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MYCOFLORA OF POULTRY FEEDS AND MYCOTOXINS PRODUCING POTENTIAL OF *ASPERGILLUS* SPECIES

MUHAMMAD KASHIF SALEEMI[1], MUHAMMAD ZARGHAM KHAN[1*], AHRAR KHAN[1] AND IJAZ JAVED[2]

[1]Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, 38040, Pakistan
[2]Department of Physiology & Pharmacology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, 38040 Pakistan

Abstract

The aim of this study was to determine the mycoflora of poultry feeds and evaluate the aflatoxin and ochratoxins producing ability of isolated *Aspergillus* species. One hundred and nineteen samples of commercially prepared and farm mixed feeds were collected during 2005- 2007. The samples were inoculated on potato dextrose agar, Czapek dox solution agar, Czapek yeast autolysate agar and yeast extract sucrose (YES) agar. Mycotoxins analysis of plate cultures was performed by a HPLC technique. Fungal contamination of commercially prepared and farm-mixed and total feeds was 69.66 and 83.33 and 73.10%. *Aspergillus* species were the most predominant followed by *Penicillium*, *Fusarium* and *Alternaria*. Among the *Aspergillus* isolates, *A. niger* aggregates (37.74%) was most frequently isolated species followed *by A. flavus* (22.64), *A. ochraceous* (16.98%), *A. parasiticus* (13.21%), *A. carbonarius* (3.77%), *A. fumigatus* (3.77%) and *A. oryzae* (1.89%). Proportion of toxigenic fungi among *Aspergillus* isolates was 73.58%. Aflatoxigenic isolates of *A. flavus and A. parasiticus* were 83.33% and 85.71% while ochratoxigenic isolates of *A. carbonarius*, *A. niger* aggregates and *A. ochraceous* were 50, 65 and 100%, respectively. On YES medium toxigenic *Aspergillus* isolates produced aflatoxins varying from 0.00095 to1.9807 µg/g whereas ochratoxin A production varied from 0.00136 to 16.7168 µg/g.

Introduction

Agricultural products including cereals and oilseeds meals constitute a major component of poultry feed ingredients. Mould contamination is wide spread in tropical countries where poultry production and processing are expanding rapidly (Van den Bergh *et al*., 1990; Okoli *et al*., 2006). The contamination of agriculture commodities with toxigenic fungi under favorable conditions may lead to mycotoxin buildup reaching to injurious levels for farm animals and human health. The production of mycotoxins is often species specific; therefore, identification of fungi is of prime importance. Among different mycotoxins, aflatoxins (AF) and ochratoxin A (OTA) are the most important contaminants of poultry feeds. The presence of OTA in feeds raises concerns in livestock industry due to subclinical intoxications and poor growth in animals (Gentles *et al*., 1999). AF are the most studied group of mycotoxins which apart from producing clinical toxicosis also reduce the resistance to diseases and interfere with vaccine induced immunity in poultry birds (Sharma, 1993).

Regular monitoring of toxigenic mycoflora of the agricultural based feeds and foods is an essential pre-requisite for development of strategies to control or prevent mycotoxins exposure of feed animal and human population. Study of prevalence of toxigenic mycobiota of animal/poultry feeds is regularly and frequently reported from many countries including Brazil (Olivia *et al*., 2006; Rosa *et al*., 2006), Argentina (Dalcero *et al*., 1997), Nigeria (Osho *et al*., 2007) and Spain (Accensi *et al*., 2004). In Pakistan limited studies have been

*Corresponding author E-mail: mzargham2000@yahoo.com

reported upon presence of mycotoxins in poultry feeds and agricultural products (Hanif *et al*., 2006; Saleemullah *et al.,* 2006; Russel & Peterson, 2007). *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus* fungi were isolated from the maize in Swat Valley of Northwest Frontier Province (Shah *et al.,* 2008). Afzal *et al.,* (1979) reported aflatoxins and aflatoxigenic fungi in animal feedstuffs samples (*n*=54) collected over a six-month period. Since then no published work is available upon the presence of toxigenic fungi in farm animal or poultry feed stuffs, the present study was designed with the aims to: (i) determine the mycoflora of poultry feeds and (ii) evaluate the ability of *Aspergillus* species to produce aflatoxins and ochratoxin. Agricultural products grown in Pakistan constitute the major component of poultry feeds; hence this study will also indirectly highlight the toxigenic mycobiota of the agricultural products of the region.

