International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.10, pp 81-88, 2016

Frequency of fungal and aflatoxin B_1 contaminants in cattle feed

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Abstract : Background Fungal contamination of animal feed is extensively widespread as those fungi are ubiquitous in nature. Among those fungi is *Aspergillus* which produce aflatoxins when favored conditions of temperature and humidity are available. There are four major types of aflatoxins B_1, B_2, G_1 and G_2. Aflatoxin B_1 which considered as the most dangerous naturally occurring toxin have carcinogenic effect on both human and animals. **Method** Sixty finished cattle feed samples from Giza governorate were examined for the presence of fungi and aflatoxin B1 contaminants. Total mould count (TMC) was performed by pour platting technique while aflatoxin B_1 detection was done using thin layer chromatography (TLC) technique. **Results** The total mould count / gm was calculated with mean ± standard error $5.58X10^4 \pm 2.96X10^4$. Our results showed that the most commonly isolated fungal genera was *Aspergillus* (85%). Among *Aspergillus* genus, *A. flavus* was the most frequently isolated species as it was isolated from 71.7% from total samples. TLC analysis of aflatoxin B1 revealed its presence in 18.3% from the total examined samples with range between 1.5-72.4 ppb and finally the mean ± standard error was 24.15 ± 8.16 **Conclusions** In conclusion, Regular monitoring for the presence of aflatoxin B1 in animal feed is crucial for implementing perfect feed safety programs as aflatoxin B1 consumption can increase susceptibility to diseases, impair the reproductive performance and it can be excreted in milk in the form of aflatoxin M_1 which considered of major public health concern.
Keywords: Animal feed, Total mould count, Aflatoxin B_1, TLC.

Introduction

Contamination of feed with fungi can lead to nutrient losses and adverse effects on animal health and production [1]. Some moulds have been found to produce highly toxic chemical secondary metabolites known as mycotoxins which consider a potential real risk to public health due to induction of tumors and organ damage [2].

Globalization of the trade in agricultural commodities has contributed significantly to potential hazards that require knowledge and awareness about mycotoxins. Availability of sophisticated methods for testing residues and undesirable substances at all points of the supply chain made safety awareness in food and feed production strongly applied [3].

Mycotoxins are secondary fungal metabolites that considered toxic to human and animals. Toxigenic fungi often grow on edible plants as a result they able to contaminate food and feed [4].

On a universal scale, It is estimated that more than 25% of the world's crops are contaminated with mycotoxins that incriminate on extreme economic losses and public health threat [5]. Mycotoxins are carcinogens

and teratogens, and may be transmitted to man in meat and milk. They are produced mainly by three genera of moulds: Aspergillus, Penicillium and Fusarium [6].

As Aspergillus, Penicillium, and Fusarium genera frequently contaminate crops, mycotoxins such as aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), and zearalenone (ZEA) are found in food and feed in a wide range of concentrations, depending on environmental and storage conditions [7].

Consumption of aflatoxin contaminated feeds can cause aflatoxicoses in livestock. In fact, all feeds are susceptible to mycotoxin contamination as long as conditions that permit mould colonization are available [8]. Aflatoxicoses in dairy cows is considered a potential risk for public health, particularly in children, due to the production of aflatoxin M_1 in milk[9].

It is estimated that about 5 billion people worldwide suffer from uncontrolled exposure to aflatoxins [10]. Moreover, aflatoxins can cause serious economic losses by reduction of grain nutritive value and animal production [11]. In addition, Aflatoxin is one the most widely occurring and dangerous mycotoxin that mainly produced by toxinogenic strains of *Aspergillus flavus* and *A. parasiticus*. They able to produce four major types (B1, B2, G1 and G2) [8]**.** Among the aflatoxins, aflatoxin B1 (AFB1) is one of the most toxic and carcinogenic compounds [12]. Aflatoxin B1 is carcinogenic toxin for human and animals as it is produced mainly by Aspergillus flavus in food and feed [13].

Aflatoxin B1 (AFB1) is well known as the most prevalent and toxigenic mycotoxins and the International Agency for Research on Cancer (IARC) has classified AFB1 as Group 1 of human carcinogen [14].

Regular and routine monitoring of aflatoxin in animal feeds are crucial to reduce animal and consequently human exposure[15].

Materials and methods:

This study was carried out in the Department of Microbiology and Immunology– National Research Center – Dokki – Egypt.

