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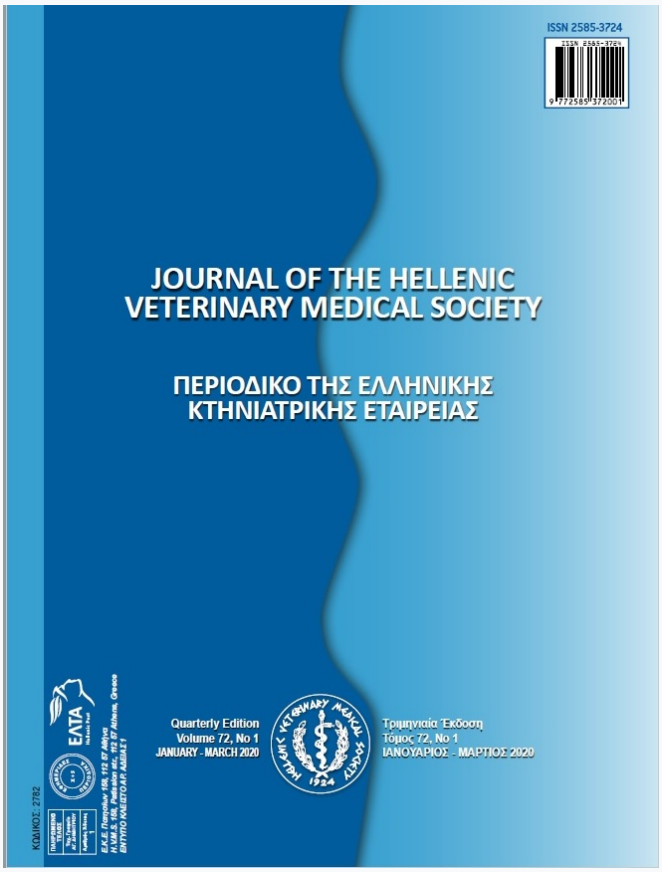

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Research article
Ερευνητικό άρθρο

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, Fumonisins 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 Fumonisins (92%) were found in direct examination of feed samples. Toxins 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 lossesfor 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.) (Capriottiet 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, Fumonisins, 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 10mLof 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 ^{0}C) for 3-7 days. After incubation isolated colonies of fungi were sub-culturedon 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)wasused by sub-culturing therepresentative 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 redcolor 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 mLChloroform: 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 wavelengthafter 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 wasstudied for the mycotoxin presence.

Detection of Aflatoxins in Feed by Agra Strip test

The 2nd part of the feed sampleswas directly used for mycotoxin presence, where Aflatoxin test kits AgraStrip® was used having 4ppb Cut-offvalue. 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. andused for aflatoxin detection. An amount 50µL diluent assay was added into themicro-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 5minutes 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 bagand buffer bag was added followed by the addition of 30mL distilled water was added and mixedfor 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 ^{0}C Bands were observed after 3 minutes of incubation.

Detection of mycotoxins infeed by Enzyme Linked Immunosorbent Assay (ELISA)

A competitive ELISA was performed with the help of Aflatoxin and Fumonisin ELISA test kit AgraQuant® as per manufacturerinstructions.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 werethoroughlymixed and 100µL of it was transferred tothe 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 againat room temperature for 5 minutes. 100µL of stop solution to each well was added and mixed slightly andthe 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 withspotted samples. TLC plate wasthen 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 thesolvent finishing line was recorded. Plate was dried in the air and visualized under UV lamp at 364 nm wavelength. Spots withblue 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-Hernándezet 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 highestfrequency 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 preliminary confirmedwith the help of ammonium vapors test in YEA medium (Figure1).Production of mycotoxins (Aflatoxins and Fumonisins) were furtherconfirmed with the help of AgraStrip® for total Aflatoxin and Fumonisins and ELISA. Direct examinationof feed samples for mycotoxins by AgraStrip and ELISA revealed that the majority of the feed samples was contaminated with Aflatoxins (77%) and Fumonisins (92%).

Table 1. Prevalence of different fungi in feed samples

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

The quantitative analysis of mycotoxins through ELISA revealed that the aflatoxin quantities in different samples vary between 0-40 PPB while that of Fumonisin between 0-500 PPB. The concentration values of Aflatoxins and Fumonisins in feed samples were determined from a calibration curve prepared by using known concentrations.Aflatoxin produced byfungal isolatesin 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−1for 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 CH3, 1228-1216 cm−1 for PO_2asymmetric 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).

