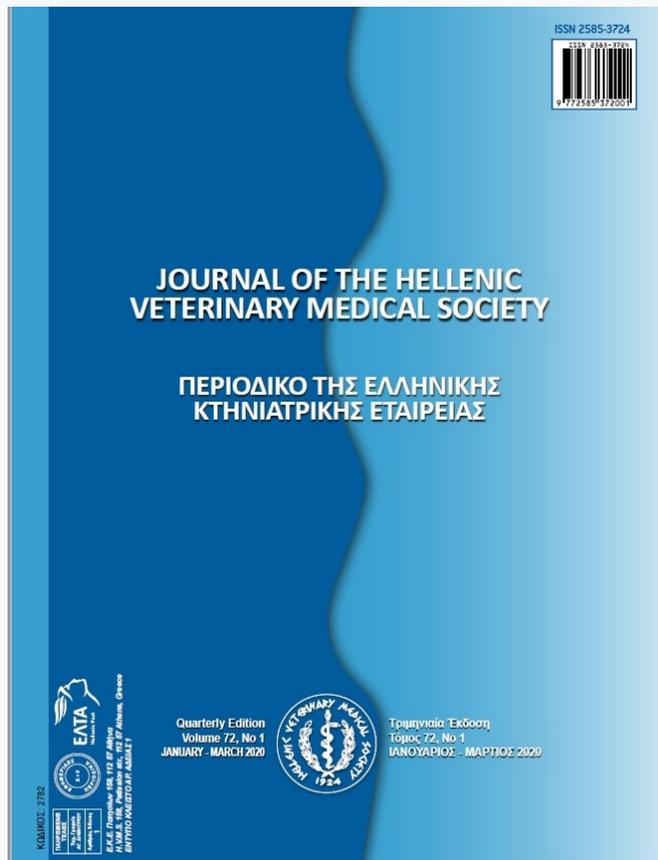


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Detection of Mycotoxigenic Fungi and Mycotoxins in Poultry Feed of Balochistan Pakistan

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ABSTRACT: Mycotoxins are secondary toxic metabolites of fungi, commonly associated to agriculture commodities. Aspergillus, Fusarium, Penicillium and Claviceps genera are the most common mycotoxigenic fungi and the most important types of mycotoxin are Aflatoxins, Zearalenone, Ochratoxin, Fumonisin and Trichothecenes. Mycotoxins can be carcinogenic, cytotoxic, mutagenic, teratogenic, neurotoxic, nephrotoxic, estrogenic and immunosuppressant. This study was conducted to examine the presence of mycotoxigenic fungi and mycotoxins in poultry feed used by the poultry farmers of Balochistan. Samples (n=100) were analyzed and found contaminated with fungi such as Aspergillus, Mucor, Rhizopus and Fusarium genera, amongst them Aspergillus species was observed highly prevalent (89%). Aflatoxin production in culture plates were initially screened with the help of ammonia hydroxide vapor test, and subsequently analyzed with the help of AgraStrip®. The Aflatoxins (77%) and Fumonisin (92%) were found in direct examination of feed samples. Toxin presence was also confirmed with the help of ELISA using AgraQuant® kits. Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) were used for characterization and quantification of aflatoxins. It was concluded that the feed samples were abundantly contaminated with aflatoxins and fumonisins and the fungi responsible for their production. This contaminated feed is one of the reasons for economic losses for poultry farmers of the region.

Keywords: Aspergillus; Aflatoxins; Fumonisin; Chromatography; ELISA

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INTRODUCTION

Mycotoxins are structurally diverse low molecular weight secondary fungal metabolites produced mostly by the fungal species of *Penicillium*, *Fusarium*, *Aspergillus*, *Alternaria* and *Claviceps* (Flores-Flores *et al.*, 2018). Exposure of humans to mycotoxins occurs through the consumption of contaminated agriculture products (rice, cereals, vegetables etc.) or through the intake of contaminated animal products (milk, eggs, etc.) (Capriotti *et al.*, 2012). Contamination of food and feed by fungi and their mycotoxins can occur at every step of the food supply chain from agricultural production, storage and processing (Khan *et al.*, 2020). Several environmental factors including storage time, temperature, pH, oxygen, moisture and humidity promote the growth of fungi and subsequently the production of mycotoxin (Kana *et al.*, 2013).