1- Samples

A total of sixty finished cattle feed samples and they consisted of (corn, soybean meal, wheat bran, cotton seed cake and other additives of lime stone, minerals and salt). These samples were collected from different farms in Giza governorate.

2- Isolation, identification and count of mould from the tested samples[16].

a- Preparation of sample homogenate

Twenty five grams of each sample were aseptically homogenized in a blinder containing 225 ml of 1% sterile buffered peptone water and mix it for 30-60 seconds to give 0.1 dilution.

b- Serial dilution

Pipette 1ml of food homogenate into a tube containing 9 ml of the diluent. From the first dilution transfer 1 ml to the second dilution tube containing 9 ml of the diluent and so on until the desired dilution was obtained.

c- Pour plating

Pipette 1 ml of the sample homogenate and of such dilutions which have been selected for plating into a Petri dish in duplicate. Pour into each petri dish 10 to 20 ml molten SDA (cooled to 42-45^{o}C).Mix the media and dilutions by swirling gently clockwise and anti-clockwise and allow setting.

d- Incubation

Inoculated plates were left to solidify at room temperature. The plates were inverted to prevent spreaders and incubated at 25°C for 5-7 days. During the incubation period, the plates were examined daily for the star- shaped mould growth were counted separately using a colony counter and mould count/gram [17]

3- Determination of aflatoxin residues in the examined samples by TLC technique[18]

a- Preparation and extraction of aflatoxins

50 Gram of each sample were each finally ground and homogenized and then it is extracted with 250 ml of methanol: water solution (55:45 V/V) and 10 ml of hexane was added thrice after being well shaken. The suspension was filtered with whatman no.1 filter paper. The filtrate was extracted twice with 50 ml chloroform in separating funnel, the chloroform layer was drained and passed over a thin layer of 10 gram of anhydrous sodium sulphate. The extract was evaporated till dryness.

b- Purification and clean up extracted filtrate

Each vial of sample was dissolved in 2-3 ml of chloroform and purified using the column chromatography. Then the extract left to dry and cooled at 0 C till examination.

c-Thin layer chromatographic

I- Preparation of aflatoxin standard solution

Benzene - acetonitrile (9-1) was added to the container of dry aflatoxin and the concentration calculated to give a 8-10µg/ml. the solution was agitated for one minute and transferred into a glass stopered flask. By using the automatic pipette a portion of the stock standard aflatoxin solution was diluted with benzene-acetonitrile (9-1) to obtain a concentration of 0.5 µg aflatoxin B_1 and the flask containing the stock solution was weighed, wrapped tightly in aluminum foil and stored at 0 ºC till used.

II- Detection of aflatoxins by TLC

Resolutions of reference aflatoxin B_1 solution was prepared to give a final dilution with Benzene - acetonitrile (9:1) 0.5 µg aflatoxin B_1.

A vial of sample extract residue was uncapped and 0.1 µl Benzene - acetonitrile (9:1) was added and mixed. Activation of thin layer plates for one hour in hot air oven at 110 ºC and removed immediately to the dessicator to cool.

A known volume of the sample solution spots of (5, 10, 20 and 40 µl) was spotted on an imaginary line from the bottom edge of the plate. Standard solution was spotted on the plate with known concentration using 10-20 µl capillary pipette.

The plates was developed with toluene: ethyl acetate : 90 % formic acid (5, 4, 1 : V/V/V) in an equilibrate jar or developing tank for 30 minutes. When the solvent travels about 12 cm front, the plates were removed from the jar, air dried and inspected under long wave ultraviolet light lamp (360 nm) for examining the tested and standard spots matches. Aflatoxin was calculated by the following equation or formula.

$$\mu g/kg = \frac{SxYxV}{ZxW}$$

S= µl aflatoxin standard which matches the unknown (spot from the sample extract).
Y= concentration of aflatoxin standard in µg/ml.
V= µl of final dilution of sample extract.
Z= µl of sample extract giving a spot fluorescent intensity equal to the standard (S).
W= weight of the sample in gram

4- Statistical analysis

Data obtained were analyzed statistically for descriptive statistics (mean, maximum minimum and standard error) using SPSS 14.[19]

Results and Discussion:

Feed contamination with fungi can lead to nutrient losses and detrimental effects on animal health and production [1]**.**

Advanced countries considered the mould counts as a standard test for hygienic condition due to its economic and public health effects[13].

