 *A. flavus* from grains and feed samples and screened 11 produced both AFB_1 and AFB_2. The AFB_1 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 AFB_1 (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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