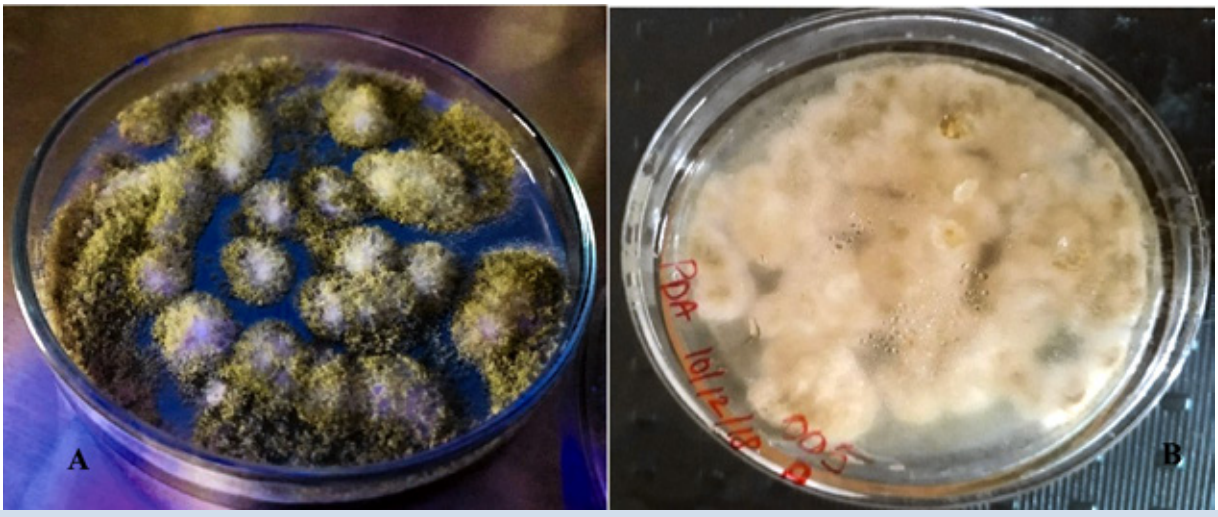

Figure1. (A) Shows aflatoxigenic nature under UV light at 365 nm,(B)Ammonia vapor