To ensure the hygienic quality of animal feed, the total fungal counts of samples must not exceed the value proposed as a limit which is (1×10^4 cfu/gm). These high levels could reduce the nutrient adsorption [20] and palatability [21]**.**

The current study presented the results of total mould counts/ gm in the examined samples. Total mould count / gm was calculated with min. $6.00X10^2$ whereas the max. $1.12X10^5$ and finally the mean ± standard error was $5.58X10^4 \pm 2.96X10^4$. These results are quite similar to those obtained by [22] as they calculated the mean value of TMC/gm in finished cow feed samples and it was 4.2×10^4 also our results are to some extend similar with others in poultry, pig and horse feeds as the fungal counts were between 4×10^3 and 42×10^3cfu/g in poultry feed [23] whereas in equine feeds [24] ranged from not detectable (ND) to $1.3x10^6$ CFU/g but all feed samples of pigs was much higher as their count exceed the feed hygienic quality limit ($1x10^4$) . [25,26]

A total of 99 mould strains belonging to 6 genera were isolated and identified from the feed samples. The results given in **Table (1)** and **Figure (1)** showed that the most commonly isolated fungal genera were *Aspergillus* (85%), *Penicillium* (36.7%), *Rhizopus* (18.3%), *mucor* (11.7%) and *Fusarium* (8.3%) which was nearly similar to results obtained by [27] as they found that (54.4%) of feeds analyzed contained *Aspergillus* also [28] and [29] assured also that *Aspergillus* species was the most prevalent fungal contaminant found in feed samples and those results were in concordance with results obtained by[1] as they isolated *Aspergillus* (56%), *Mucor* (17%), *Penicillium* (15%), *Fusarium* (6%), *Cladosporium* (2%) and [30] as they isolated aspergilli in the rate of (53.57%) from compound animal feed, Whereas [31] found that *Fusarium* was the most frequently isolated genus but [32] found that *Rhizopus* was the most prevalent one (56.41%), followed by *Aspergillus* (43.66%) and *Fusarium* (14.97%). On the other hand [33] found that *Penicillium* was the most frequently recovered genera from animal feed.

Table (1): Incidence of the most commonly isolated mould genera from examined cattle feed samples. (n=60)

Mould Genera	**Cattle feed samples**	
	No.	**%**
Aspergillus	51	85
Penicillium	22	36.7
Rhizopus	11	18.3
Mucor	7	11.7
Fusarium	5	8.3
Alternania	3	5

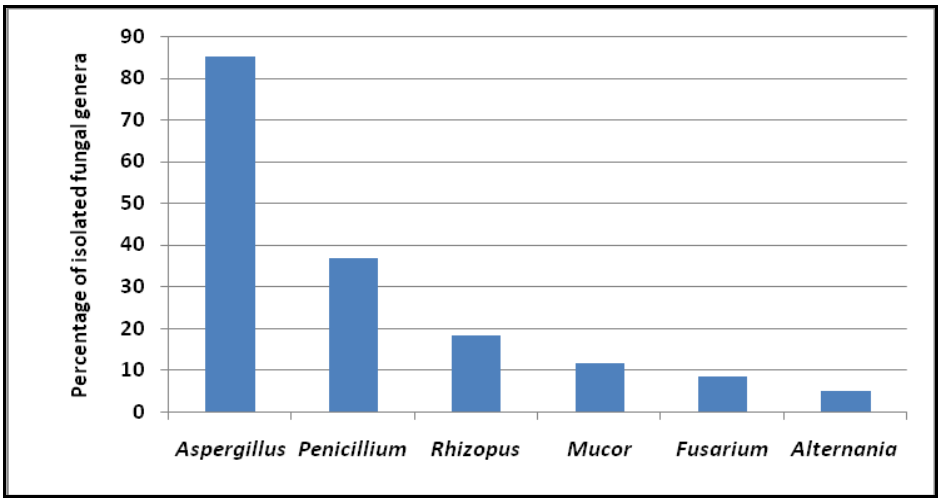

figure (1) Incidence of the most commonly isolated mould genera from examined cattle feed samples. (n=60)

The presence of *Aspergillus* is not only of economic important but also represents a real health hazard. They have allergic, toxigenic and pathogenic effect through the production of mycotoxins. [34]

Among *Aspergillus* genus, *A. flavus* was the most frequently isolated contaminant as it was isolated from 71.7% from total samples as shown in **Table (2)** and **Figure (2)** . Other fungal strains were *A. niger*, *A. fumigatus*, *A. terreus*, *A. candidus*, *A. ochraceus* and *A. parasiticus* and were isolated up to 58.3, 41.7, 16.7, 13.3, 5, 3.3 from the samples, respectively. Those results were in concordance with other results obtained by [35] and [22] along with [27] as they isolated *A. flavus* in the rate of 34% from total isolated aspergilli also [32] found that *Aspergillus flavus* was the most common species of *Aspergillus* genus by 36.69% however [1] isolated *Aspergillus flavus* from 48% of the tested samples furthermore [28] observed that *A. flavus* isolated from more than 90% of the their samples.

