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RESEARCH ARTICLE

Molecular Characterization of Toxigenic *Aspergillus flavus* Isolated from Sick Broiler Lungs and Risk Factors Analysis

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A B S T R A C T

Brooder's pneumonia is a respiratory disease of poultry caused by fungi of genus *Aspergillus. Aspergillus flavus* is a toxigenic fungus, which sporulates easily in poor quality bedding and contaminates indoor air. The objective of present study is to isolate *A. flavus* from diseased birds, characterize toxigenic potential/variability and analysis of the risk factors associated with brooder's pneumonia caused by *A. flavus.* Litter, air and morbid Cobb 500 strain from poultry farm (n=10) were selected for sampling. Moisture contents detected in bedding of saw dust and rice husk were 73 to 46.4 and 20.06 to 23.26% respectively. Poultry farms used saw dust were found more contaminated (CFU/30minutes 80±2.6 to 160±6.0) in comparison to those having rice husk (CFU/30minutes 28 ± 3^{a} 50 ± 2.6^{b}) as bedding material. *A. flavus* was isolated from lungs of all infected birds and identified based upon colony and microscopic morphology. Infected lung tissues containing fungal plaques were processed for histopathological examination. Tissue was found necrotized and contaminated with fungal hyphae. Purified *A. flavus* species were evaluated for aflatoxins (Total aflatoxins and AFB_1) production potential by Enzyme linked immunosorbent assay (ELISA). All species were found toxigenic with production of total aflatoxin ranged from 1.51 to 8.77ppm and AFB_1 from 0.33 to 3.0 ppm. Molecular analysis by Multiplex Polymerase chain reaction (PCR) revealed genetic variability in aflatoxins biosynthetic pathway. *AflR* gene was amplified in all species with 1032bps amplicon size. Multiplex PCR amplified the *omt-A* gene in 40, *ver-1* in 70 and *nor-1* in 60 percent toxigenic isolates. It is concluded that toxigenic *A. flavus* is emerging as major pathogen in Brooder's pneumonia associated with poor litter quality.

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INTRODUCTION

Aspergillosis (brooder's pneumonia) is a common mismanaged problem of poultry mainly caused by *Aspergillus*, saprophytic fungi dwelling in soil. This saprophytic fungus may switch to pathogen depending upon certain factors. Among the major pathogens *A. fumigatus* stands first followed *by A. flavus, A. niger, A. glaucus* and *A. terreus* (Girma *et al*., 2016). However, different aspergilli may co-infect. Mixed infections are reported in parrots, ostriches and falcons (Arné *et al.*, 2021). In birds, aspergillosis is either acute or chronic. Birds at early age are more susceptible to acute aspergillosis leading to high morbidity and mortality. On other hand, older birds suffered from chronic aspergillosis due to compromised immune system results in less morbidity (Girma *et al*., 2016). The disease involves the respiratory tract and may be systemic. In case of systemic it affects viscera and brains with the development of white to yellow caseous nodules and plaques in tissue. Neurological signs may be observed in young birds in case of acute aspergillosis (Hauck *et al*., 2020).

Poor husbandry conditions along with presence of *Aspergillus* conidia contribute to the disease in birds. The disease is more prevalent in environment having poor sanitization, poor ventilation, malnutrition, respiratory irritants, overuse of certain medications (corticosteroids) and mycotoxins in feed. Type of litter is also important. Some bedding materials having higher water content are prone to fungal growth (Sultana *et al*., 2015). The moldy

litter is considered a main source of aspergillosis. Fungi require hot and humid environment to grow. Dispersal of fungal spores due to bird's movement, density of birds, refreshing process of the bedding contaminate air, litter and eggs in poultry shed (Arné *et al*., 2011). Transmission of aspergilli is through inhalation of spores. However, presence of pre-disposing factors leads to the development of aspergillosis in birds after exposure (Girma *et al.*, 2016). El-Ghany described it as occupational zoonotic disease. The farm workers with immunocompromised conditions may expose via direct or indirect contact of birds, handling of contaminated litters and inhalation of dust containing fungal spores from contaminated farm environment (Abd El-Ghany, 2021).