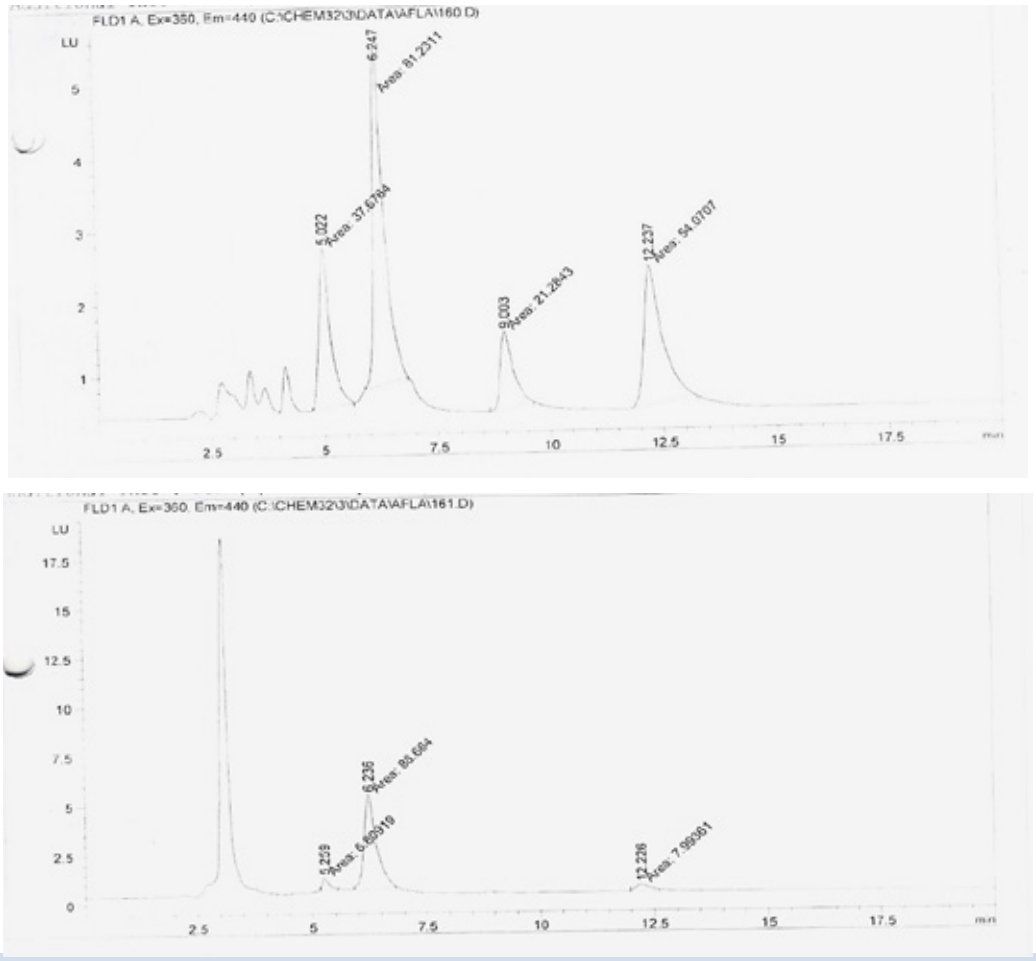

Figure2. Aflatoxins chromatogram of HPLC detection,(A) Standard (B) Sample

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 followingthe figures reported by Pacinet al.,(2003) from Ecuador. The *A. flavus* was found the most dominant fungi,most likely due to its ability to tolerateharsh environment and high temperature (Battilaniet 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 foraflatoxigenic 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

35.94µg/g and that of AFB2 was 2.53µg/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.

REFERENCES

Ahmed DA, Al-Khafaji NJ, Ahmed LT(2017) Isolation and molecular identification of Aspergillus spp. collected from different sources of animals feed. Int J Curr Microbiol App Sci*6*(6):1792-1797.

Ali FS, Akbar A,Prasongsuk S, Permpornsakul P, Yanwisetpakdee B, Lotrakul P, Punnapayak H, Asrar M, Ali I(2018) Penicillium Imranianum, A new species from the man-made solar saltern of Phetchaburi province, Thailand. Pak J Bot 50(5):2055-2058.

Alvarado-Hernández JR, Carvajal-Moreno M, Rojo-Callejas F, Ruiz-Velasco S(2016) Aflatoxins in natural peanuts (*Arachishypogaea* L.) of Mexico: Validation of the biochemical methods for extraction and quantification. J Plant BiochemPhysiol 4:1000168.

Anjum MA, Khan SH, Sahota AW, Sardar R(2012) Assessment of aflatoxin B1 in commercial poultry feed and feed ingredients. J Anim Plant Sci 21: 117-120.

Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P,Kozakiewicz Z(2003) Occurrence of ochratoxin A–producing fungi in grapes grown in Italy. J Food Prot 66(4):633-636.

BeyeneAM, Du XE, Schrunk D, Steve E,Rumbeiha KW(2019) High-performance liquid chromatography and Enzyme-Linked Immunosorbent Assay techniques for detection and quantification of aflatoxin B1 in feed samples: a comparative study. BMC Res Notes 12: 492. https://doi.org/10.1186/s13104-019-4538-z

Capriotti AL, Caruso G, Cavaliere C, Foglia P, Samperi R,LaganàA(2012) Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/ mass spectrometry. Mass Spectr Rev 31(4):466-503.

Dalcero A, Magnoli C, Chiacchiera S, Palacios G, Reynoso M(1997)Mycoflora and incidence of aflatoxin B1, zearalenone and deoxynivalenol in poultry feeds in Argentina. Mycopathologia 137(3):179-184.

da Rocha EBM, Freire FCO, Maia FEF, Guedes FIF, Rondina D (2014). Mycotoxins and their effects on human and animal health. Food Cont 36(1):159-165. DOI: http://doi.org/10.1016/j.foodcont.2013.08.021

FAO (2002) The State of Food Insecurity in the World (SOFI), Food and Agriculture Organization of the United Nations. http://www.fao.org/docrep/005/y7352e/y7352e0 0.htm.

Flores-Flores ME, González-Peñas E(2018) Analysis of mycotoxins in Spanish milk. J Dairy Sci 101:113–117

Gherbawy YA, Shebany YM,Alharthy H (2019) Aspergilli and Their Aflatoxins Contamination of Poultry and Animal Feedstuff Samples in Western Region of Saudi Arabia. Sains Malaysiana48(4):765-771.

Greco MV, Franchi ML, Rico Golba SL, Pardo AG, Pose GN(2014) Mycotoxins and mycotoxigenic fungi in poultry feed for food-producing animals. Sci World J 2014.