Around the world, it is estimated that about 25% of the crops are contaminated with mycotoxins (FAO, 2002). More than 480 mycotoxins are currently known, and aflatoxins are of the greatest importance. Among the reported mycotoxins, the most economically important are Aflatoxins, Fumonisin, Ochratoxin A, Trichothecenes, Zearalenone, and Citrinin (Jajić *et al.*, 2018; Shi *et al.* 2018).

Metabolic function and activity of mycotoxins has not been reported yet (da Rocha *et al.*, 2014). Though mycotoxins have not been extensively studied in Pakistan, their presence in poultry feed is reported by some researchers (Rashid *et al.*, 2012). Mycotoxins such as aflatoxins are also regularly detected in poultry feed from countries around the globe, such as Brazil, Nigeria, Colombia, Malaysia, Saudi Arabia, and India, (Anjum *et al.*, 2012).

As poultry feed can contain toxigenic fungi, it is therefore important to regularly monitor feed for their presence, to ensure food safety and prevent its spread to the human food chain. This study was designed to investigate the presence of mycotoxigenic fungi and mycotoxins in poultry feed used by the poultry farmers of Balochistan.

Balochistan is largely consist of arid and semi-arid lands, with a human population scattered in rural areas, dependent mostly on livestock and poultry farming (Mirza *et al.*, 2009). Our study is focused on small scale poultry farming, mostly practiced in the province due to limited facilities and economic constrains.

MATERIALS AND METHODS

Sample Collection

Poultry feed samples were collected (n=100) from different poultry farms of Quetta, a region of Balochistan. Feed samples were stored in sterile polythene bags and transported to the Food Microbiology and Bioprocess Technology Laboratory, Department of the Microbiology University of Balochistan, Quetta. The samples were divided into two parts each for mycotoxigenic fungal culture isolation and mycotoxins detection.

Isolation and identification of fungi

For each poultry feed sample, 10-fold serial dilution of one-gram feed was performed by adding it to 10mL of sterile distilled water. One ml from each dilution was spread over Sabouraud Dextrose Agar (SDA) supplemented with Chloramphenicol. The Petri plates were incubated at room temperature (25±1 °C) for 3-7 days. After incubation isolated colonies of fungi were sub-cultured on Potato Dextrose Agar (PDA). The preliminary identification was performed by the assessment of morphological, cultural and microscopic characteristics (Ali *et al.*, 2018).

Mycotoxigenic potential determination

For the determination of mycotoxigenic potential of the fungi isolates, a specific culture media Yeast Extract Agar (YEA) was used by sub-culturing the representative fungal colonies of the isolates from PDA over YEA and incubated at room temperature for 7 days (Shekhar *et al.*, 2017).

Ammonia vapor test for mycotoxin

Concentrated solution of ammonium hydroxide (1-2 drops) were dropped over the surface of *A. flavus* grown on YEA and left for 10–15 minutes. The appearance of pink or red color in the culture medium showed the production of mycotoxin (Shekhar *et al.*, 2017).

Detection of mycotoxins production by Agra strip and Thin Layer Chromatography

Fungal culture (45 days) on Potato Dextrose Broth (PDB) was mixed with extractant 10 mL Chloroform: Acetone (85:15 v/v) and incubated at room temperature for 15-20 minutes with vigorous stirring for 5 minutes. The extracts were then filtered with Whatman No.1 filter paper and the filtrates were evaporated. The residues were resuspended in 500µL of methanol and aseptically filtered. The filtrates were

then collected for aflatoxin strip test. Part of the fungal culture extracts were processed for TLC by spotting it on TLC plates along with aflatoxin standards. Plate was placed in chromatographic tank using Toluene and Acetone as mobile phase (1:1). The plates were observed in UV lamp at 365 nm wavelength after completion of the process (Yazdani et al., 2010).

Fourier Transform Infrared Spectroscopy

Fungal culture (45 days) on PDB was mixed with extractant 10 mL chloroform: acetone (85:15 v/v) and incubated at room temperature (25°C) for 15-20 minutes with vigorous stirring for 5 minutes (Yazdani et al., 2010). The extracts were filtered through Whatman No.1 filter paper and the filtrates were evaporated. The residues were resuspended in 500µL of methanol and filtered aseptically for FTIR analysis. The samples were then process for FTIR in the range of 3500-500 cm⁻¹. The peak was studied for the mycotoxin presence.