Among the causative agent of aspergillosis, *A. flavus* is second one and common in Asia, Africa and Middle East. However, there is report on increasing incidence of *A. flavus* in aspergillosis. Among the ten deadly fungi, *A. flavus* is ranked at 5th due to its disease development in human, animals, crops and mycotoxins related toxicities (Rudramurthy *et al*., 2019). *A. flavus* is responsible for production of two naturally occurring aflatoxins (AFs) include aflatoxin B_1 (AFB_1) and aflatoxins B_2 (AFB_2) (Pfliegler *et al.*, 2020). There is a genetic diversity among *A. flavus* for aflatoxins production. The biosynthetic pathway is comprised of 23 enzymes encoded by 25 genes located in 70kb region on chromosome III. This biosynthetic pathway is regulated by gene cluster including *aflR*, *aflS* and *aflP*; other genes are *aflQ*, *aflD*, *aflM* and *aflO genes*. The genetic diversity in *A. flavus* aflatoxins production can be determined by molecular approaches like polymerase chain reaction (Acur *et al*., 2020).

The mycotoxins pose a serious health hazards to animal, human and poultry collectively called mycotoxicosis. If caused by aflatoxins, it is named as aflatoxicosis. The main route of aflatoxins transmission of ingestion but inhalation and dermal exposure is also reported. Poultry are among the most susceptible specie to aflatoxicosis. The major target organ is liver. Respiratory exposure to AFs is related with the respiratory tumor. AFs cause immunosuppression and make the birds vulnerable to bacterial, viral and parasitic infection (Lakkawar *et al*., 2017). AFs exposure disturb the growth and performance parameters of birds and causes reduction in weight gain and feed conversion ratio, deleterious effects on male and female reproductive system and effects the egg quality in laying birds. However, mortality is also linked in acute aflatoxicosis (Abedi and Taaebi, 2015).

To control the risk associated with *A. flavus*, different available chemical compounds are being utilized including dithiocarbamates, surface disinfectant and antifungal (Azoles) for treatment. However, these pose a public health concern (Zhang *et al.*, 2022). Low safety margin of antifungals to treat the aspergillosis, development of antifungal resistance and lack of interest in vaccine development against aspergillosis are hurdles to control disease in birds. So, the best prevention strategy so far is to improve the management practices (Hauck *et al*., 2020).

Current study was carried out to find the occurrence of *A. flavus* in aspergillosis. Later aflatoxins production potential and genetic diversity was assessed. The study also emphasized on association of risk factors with aspergillosis caused by *A. flavus.*

MATERIALS AND METHODS

Sampling: Samples for isolation of fungi were collected from morbid chicks. A total of 10 broilers (Cobb 500 strain, 5-20 days old) were selected on the basis of history of respiratory distress (dyspnea and gasping). The flocks selected for sampling were having poor biosecurity, wasting and litter used of saw dust, rice husk.

Gross examination of morbid birds was performed. Necroscopic examination of birds was carried out for presence of fungal plaques or granulomas on lungs, liver and respiratory passages. Infected lung tissues containing fungal plaques were collected aseptically, fragmented (measuring 0.5-1 cm^3) and stored immediately in 10% buffered formalin for histo-pathological examination. For mycological and toxicological examination samples were stored at -25°C. For indoor air quality and moisture contents in litter, air and litter were collected as samples.

Indoor air exposure sample: For determination of fungal spore's load in indoor air was determined by exposure on Sabouraud's Dextrose Agar (SDA) medium containing 0.05g/L chloramphenicol. Plates were exposed to air passively. SDA open plate were placed at 0.5-meter height from floor for 30 minutes in 3 different places of poultry shed for direct exposure of floating fungal spores at chicken nostril level. After that exposed plates were transferred to laboratory and incubated at 27.5 °C for 5-7 days. Fungal colonies from plates were counted and expressed as colony forming units (CFU)/30 minutes (Shokri, 2016). Data were analyzed statistically by one-way ANOVA (analysis of Variance) using SPSS (Statistical package for Social Sciences) software version 21 to compare mean counts.

Litter moisture: Moisture level in litter was evaluated. For this Saw dust (n=7) and rice husk (n=3) was used as litter materials in poultry shed were collected. Each sample (10 g) was collected from 5 places (side walls-2, middle-1, beside drinker-1) and moisture content was measured by dry heat protocol according to direction of Fairchild and Czarick (2011) guidelines.