Materials and Methods

Study region: Faisalabad, the third big city of Pakistan is located in the central Punjab province. Faisalabad itself and its adjacent towns constitute a major region of broiler and layer poultry farming. Agricultural based poultry feed ingredients are produced and also brought in this area from all parts of the country. All the commercial feed manufacturing units located in different parts of the country supply prepared feed to this region. Hence, a study conducted in Faisalabad could be a representative of the whole country.

Sample collection: Poultry feed samples (*n*=119) comprising of commercially prepared feed (*n*=89) and farm mixed feed (*n*=30) were collected over a period of two years from August 2005 to August 2007. Samples were collected at regular intervals evenly spread over the study period. The samples of poultry feeds were collected from feeds present at the farms. For each sample, 3 kg feed was collected from each of the three different areas of a lot. After thorough mixing a composite sample of 1kg was kept in a polythene bag and stored in refrigerator prior to inoculation onto culture media every week.

Isolation and identification of fungi: Feed samples were initially inoculated onto potato dextrose agar (Samson *et al*., 2004) and then sub-cultured on Czapek dox solution agar (Pitt & Hocking, 1997), Czapek yeast autolysate agar (Pitt & Hocking, 1997) and yeast extract sucrose agar (Samson *et al*., 2004). The plates were incubated at 27 ºC in dark for 10 days. Slide cultures were prepared for microscopic examination. Fungi were identified (Klich & Pitt, 1988; Singh *et al*., 1991; Pitt & Hocking, 1997; Klich, 1995). The fungal isolates were also cultured on PDA slants, incubated at 27ºC for 10 days and stored at 4ºC in refrigerator for future studies. The isolation frequency (Fr) and relative density (RD) of species were calculated according to Gonzalez *et al.,* (1995) as follows:

$$\text{Fr (\%)} = \frac{\text{Number of samples with a species or genus}}{\text{Total number of samples}} \text{ X } 100$$

$$\text{RD (\%)} = \frac{\text{Number of isolates of a species or genus}}{\text{Total number of fungi isolated}} \text{ X } 100$$

Mycotoxins analysis: Each *Aspergillus* isolate was inoculated on yeast extract sucrose agar medium (YES) at three equidistant points on a Petri plate and incubated at 27ºC for seven days in dark. Fungal cultures were extracted by micro-scale extraction (Smedsgaard, 1997) with a modification that a total of 18 plugs (6 mm diameter) were cut from each plate in equal number from the middle, rim and areas between the colonies.

These plugs were transferred to a 10 ml glass screw-capped vial containing 3 ml solvent mixture comprising of methanol-dichloromethane-ethyl acetate (1:2:3) containing 1% (v/v) formic acid and were extracted ultrasonically for 60 minutes. A 0.5 ml of the extract was shifted to a glass vial and evaporated to dryness under a gentle stream of nitrogen. The evaporated residues of 0.5 ml extract were re-dissolved ultrasonically for 10 minutes in 400 µl methanol containing 0.6% (v/v) formic acid, 0.02% v/v) hydrochloric acid and 2.5% (v/v) water. Analysis of AF and OTA was performed on the high pressure liquid chromatography system (Prominence ™, Shimadzu®) equipped with florescent detector RF-10AXL® (Shimadzu) by using C-18 column**,** Mediterranean Sea18® 5µm 25cm x 0.46 (Teknokroma, Spain).

For AF analysis part of the extract was derivatized (Anon., 2000). Mobile phase consisted of a mixture of acetonitrile: methanol: water (22.5: 22.5: 55) with the flow rate of 1.0 ml /min, 30˚C. The emission and excitation wavelengths were 360 nm and 440 nm, respectively.