Detection of Aflatoxins in Feed by Agra Strip test

The 2nd part of the feed samples was directly used for mycotoxin presence, where Aflatoxin test kits AgraStrip® was used having 4ppb Cut-off value. The extraction procedure was performed according to manufactures protocol. Briefly, 10g of ground samples, 60mL of ethanol and distilled water were added to the flask and covered entirely. The Samples were extracted at ratio of 1:2 and mixed vigorously for 1 minute using vortex. Extracts were filtered by Whatman filter paper No.1. and used for aflatoxin detection. An amount 50µL diluent assay was added into the micro-well of aflatoxins detection kit. Sample 50µL was mixed into a well through sterile pipette. Aflatoxin 4ppb strip was added into well and left for 5 minutes and results were observed subsequently.

Detection of Fumonisin from Feed by AgraStrip test

Manufactures protocol was followed for extraction. In brief, each sample, 10g of ground sample was added in whirl-pak filter bag and buffer bag was added followed by the addition of 30mL distilled water was added and mixed for 5 minutes. Then 1mL of buffering agent and 50µL sample was added in yellow tube and mixed vigorously. The dip strip was placed in the well and incubated for 3 minutes at 40-45 °C Bands were observed after 3 minutes of incubation.

Detection of mycotoxins in feed by Enzyme Linked

Immunosorbent Assay (ELISA)

A competitive ELISA was performed with the help of Aflatoxin and Fumonisin ELISA test kit Agra-Quant® as per manufacturer instructions. In brief, 5g of ground poultry feed sample was extracted in 2 mL of methanol (70%). Mixed vigorously with vortex stirrer for 5 minutes and filtered by Whatman filter paper No. 1. The extracts were diluted with distilled water in 1:1 ration for aflatoxins and 1:14 for fumonisins. 50µL of the diluted filtrate was used. An amount 200µL of conjugate and 100µL of each standard were added in dilution wells. Then contents were thoroughly mixed and 100µL of it was transferred to the well coated with antibody and then well were incubated for 10 minutes at room temperature. The incubated contents were washed out with distilled water 5 times and 100µL of substrate was added to wells coated with antibodies and incubated again at room temperature for 5 minutes. 100µL of stop solution to each well was added and mixed slightly and the absorbance measured by using Microplate reader at 450 nm.

Detection of aflatoxins in feed by TLC and HPLC

Feed samples were processed by thin-layer chromatography. Briefly, for each feed sample 10g of ground, 90mL chloroform 10mL distilled water and 2g sodium chloride was added in the flask. Contents were shaken at room temperature on an orbital shaker for 5 minutes. The coated TLC plate was spotted with Aflatoxin standard on the baseline along with spotted samples. TLC plate was then placed in a TLC chamber filled with solvent mixture of Toluene: Acetone (1:1). The TLC plate was removed when the solvent front raised, and the solvent finishing line was recorded. Plate was dried in the air and visualized under UV lamp at 364 nm wavelength. Spots with blue fluorescence were encircled and RF value was measured and matched against the standard (Rafique et al., 2018).

Aflatoxins in feed samples were quantified by HPLC according to the procedure defined 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 minute.

RESULTS

The fungal isolates were morphologically identified based on its cultural characteristic and microscopic morphology. It was found that almost all poultry feed samples were contaminated with at least

one fungus, where as *Aspergillus* spp., *Mucor* spp., *Rhizopus* spp., and *Fusarium* spp. were abundantly found. Among these genera the highest frequency was recorded for *Aspergillus* genus (Table 1). In the present study, *Aspergillus flavus* showed the highest prevalence rate (46%), followed by *Aspergillus niger* (22%), *Aspergillus parasiticus* (21%), *Mucor* (6%), *Rhizopus* (4%) and *Fusarium proliferatum* (1%). Aflatoxigenic properties of the fungal isolates were preliminarily confirmed with the help of ammonium vapors test in YEA medium (Figure 1). Production of mycotoxins (Aflatoxins and Fumonisin) were further confirmed with the help of AgraStrip® for total Aflatoxin and Fumonisin and ELISA. Direct examination of feed samples for mycotoxins by AgraStrip and ELISA revealed that the majority of the feed samples was contaminated with Aflatoxins (77%) and Fumonisin (92%).