Isolation of *Aspergillus flavus*: The lung tissue fragments were directly inoculated into Sabouraud's dextrose agar (Bioxont BD) containing streptomycin and penicillin (20:40) and incubated at 25±2°C for 7 days. The characteristic colony of *Aspergillus flavus* (*A. flavus*) was sub-cultured onto modified Czapek Dox agar (Oxoidt, UK) and colony morphology was observed (Germain and Summerbell, 2011). Then mycological identification was done according to Ridell technique (Ridell, 1951) by serial micro-cultures of 24, 48, and 72 hrs. *A. flavus* was preserved by suspending conidia into phosphate saline solution and placed at 4°C for further study.

Histopathological examination: The lung tissue samples were sent to commercial diagnostic laboratory for

histopathological examination. Tissues were cut into 3 µm sections and hematoxylin and eosin (HE) was performed using standard procedure (Luna, 1968).

Aflatoxin production potential: The ability of aflatoxin production of identified isolates was measured by competitive enzyme link immunosorbent assay (ELISA). Total aflatoxins (AFs) and aflatoxin B_1 (AF B_1) as measured from fungal cultured on Czapek Dox Agar (Oxoid, UK) medium. Aflatoxins were extracted using 70% methanol. The ELISA was performed according to manufactures instructions. For quantification of total AFs and AF B_1, Romar Lab, ELISA Kits of Singapore and Sigma, US were used. Manufacturer-supplied Software was used for the analysis of ELISA results according to guidelines provided.

Genetic Diversity of Toxigenic isolates: Genomic DNA was extracted from fungal mycelia of *A. flavus* by QIAamp® DNA Mini Kit (Quiagen®, USA) according to manufacturer protocol. The isolated *A. flavus* from lung tissues were cultured into Sabouraud's dextrose broth (SDB) in shaking incubator at 25ºC for 72 hours. Then the mycelia were harvested by filtration, frozen into liquid nitrogen and grinded by pestle mortar. The ground mycelia were suspended in lysis buffer and heated at 68°C for 15 minutes followed by centrifugation at 15000× g for 15 minutes. Genomic DNA was purified using affinity column provided in the kit. The purified DNA was stored in elution buffer.

The multiplex polymerase chain reaction (PCR) of four aflatoxins enclosed genes (*aflR, omt-A, ver-1* and *nor-1*) for the aflatoxin biosynthesis pathway were analyzed by primers described by Criseo *et al*. (2001) (Table 1). The reaction mixture (25 µl) was prepared using QIAGEN Multiplex PCR Master Mix kit (Qiagen, USA). The reaction mixture with PCR conditions is described in Table 2. Then amplified products were visualized by UV illumination after electrophoresis on 1% agarose gel (Thermo Fisher Scientific, USA) with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA).

RESULTS

Indoor air exposure sample: Indoor air quality of poultry farm was determined by the fungal counts (CFU/30 minutes) indicated in table 1 and represented in Fig 1 & 2. Poultry farms with saw dust as bedding material were fund more contaminated with fungal spores in comparison to those having rice husk as bedding material. As far as moisture contents of litter are concerned, saw dust having more water content. It could be more suitable for fungal if hot and humid environment available. Heavy contamination poses high risk for development of disease. *Aspergillus flavus* were also included in fungal counts. Moisture contents for poultry litter are recommended 30 percent to 40 percent. If contents are higher, causes litter caking and ammonia emission while low moisture contents potentiate the respiratory problems due to higher dust level if contaminated with microorganism including fungal spores. Litter (6/10) having higher moisture contents than recommended level.

Isolation and identification of *Aspergillus flavus*: *A. flavus* was isolated from all infected lung tissues (n=10). It was purified and identified based on colony morphology and microscopic morphology. Initial growth was white with cottony texture and on maturation growth turned to yellowish green on obverse side of the plate. No diffusible pigment was observed. As far as microscopic structures are concerned, septate hyaline hyphae, conidia (circular chains) on periphery of vesicle were observed under microscope at 100× (Fig. 2). The identified isolates were assigned number, (AFPF-1-, AFPF-2, AFPF-3, AFPF-4, AFPF-5, AFPF-6, AFPF-7, AFPF-8, AFPF-9 and AFPF-10).