OTA Analysis was performed using a mixture of acetonitrile: water: acetic acid (57: 41: 2) as mobile phase with a flow rate of 1.0 ml/min at 40˚C. The emission and excitation wavelengths were 333 nm and 477 nm, respectively. OTA was confirmed by methyl ester formation (Zimmerli & Dick, 1995).

Results

Mycological isolation and identification: Out of total (*n*=119), commercially prepared (*n*=89) and farm-mixed (*n*=30) feed samples, fungi could be isolated from 87 (73.11%), 62 (69.66%) and 25 (83.33%) samples, respectively. Total isolates from these feed samples were 92, 65 and 27, respectively. Table 1 presents the mycological data of poultry feeds. In commercially prepared poultry feeds, Fr for *Aspergillus, Alternaria, Fusarium and Penicillium* was 43.82, 1.12, 5.61 and 22.47%, respectively. RD of *Aspergillus, Alternaria, Fusarium* and *Penicillium* was 60.0, 1.54, 7.69 and 30.77%, respectively. In farm-mixed poultry feeds, Fr for *Aspergillus, Alternaria, Fusarium and Penicillium* was 46.66, 10, 10 and 23.33% whereas RD for these fungi was 51.85, 11.11, 11.11 and 25.93%, respectively. Data of total samples of commercially prepared and farm-mixed feeds also showed similar trends of Fr and RD as observed in either commercially prepared or farm-mixed feeds.

***Aspergillus* isolates of feed samples:** RD of different *Aspergillus* isolates has been presented in Table 2. Based upon feed samples, fungal isolates or *Aspergillus* isolates, the most frequently isolated fungi were *Aspergillus niger* aggregates followed by *A. flavus, A. ochraceous, A. parasiticus, A. carbonarius, A. fumigatus* and *A. oryzae*.

Aflatoxigenic and ochratoxigenic *Aspergillus* isolates: Out of 53 isolates of *Aspergilli*, 39 (73.58%) were found toxigenic capable of producing either AF or OTA. AF produced by different isolates of *Aspergillus* species has been presented in Table 3. Among *A. flavus* and *A. parasiticus* the aflatoxigenic characters were observed in 83.33 and 85.71% isolates. Among toxigenic *A. flavus* isolates (10/12), six produced four AFs (AFB_1, AFB_2, AFG_1 and AFG_2), two produced AFB1, AFB2, AFG1, one produced AFB1, AFB2, AFG2 and one isolate produced AFB1 and AFB2. Among aflatoxigenic isolates of *A. parasiticus* (6/7), five produced four AFs (AFB1, AFB2, AFG1, AFG2) while one produced three AFs (AFB1, AFB2, AFG1). Among *A. carbonarius, A. niger* aggregates and *A. ochraceous* the ochratoxigenic character was observed in 50, 65 and 100% isolates, respectively (Table 4). The only isolate of *A. oryzae* was atoxigenic.

Table 1. Isolation frequency (Fr) and relative densities (RD) of different genera of fungi isolated from poultry feeds.

Fungi isolated	*Aspergillus*	*Alternaria*	*Fusarium*	*Penicillium*
	Commercially prepared poultry feeds (n=89)			
Isolates No.	39	1	5	20
Fr (%)[1]	43.82	1.12	5.61	22.47
RD (%)[2]	60	1.54	7.69	30.77
	Farm-mixed poultry feeds (n=30)			
Isolates No.	14	3	3	7
Fr (%)	46.66	10	10	23.33
RD (%)	51.85	11.11	11.11	25.93
	Total poultry feeds (n=119)			
Isolates No.	53	4	8	27
Fr (%)	44.54	3.36	6.72	22.68
RD (%)	57.61	4.35	8.70	29.35

[1] Fr = Isolation frequency
[2] RD = Relative density

Table 2. Isolation frequency and Relative density of *Aspergillus* fungi isolated from feed samples (n=119).