Table 1. Prevalence of different fungi in feed samples

Fungal Genera	Growth (%)
<i>Aspergillus flavus</i>	46%
<i>Aspergillus niger</i>	22%
<i>Aspergillus parasiticus</i>	21%
<i>Mucor</i> spp.	6%
<i>Rhizopus</i> spp.	4%
<i>Fusarium proliferatum</i>	1%

The quantitative analysis of mycotoxins through ELISA revealed that the aflatoxin quantities in dif-

ferent samples vary between 0-40 PPB while that of Fumonisin between 0-500 PPB. The concentration values of Aflatoxins and Fumonisin in feed samples were determined from a calibration curve prepared by using known concentrations. Aflatoxin produced by fungal isolates in culture media and extracted from feed samples was also confirmed with Thin Layer Chromatography. Blue/green fluorescence bands under UV light confirmed the presence of aflatoxins.

The FTIR spectroscopy studied in the wavelength range of 3500–500 cm^{-1} for the toxins present showed different peaks for functional groups indication confirming the presence of Aflatoxins and Fumonisin. The FTIR peaks (spectra) were observed at frequency of 3280.10 cm^{-1} correspond for N-H and O-H stretching, 2969 cm^{-1} for CH_2 asymmetric stretch composed of lipids with a little contribution of proteins, carbohydrates and nucleic acids, 1737 cm^{-1} for Ester C=O, 1651 cm^{-1} for C=O, 1365 cm^{-1} for CH_3 , 1228-1216 cm^{-1} for PO_2 asymmetric stretching mainly composed of nucleic acids with little contribution from phospholipids and for PO_2 symmetric stretching nucleic acids and phospholipids stretch of C-O glycogen 1044 cm^{-1} were observed.

The HPLC analysis of selected samples confirmed the presence of aflatoxins and the aflatoxins B1 and B2 in the samples were quantified as 35.94 μg and 2.53 μg respectively (Figure 2).



Figure 1. (A) Shows aflatoxigenic nature under UV light at 365 nm, (B) Ammonia vapor