Table 1: Primer Sequence and Amplicons size of toxin genes

Primer name	Target gene	Primer sequence (5´-3´)	Amplicons size (bps)
NorF	*nor-1*	ACCGCTACGCCGGCACTCTCGGCAC	400
NorR		GTTGGCCGCCAGCTTCGACACTCCG	
VerF	*ver-1*	GCCGCAGGCCGCGGAGAAAGTGGT	537
VerR		GGGGATATACTCCCGCGACACAGCC	
OmtF	*omt-A*	GTGGACGGACCTAGTCCGACATCAC	797
OmtR		GTCGGCGCCACGCACTGGGTTGGGG	
AflrF	*aflR*	TATCTCCCCCCGGGCATCTCCCGG	1032
AflrR		CCGTCAGACAGCCACTGGACACGG	

The *A. flavus* genome was evaluated for presence or absence of genes involved in aflatoxin biosynthetic pathways. Table 1 is representing the gene sequence of primers used for amplicons of genes involved in biosynthesis of aflatoxins. The region selected for *AflR* produced an amplicon of 1032bps. The PCR product of *nor-1, ver-1* and *omt-A* was of 400, 537 and 797 bps.

Table 2: Multiplex PCR reaction mixture and conditions

Components	Volume (µl)	Multiplex PCR conditions
QIAGEN Multiplex PCR Master Mix (2X)	12.5	Initial Denaturation: 94°C for 5 mins
Primer mix	2	Denaturation: 94°C / 30 sec
RNase-free water	5.5	Annealing 67°C / 30 sec
Template DNA	5	Extension: 72°C / 30 sec
Total Volume	25 µl	Final Extension: 72°C / 10 mins
		No of cycles: 35

The table 2 describes the recipe of reaction mixture of multiplex PCR for aflatoxin biosynthetic pathway of *A. flavus* and condition for PCR process. The master mix (dNTPs, buffer, polymerase, $MgCl_2$) was 2X, so in 25uL reaction, 12.5uL of it were used. It was mixed with 4 different set of primers mentioned in table 1, 2uL of primer mix (combination of all primers), and 5uL of template DNA (extracted from *A. flavus*). The three steps (Denaturation, Annealing and extension) of PCR process were repeated for 35 times. Two steps; initial denaturation and final extension were performed for just one time.

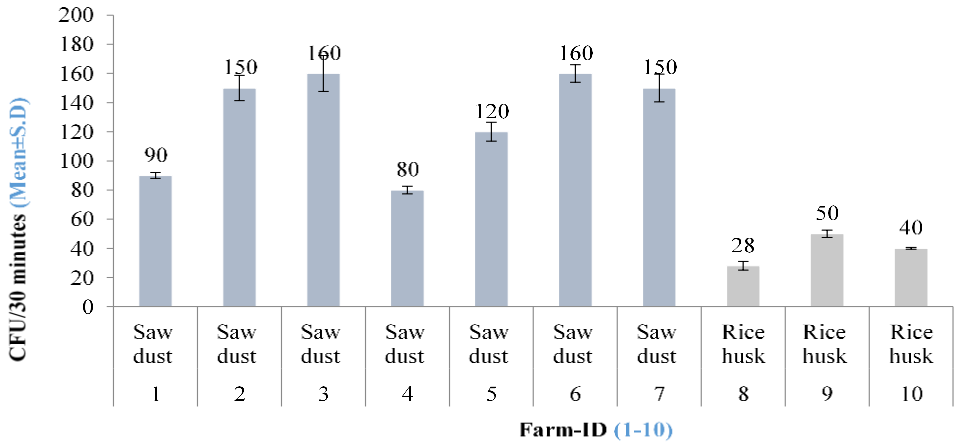

Fig. 1: Fungal counts in poultry farms Enviorment. Farm ID (1-7): Counts of air have saw dust as bedding material. Farm ID (8-10): Counts of air have saw dust as bedding material. Data is expressed as Mean± S.D (P=0.00) compared between ten poultry farms