Fungi isolated	No. of isolates	Isolation frequency (%)	Relative density (%) based upon total isolates (n=92)	Relative density (%) based upon *Aspergillus* isolates (n=53)
A. carbonarius	2	1.68	2.17	3.77
A. flavus	12	10.08	13.04	22.64
A. fumigatus	2	1.68	2.17	3.77
A. niger aggregates	20	16.80	21.74	37.74
A. ochraceous	9	7.56	9.78	16.98
A. oryzae	1	0.84	1.09	1.89
A. parasiticus	7	5. 88	7.61	13.21
Total *Aspergillus*	**53**	**44.53**	**57.61**	

Table 3. Aflatoxigenic characters of *Aspergillus* species isolated from poultry feeds.

Fungal species		*Aspergillus flavus*	*Aspergillus parasiticus*
No. of isolates		12	7
Aflatoxigenic isolates No. (%)		10 (83.33)	6 (85.71)
Aflatoxin levels (µg/g)	AFB_1	0.00157-1.9887	0.0135-1.357
	AFB_2	0.0003-0.3279	0.000395-0.00124
	AFG_1	0.000127-0.06565	0.00034-0.01147
	AFG_2	0.00008-0.00073	0.00045-0.00160
	AF total	0.0097-1.9807	0.00095-1.3700454

Table 4. Ochratoxigenic characters of *Aspergillus* species isolated from poultry feeds.

Fungal species	Total isolates	Ochratoxigenic No. (%)	Ochratoxin A levels (µg/g)
A. carbonarius	2	1 (50)	0.0-0.0056
A. niger aggregates	20	13 (65)	0.00136-0.060
A. ochraceous	9	9 (100)	0.00179-16.7168

Discussion

Fungal contamination was present in a high proportion of the feed samples (73.10%) which was in line with the reports of isolation of fungi from poultry feeds in other regions including Brazil (Oliviera *et al*., 2006; Rossa *et al*., 2006), Argentina (Dalcero *et al*., 1997) and Nigeria (Osho *et al*., 2007). Different genera of contaminating fungi in the present study ranked according to their isolation frequency were *Aspergillus, Penicillium, Fusarium* and *Alternaria*. From Pakistan 39 fungal isolates from animal feed stuffs including *Aspergillus* (21/39), *Mucor* (7/39), *Rhizopus* (6/39), *Fusarium* (4/39) and *Penicillium* (1/39) have been reported (Afzal *et al.,* 1979). A recent report described that 70% of the maize samples collected from different parts of Paksitan were infested with *A. flavus, A. niger, A. wentii* and *Penicillium* spp., (Niaz & Dawar, 2009). High isolation frequencies of *Aspergillus* and *Penicillium* fungi from mixed poultry feeds have been reported in Brazil (Simas *et al*., 2007), Argentina (Dalcero *et al*., 2008) and Spain (Abarca *et al*., 1994; Bragualt *et al*., 1995). Highest isolation frequency of *Aspergillus* in mixed poultry feed and component raw materials have also been reported in Sapin (Accensi *et al*., 2004). Different cereals constitute a major component of poultry feed thus, cereal mycoflora may also be a reflection of fungal contamination of poultry /animal feeds. High frequency of *Aspergillus* fungi was present in maize in Italy (Giorni *et al*., 2007) and Ghana (Kpodo *et al*., 2000). In Algerian wheat samples high levels *Aspergillus* (66 to 84%) were followed by *Penicillium, Fusarium, Alternaria* and *Mucor* (Riba *et al*., 2008). High contamination level of *Aspergilli* might be due to their high temperature tolerance character (Battilani *et al*., 2003), which might also be responsible for the high frequency of *Aspergillus* in poultry feeds in Pakistan.