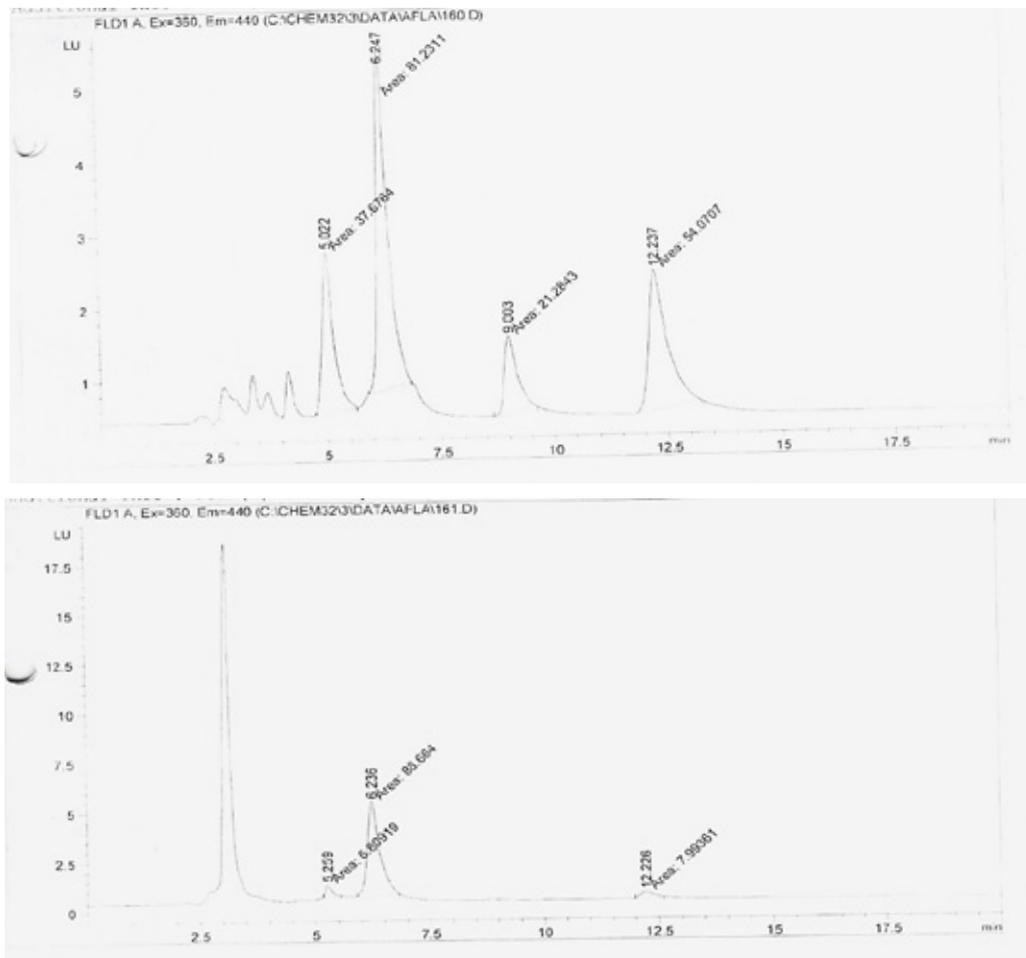


Figure2. Aflatoxins chromatogram of HPLC detection,(A) Standard (B) Sample

DISCUSSION

Occurrence of fungi in poultry feed is not only affecting the organoleptic properties of feed but some of them are lethal toxin producers (Greco et al., 2014). In our study, 100% of the feed samples were found contaminated with fungal genera. Similar results were reported in other regions, such as Brazil (Rosa et al., 2006), Argentina (Dalcero et al., 1997), Nigeria (Osho et al., 2007) and in Pakistan (Sana et al., 2019). Our study showed a prevalent *A. flavus* (46%) contamination, a finding that is in compliance with the study conducted in Saudi Arabia by Gherbawy et al. (2019) that reported a prevalence of 59% *A. flavus* in poultry feed Rafique et al. (2018) reported 34% *A. flavus* in Pakistan, lower than our study. We found 22% *A. niger*, which follow the study conducted by Raju et al. (2018) in Kerala India. Compared to our results regarding *A. parasiticus* (21%), Ahmed et al. (2017) reported 51% *A. parasiticus* presence in their study conducted in Iraq. In our study the presence of *Mucor* (6%), *Rhizopus* (4%) and *Fusarium proliferatum* (1%) were reported, which are following the figures

reported by Pacinet al., (2003) from Ecuador. The *A. flavus* was found the most dominant fungi, most likely due to its ability to tolerate harsh environment and high temperature (Battilani et al., 2003).

Aflatoxins are mostly produced by *Aspergillus* species include *A. parasiticus* and *A. flavus*. Improper storage conditions in poultry feed lead to the production of Aflatoxins. Similarly to our study, Yazdani et al. (2010) performed ammonia vapor test for aflatoxigenic potential determination in their study. Aflatoxins and fumonisins showed 77% and 92% contamination confirmed by AgraStrip® and ELISA AgraQuant®. The ELISA and HPLC are equally sensitive and recommended for the detection of mycotoxins (Beyene et al., 2019).

In our study the mycotoxins production was also analyzed by using FTIR, the results were comparable to that of Kos et al. (2016) and Sieger et al. (2017). Chromatographic techniques such as TLC and HPLC were performed for the mycotoxin detection in our study. Aflatoxins were identified and quantified by HPLC which showed that the AFB1 quantity was

35.94 $\mu\text{g/g}$ and that of AFB₂ was 2.53 $\mu\text{g/g}$, lower from that found by Saleemi et al. (2012). The HPLC is now widely used technique for identification and quantification of Aflatoxin (Wacoo et al., 2014).

It was concluded from this study that majority of poultry feeds in Balochistan are contaminated with Aflatoxin and Fumonisin. This contamination is usually linked to the uses of raw materials, environmental conditions and other sources, such as storage and

transportation practices. Precautions and limitation of fungal growth at different stages of feed preparation can limit the contamination.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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