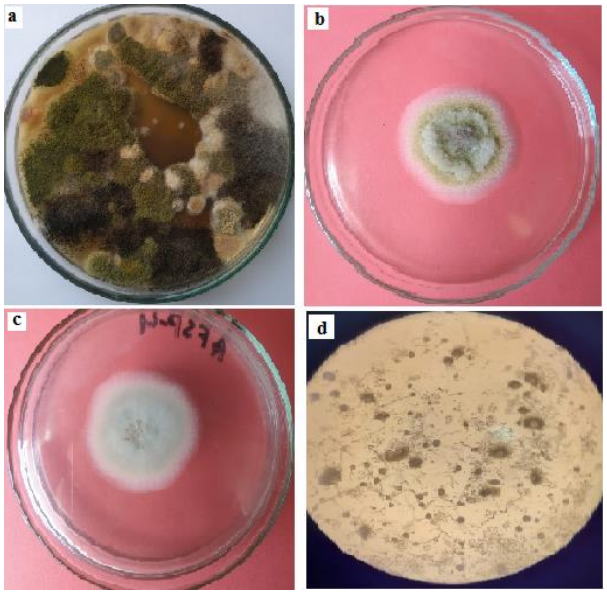

Fig 2: Representative pictures of fungal counts isolation and identifiocation (a: Fungal count plate with predominant *Aspergillus flavus* colonies, b: Obverse View of pure culture of *Aspergillus flavus* showing lime green color of spores, white mycelia and cottony texture on Sabouraud's dextrose agar plate, c: Reverse view of *Aspergillus flavus* on Sabouraud's dextrose agar plate, d: Microscopic View (100X total magnification) showing hyphae, vesicle and conidia of *Aspergillus flavus*).

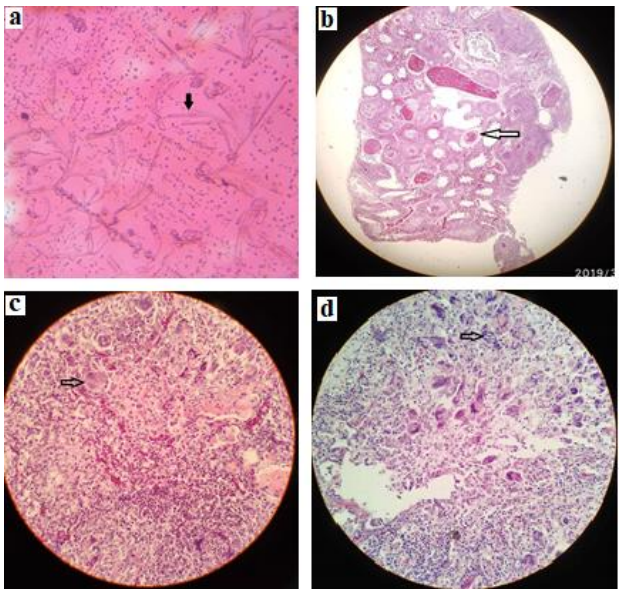

Fig 3: Microscopic examination of lungs of diseased birds. (**a:** fungal hyphae in tissue, **b:** Cagulative necrosis, **c:** Caseous necrosis, **d:** necrotic foci).

Table 3: Moisture contents of litter and fungal counts in indoor air

Farm-ID	Type of Litter	%age moisture	CFU/30 minutes	CFU/30 minutes	CFU/30 minutes	Mean CFU±S.D	*p* value
1	Saw dust	33.52	90	92	88	90 ± 2^{c}	
2	Saw dust	41.91	140	155	155	150 ± 8.66^{e}	
3	Saw dust	44.32	174	150	156	160 ± 12.4^{e}	
4	Saw dust	29.73	81	77	82	80 ± 2.6^{c}	
5	Saw dust	36.1	119	127	114	120 ± 6.5^{d}	0.00
6	Saw dust	46.4	153	164	163	160 ± 6.0^{e}	
7	Saw dust	45.71	156	155	139	150 ± 9.5^{e}	
8	Rice husk	20.06	28	25	31	28 ± 3^{a}	
9	Rice husk	23.26	52	51	47	50 ± 2.6^{b}	
10	Rice husk	21.21	39	40	41	40 ± 0.57^{b}	

The fungal counts of ten different farms are mentioned in table 3 with two different bedding materials. Out of 10 selected farms 7 were using saw dust and 3 were using rice husk as bedding material. The counts are presented in Colony forming units (CFU) per 30 minutes' exposure of SDA plate to air of farm. In table counts are mentioned as mean± standard deviations as three places were selected for air sampling in same farm. There are higher number of counts in farms having saw dust but statistically significant variations were recorded (P=0.00).