Jajić I, Glamočić D, Krstović S, PolovinskiHorvatović M (2018) Aflatoxin M1 occurrence in Serbian milk and its impact on legislative. J Hellenic Vet Med Soc 69(4):1283-1290.

KanaRK, Gnonlonfin BGJ, Harvey J, Wainaina J, Wanjuki I, SkiltonRA, Teguia A(2013) Mycobiota and toxigenicity profile of Aspergillus flavus recovered from food and poultry feed mixtures in cameroon. J Ani Poult Sci *2*(4):98-107.

Khan GI, Akbar A, Anwar M, Shafee M, Sadia H, Razzaq A, Rehman FU (2020) Eco-friendly bacterial biodegradation of mycotoxins. Pure Appl Biol 9(2):1708-1717. http://dx.doi.org/10.19045/bspab.2020.90181

Kos G, Markus S, David M, Celine Z, Michael S, Tuba O, Boris M, Rudolf K(2016) A novel chemometric classification for FTIR spectra of mycotoxin-contaminated maize and peanuts at regulatory limits. Food AdditCont: Part A33:10:1596-1607. DOI: 10.1080/19440049.2016.1217567

Mirza SN, Athar M, Qayyum M (2009) Eff ect of Drought on Rangeland Productivity and Animal Performance in Dryland Region of Balochistan, Pakistan. Agricul Conspectus Sci 74(2):105-109

Osho IB, Awoniyi TAM, Adebayo AI(2007) Mycological investigation of compounded poultry feeds used in poultry farms in southwest Nigeria. Afri J Biotech *6*(15).

Pacin AM, Gonzalez HHL, Etcheverry M, Resnik SL, Vivas L,Espin S(2003) Fungi associated with food and feed commodities from Ecuador. Mycopathologia 156(2):87-92.

Rafique A, Javed MT, Ali SS(2018) Contamination of commercial poultry feeds and feed ingredients with Aspergillus flavus and aflatoxin B1 in Punjab Pakistan. J Ani Plant Sci *28*(4):1021-1026

Raju S, PadamadanCJ,Shenpagam NH(2016) Mycotoxin production by fungi isolated from commercially prepared livestock feed in Kerala. Int J Appl Res*2*(5):154-159.

Rashid N, Bajwa A, Rafeeq M, Khan MA, Ahmad Z, Tariq MM, Wadood A, Abbas F(2012) Prevalence of aflatoxin B1 in finished commercial broiler feed from west central Pakistan. J Anim Plant Sci 22: 6-10.

Rosa CDR, Ribeiro JMM, Fraga MJ, Gatti M, Cavaglieri LR, Magnoli CE, Lopes CWG(2006) Mycoflora of poultry feeds and ochratoxin-producing ability of isolated Aspergillus and Penicillium species. Vet Microbiol 113(1-2):89-96.

Saleemi MK, Khan MZ, Khan A, Javed I, Hasan ZU, Hameed MR, Mehmood MA (2012) Occurrence of toxigenic fungi in maize and maize-gluten meal from Pakistan. PhytopathologiaMediterranea 51(1).

Sana S, Anjum AA, Yaqub T, Nasir M, Ali MA, Abbas M(2019) Molecular approaches for characterization of aflatoxin producing Aspergillus flavus isolates from poultry feed. Pak Vet J 39(2):169-174.

Shekhar M, Singh N, Dutta R, Kumar S, Mahajan V (2017) Comparative study of qualitative and quantitative methods to determine toxicity level of Aspergillus flavus isolates in maize. PLoS ONE 12(12): e0189760. https://doi.org/10.1371/journal. pone.0189760

Shi H, ShengliL, Yueyu B, Luciana LP, Yaogeng L,Peiqiang Y (2018) Mycotoxin contamination of food and feed in china: occurrence, detection techniques, toxicological effects and advances in mitigation technologies. Food Cont 91, 202–215.

Sieger, Gregor MK, Michael S,Matthia G, Rudolf K, Boris M(2017) Portable Infrared Laser Spectroscopy for On-site Mycotoxin Analysis. Sci Rep 7:44028. doi: 10.1038/srep44028.

Yazdani D, Zainal AM, Tan YH,Kamaruzaman S(2010) Evaluation of the detection techniques of toxigenic Aspergillus isolates. Afri J Biotech 9(45):7654-7659.

Wacoo AP, Wendiro D, Vuzi PC, Hawumba JF (2014) Methods for detection of aflatoxins in agricultural food crops. J Appl Chem 2014.