Fungal contamination frequency was higher in farm-mixed poultry feeds (83.33%) as compared with commercial poultry feeds (69.66%). A possible reason for low fungal contamination frequency in commercially prepared feeds might be inclusion of antifungal agents to prevent fungal growth during prolonged and varied storage conditions at farms whereas in farm-mixed poultry feeds such inclusions might be skipped due to short duration of storage. Another reason might be that commercial poultry feed is usually in pellet or crumbs form which is prepared at high temperature thus reducing its bacterial and fungal loads (Chelkowisky 1991; Dalcero *et al*., 2002) whereas farm mixed feed is invariably in mash form, not exposed to high temperatures and might have high microorganism count.

Among the *Aspergilli* isolated from feed samples, *A. niger* aggregates were the predominant species followed by *A. flavus, A. ochraceous* and *A. parasiticus*. These results differ from some reports describing *A. flavus* as the predominant species followed by *A. niger* aggregates (Accensi *et al*., 2004; Rosa *et al*., 2006; Somashekar *et al*., 2004). Similarly *A. flavus* was the predominant species in pet foods (Martins *et al*., 2003) and wheat samples from Algeria (Riba *et al*., 2008). A higher occurrence of black *Aspergilli* reported in grapes in France during 2001-2003 was incriminated to temperatures higher than 37°C (Bejaoui *et al*., 2006). In Pakistan high temperature and humidity might be responsible for higher frequency of *A. niger* aggregates in poultry feeds compared with other species of *Aspergillus*.

Out of 53 isolates of *Aspergilli*, 39 (73.58%) were found toxigenic capable of producing either AF or OTA. Rashid *et al*., (2008) reported one toxigenic isolate of *A. parasiticus* out of 157 *A. flavus* and 36 *A. parasiticus* strains isolated from stored wheat grains from Punjab, Sindh and NWFP provinces of Pakistan. The AF producing potential of toxigenic isolates on YES medium varied from 0.0009 to 1.9887 µg/g which were in line with similar results in poultry feed (Frage *et al*., 2007) and pet foods (Campos *et al*., 2008). Some isolates of aflatoxigenic fungi produced all four toxins (AFB1, AFB2,

AFG1, AFG2) while others produced either three or two AFs. Fraga *et al*., (2007) also reported that among aflatoxins producing species isolated from poultry feeds, 63% species produced AFB_1, AFB_2, AFG_1 and AFG_2 whereas 27% belonging to *A. flavus* and *A. candidus* strains produced AFB_1, AFB_2, and AFG_1 but no AFG_2. Similarly isolates of *A. flavus* from cocoa beans varied in their ability to produce different AFs (Sanchez-Hervas *et al*., 2008).

Ochratoxigenic *Aspergilli* isolates in the present study (*A. niger* aggregates, *A. carbonarius and A. ochraceous*) varied in OTA producing abilities from 0.0014 to 16.72 µg/g. In Brazilian poultry feeds ochratoxigenic species included *A. niger*, *A. carbonarius*, *A. ochraceous*, *A. melleus* and *P. verrucosum*. The OTA production of these isolates on CYA medium ranged from 25 to 120 µg/kg (Rosa *et al*., 2006). *Aspergilli* isolated from cocoa beans included *A. niger* aggregates and *A. carbonarius* with OTA production ranging from 0.5 to 90 µg/g (Sanchez-Hervas *et al*., 2008).

The present study has provided information about the contaminating toxigenic mycoflora of poultry feeds in Pakistan. It is the first report describing isolation of OTA producing Aspergilli in poultry feeds in Pakistan. *Aspergillus* fungi should be of concern because of their association with AFs and OTA. Presence of other contaminating fungi like *Fusarium* and *Penicillium* suggested the possible contamination by several mycotoxins including fusrio-toxins and fumonisins. Keeping in view the tropical climatic conditions of the study area, these fungi may propagate to produce AF and OTA to the levels injurious to poultry birds resulting in mycotoxicosis. The presence of toxigenic fungi in poultry feeds in Pakistan warrant the adoption of effective measures to avoid their propagation at pre-harvest, post-harvest stage of cereal crops and storage of ingredients and prepared feeds to prevent build up of mycotoxins to the hazardous levels.

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