Histopathological examination: Histopathological examination revealed the presence of fungal hyphae in lung tissue, coagulative necrosis, caseous and necrosis and necrotic foci were observed (Fig. 3).

Aflatoxin production potential genetic diversity in toxigenic *Aspergillus flavus*: The identified *A. flavus* isolates were evaluated for aflatoxins production potential by ELISA. Total aflatoxins and AFB_1 were targeted. All isolates (100%) were found toxigenic and positive for AFB_1 production. The highest total aflatoxins produced by AFPF-6 were 8.77 ppm. The minimum total aflatoxins were produced by AFPF-8 and it was 1.51ppm. AFB_1 was quantified in range from 3 ppm to 0.33 ppm produced by AFPF-3 and AFPF-8.

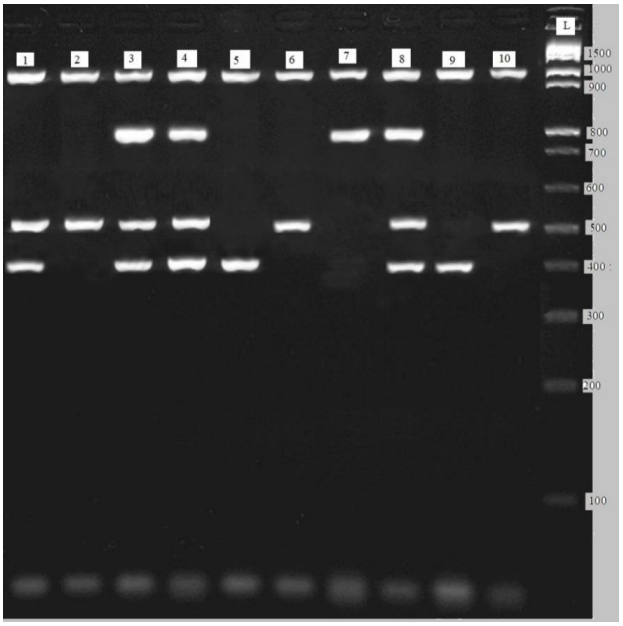

Fig 4: PCR product gel electrophoresis of aflatoxin biosynthetic pathway genes (Amplicon sizes of genes are ***nor-1***= 400bp, ***ver-1***= 537bps, *omt-A*= 797bp, and ***aflR***= 1032bp; Left-to right side: **Lane 1**: Isolate 1 positive for *nor-1*, *ver-1*, *aflR*; **Lane 2**: Isolate 2 positive for ver-1, aflR; **Lane 3**: Isolate 3 positive for *nor-1, ver-1, omt-A, aflR*; **Lane 4**: Isolate 4 positive for *nor-1, ver-1, omt-A, aflR*; **Lane 5**: Isolate 5 positive for *nor-1, aflR*; **Lane 6**: Isolate 6 positive for *ver-1, aflR*; **Lane 7**: Isolate 7 positive for *omt-A, aflR*; **Lane 8**: Isolate 8 positive for *nor-1, ver-1, omt-A, aflR*; **Lane 9**: Isolate 9 positive for *nor-1, aflR*; **Lane 10**: Isolate 10 positive for *ver-1, aflR*; **Lane L**: 100 bps DNA Marker).