Table (2) Incidence of Aspergillus species in examined cattle feed samples. (n=60)

Aspergillus species	**positive samples**	
	No	%
A. flavus	43	71.7
A. niger	35	58.3
A. fumigatus	25	41.7
A. terreus	10	16.7
A. candidus	8	13.3
A. ochraceus	3	5
A. parasiticus	2	3.3

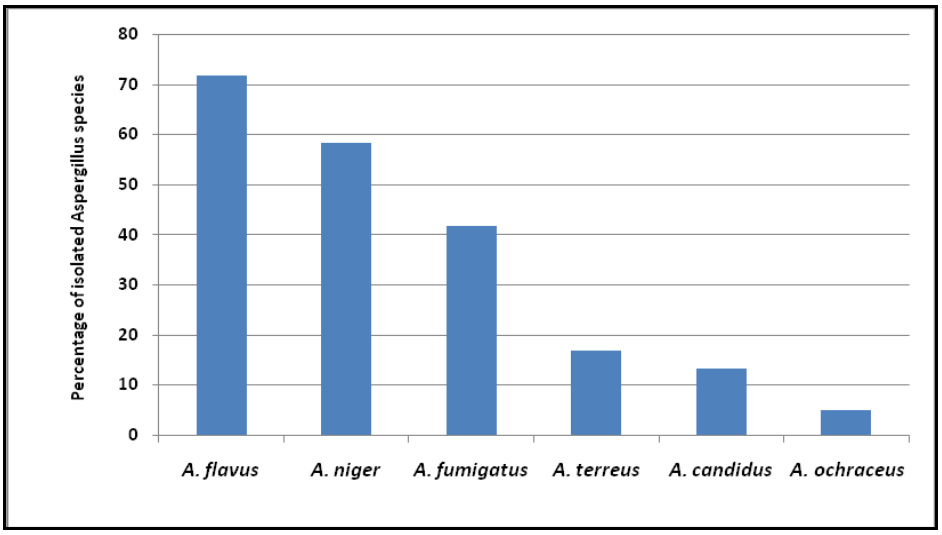

Figure (2) Incidence of Aspergillus species in examined cattle feed samples. (n=60)

The screening of samples for viable fungi is a useful practice in itself as it doesn't only act as an indicator for contamination but also supports the analysis of mycotoxins that could be present [22]

Aflatoxins are natural toxins that contaminate various types of food and feedstuff leading to health risk in both humans and animals [36]. In most countries worldwide, legislated levels for aflatoxins is 20 micro g/kg [35]

In the present study, aflatoxin B_1 analysis with TLC revealed its presence in 18.3% from the total examined samples with min. 1.5 ppb whereas the max 72.4 ppb and range between 1.5-72.4 ppb and finally the mean ± standard error was 24.15 ± 8.16. Those results were to some extend in agreement with other results obtained by [37] as they revealed the occurrence of aflatoxin in 14% of the examined samples however, [38] found that 38.2% were contaminated with AFB1 at a mean concentration of 16.5 micro g/kg and a maximum of 160.9 micro g/kg. Whereas [39] assured the presence of AFB1 in a range between 7 and 419 mug/kg while, [9] detected AFT residues in 92.5% of the feed samples and ranged from 4.82 to 24.89 micro g/kg (mean=10.84+or-5.84 micro g/kg) Moreover, [40] results revealed the presence of AFB_1 in 84.4% of the feed samples (mean 18.7+or-1.4 micro g/kg) but [41] detected the incidence of aflatoxins as it was 3.6% while [7] found AFs in 24.3% of the samples with mean value of 4.6 micro g kg^{-1} also [31] detected aflatoxin B_1 mean concentration of 22.72 micro g/kg besides [42] show that (19%) was contaminated with aflatoxins, ranging from 6.5 to 101.9 ng g^{-1}. [15] was dissimilar with our results as they isolated aflatoxin B_1 from 86% of the feed samples also [43] observed that aflatoxin B1 average in feeds was 0.412 ± 0.154 ppm.

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