Table 4: Aflatoxin production potential of *Aspergillus flavus*

Isolate no.	Aflatoxins (ug/g)-ppm		Aflatoxin biosynthesis genes			
	Total	AFB_1	*nor-1*	*ver-1*	*omt-A*	*aflR*
AFPF-1	4.39	0.51	+	+	-	+
AFPF-2	6.91	2.73	-	+	-	+
AFPF-3	8.02	3.00	+	+	+	+
AFPF-4	4.31	0.70	+	+	+	+
AFPF-5	6.49	2.07	+	-	-	+
AFPF-6	8.77	2.80	-	+	-	+
AFPF-7	7.41	2.50	-	-	+	+
AFPF-8	1.51	0.33	+	+	+	+
AFPF-9	2.25	0.48	+	-	-	+
AFPF-10	1.93	0.42	-	+	-	+
percentage	-	-	60	70	40	100

The table 4 describes the quantity of total aflatoxins (AFB_1, AFB_2, AFG1 and AFG_2) and the most potent aflatoxins (AFB_1) from fungal culture and presence or absence of regulatory gene in DNA of *A. flavus* determined by multiplex PCR. *AflR* gene was detected in all tested (10 isolates) of *A. flavus, nor-1, ver-1* and omt-A was present in 60, 70 and 70 percent of isolates as mentioned in the last row of table.

Genetic diversity exists in aflatoxins biosynthetic pathway of *A. flavus.* Multiplex PCR indicated the presence of *aflR gene* in all isolates indicated by the 1032bps band. However, variations were found in *nor-1*(400bps), *ver-1*(537bps) and *omt-A* (797bps) gene. Out of 10 isolates six showed the presence of *nor-1* gene (*AflD)*. The isolates were AFPF-1, AFPF-3, AFPF-4, AFPF-5 and AFPF-6. Out of 10, *ver-1* gene was not detected in three isolates (AFPF-5, AFPF-7 & AFPF-9). Multiplex PCR amplified the *omt-A* gene in AFPF-3, AFPF-4, AFPF-7 and AFPF-8 isolates (Fig. 4). According to the results *aflR* can be correlated the with aflatoxin production.

DISCUSSION

Fungi cause opportunistic infections due to overuse of antibiotics, presence of mycotoxins in feed. Fungal diseases are difficult to treat due to lack of vaccine and effective therapy. The best way to prevent fungal infection is via good management practices. To prevent fungal infections knowledge of risk factors and causative agent is important. Aspergillosis is mostly linked with *Aspergillus fumigatus*, which is considered as major pathogen. Mostly data is available on isolation of *A. fumigatus* in contrary to present study. *A. fumigatus* was isolated from most of the acute and chronic cases of aspergillosis in birds. This study is opposite to *A. fumigatus* isolation from commercial broiler flocks and broiler breeders farm (Musa *et al.*, 2014). Current findings are contradictory to Glare *et al*. (2014) findings in which Aspergilli from litter and other material in birds shed of brown kiwis were isolated. The predominant specie was *A. fumigatus* (Glare *et al*., 2014). The collected data of this study is also opposite to *A. fumigatus* isolation from broiler chicks by Zafra *et al*. (2008). In contrast to above mentioned studies, this study indicated the presence of *A. flavus* as a causative agent of aspergillosis associated with contaminated litter strengthen by Martin *et al*. (2007), who isolated *A. flavus* from pullets of broiler breeders. In Nigeria *Aspergilli* were isolated from sick birds of local poultry farms; the author reported the equal prevalence of *A. fumigatus* and *A. flavus* in diseased birds. This is in support to current study (Fagbohun *et al.*, 2020).

Mycoflora of litter, air and feed material have been investigated and showed correlation with development of aspergillosis (Arné *et al*., 2011) in agreement to current study. In current study litter was found contaminated with fungal spores. Poultry farms having saw dust was more contaminated in comparison to farms have rice husk as bedding material. Poor ventilation, malnutrition and high population density facilitate the development of disease (Musa *et al*., 2014). High fungal count in air is linked with more respirable particles. Sawdust are reported to have contamination of Aspergilli (Waziri and Kaltungo, 2017) comparable with present data, in which poultry farms with saw dust bedding are more contaminated with fungal spores. Air quality of poultry farms monitored by Chen *et al.* (2021) determined a range of fungal counts from 0.23 $\pm$ 0.02x10^3 to 2.9x10^3 at different stages of bird's age. The counts reported by Chen *et al.* (2021) are higher than the counts in current study. The results of counts are contradictory to counts (1908 CFU) reported in poultry farms air in Thailand (Wingfield *et al.*, 2021). Seasonal variations in moisture contents of litter and fungal counts of litter were also reported by Ostovic *et al*. (2021). The moisture level was ranged from 28.18±10.12 to 32.17±11.07 percent in summer to winter respectively in contrast to current data (20.06 - 46.4%). The author observed fungal counts in poultry farms in summer ranged from 3x10^2 to 1.65x10^5 with litter moisture ranged in 7.90-42.30. In winter the counts ranged from 2x10^2 to 1.58x10^5 with litter moisture from 12.60-49.00 percent (Ostović *et al.*, 2021). The counts are in contrast to current study. It may be due to the nature of sampling and author used litter as sample.

A. flavus is famous for aflatoxins production. This enhances the pathogenicity of this specie. The aflatoxins production potential can be evaluated by several methods including culture based methods, chromatography techniques, spectrophotometry, immunological techniques and molecular detection. Among all, immunological assays, ELISA, immunosensors and lateral flow immune assay are sensitive techniques and highly recommended for use (Matabaro *et al*., 2017). These techniques can be used for aflatoxins detection and quantification in artificial and natural media (Leszczynska *et al*., 2001). However, nucleic acid based methods are developed and quick tool for characterization of toxigenic fungi. So, based upon available data, ELISA and molecular approaches were used for determination of toxigenic potential of *A. flavus.* The results of current study are in contrast to Lagat *et al*. (2020). Aflatoxin production potential was quantified by ELISA ranged from 4.00±0.67 to 42.62±0.39 (Lagat *et al*., 2020). AFB_1 (68.56 ng/mL) produced by *A. flavus* quantified Shrestha and Mridha (2015) showed results different to current study. The AFB1 production of *A. flavus* was quantified in range of 0.22 to 11622.24 ng/g by HPLC by Sana *et al*. (2019) was less than present data. The variation in results may be attributed to the nature of physical and chemical parameters for fungal growth.

Biosynthetic pathways for aflatoxins are consisting of structural and regulatory genes. The most important genes reported for aflatoxin biosynthesis are *aflD, aflO, aflQ, aflM* and *aflP*. The expression of these genes is inked with aflatoxin production in *A. flavus.* The *aflR* and *aflS* genes are major regulatory genes. In current study the *aflR* gene is observed in all toxigenic isolated showing its correlation with toxin production potential and in agreement with previous studies (Okayo *et al*., 2020). In another published study *aflR* was reported in 73.3% *nor-1* in 80%, and *omtA* in 86.7% isolates of *A. flavus* contradictory to current study results (Fakruddin *et al*., 2015). Geisen reported that *A. sojae* and *A. oryzae* are lacking in *nor-1* gene in which are non-toxigenic but have close similarity with *A. flavus* (Geisen, 1996). This confirmed the genetic variation exists in different fungi with reference to aflatoxin production. Genetic variability reported by Olarte *et al*. (2012) analogous to spontaneous recombination during sexual reproduction in natural populations supports the current study. Tayler *et al*. (1999) linked this genetic diversity with high level of genetic recombination among species. Okoth *et al*. (2018) confirmed the lack of correlation between presence of *aflD, aflQ, aflP, aflR* genes and aflatoxin production particularly AFB1 and AFB2 in agreement to current study. So, both molecular tools and metabolic profile must be assessed for identification of toxigenic species.

Histopathological examination of air sacs was similar to lesions histopathological examination reported by Naobumi *et al*. (2013), Nururrozi *et al*. (2020) and Cheng *et al*. (2020). This indicated that *A. flavus* is similar to *A. fumigatus* in pathogenesis of brooder's pneumonia.

Conclusion: It is concluded that litter moisture influence mold growth and saw dust have higher moisture contents as compared to rice husk. Fungal load increased when saw dust is being used as litter. Those flocks using saw dust as litter have more chance of increase respiratory distress. So, it is recommended to avoid saw dust as bedding material. A strong relation was found between toxigenic *A. flavus* and aspergillosis and toxigenic *A. flavus* species are genetically diverse. However, *AflR* can be used as genetic marker to differentiate toxigenic and non-toxigenic species.

Authors contribution: Md Zulfekar Ali conducted the experiments, Saba Sana analyzed the data and bring into manuscript form, Ali Ahmad Sheikh helped in data analysis and write up process and Zoya Maheen assisted in formatting the paper according to the Journal.

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