The Bangladesh Veterinarian (2020) 37(1 – 2): 27 - 35

Detection of aflatoxin-producing fungi in maize

M Rafik, F Afroz* and MM Rahman
Department of Microbiology, Faculty of Veterinary and Animal Sciences, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh

Abstract

Maize samples (n = 110) were collected from five different areas of Dinajpur district from July 2018 to July 2019 for the detection of aflatoxin-producing fungi and aflatoxin. Aflatoxin-producing fungi were detected using culture and morphological features, and aflatoxin was detected using Agra Strip total aflatoxin test (20 ppb cut-off). *Aspergillus* spp. were detected in 56% of fresh and 23.2% of stored maize samples. Aflatoxin-producing fungi were detected in 28% of fresh samples and aflatoxin was detected in 10% of stored samples. Out of 110 maize samples, *Aspergillus* spp was identified in 38.2%, and aflatoxin-producing fungus was found in 18.2%. The presence of aflatoxin-producing fungus in stored maize increased significantly ($P<0.01$) with storage. In 20 samples aflatoxin level was more than 20 ppb. (*Bangl. vet*. 2020. Vol. 37, No. 1 - 2, 27 – 35)

Introduction

Aflatoxins are metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Goldblatt, 1973; Cleveland and Bhatnagar, 1992; Cotty, 1997; Yu *et al*., 2004). There are four principal types of aflatoxin: B_1, B_2, G_1 and G_2, named for their fluorescent properties. *Aspergillus flavus* produces aflatoxins B_1 (AFB_1) and B_2 (AFB_2) while *Aspergillus parasiticus* produces aflatoxins G_1 (AFG_1) and G_2 (AFG_2). Aflatoxin B1 is considered the most potent naturally occurring carcinogen. Aflatoxin M_1 is a derivative of AFB_1 that is formed and excreted in the milk of humans and animals following consumption of foodstuffs contaminated with AFB_1. AFB1 is derived from sterigmatocystin (ST), which is carcinogenic (Xu H. X. *et al*., 2000). These fungi survive in a wide range of environments and are found in soil, plants, animals, grains (including maize), peanuts, and tree nuts (Pitt, 2000). Aflatoxins cause liver disease in animals and are potent human carcinogens, probably the most significant mycotoxins worldwide (D Mello JPF *et al*., 1998).

Aflatoxin is a contaminant in agricultural and food products (Blesa *et al*., 2004; Pietri *et al*., 2004) and animal feeds (Dalcero *et al*., 1998; Sassahara *et al*., 2005). The growth of aflatoxigenic fungi in maize (*Zea Mays* L.) results in aflatoxin production (Oyebangi and Efiuvwevere, 1999). The Food and Agriculture Organization of the United Nations (FAO) estimated 25% of the world's cereal grains are contaminated with aflatoxins (Dowling, 1997). High levels of contamination of aflatoxin (>20 μg kg^{-1}) in maize has been reported by Moreno and Kang (1999). Colonization of maize crops prior to harvest by aflatoxigenic fungi often result in spoilage and aflatoxin

*Corresponding author:- E-mail: farzana.afroz2010@gmail.com

DOI: https://doi.org/10.3329/bvet.v37i1-2.59894

accumulation during storage (Resnik *et al*., 1996) and contaminated maize is prohibited from trade domestically or internationally (Ellis *et al*., 1991).

Aflatoxin-contaminated groundnut cake contributed to the death of more than 200,000 broiler chickens in 1994. A poultry farm in Chitradurgh, Karnataka, India (Jelinek *et al*., 1989) lost more than 2000 chickens as a result of aflatoxin-contaminated maize meal. In India, human disease outbreak was attributed to consumption of aflatoxin-contaminated maize (Krishnamachari *et al*., 1975). Therefore, the study was designed to detect the presence of aflatoxin-producing fungus with aflatoxin from the maize samples in Dinajpur district.

Materials and Methods

A total of 110 maize samples were collected from Fulbari, Kaharol, Birol, Birganj, Sadar areas of Dinajpur district. Sixty were collected fresh (Table 4) and 50 were stored (Table 3).

Processing of sample

About 300 gm of maize were homogenized in mortar and pestle using a sterile diluent as described by ISO (1995). Homogenate samples (10 gm) were transferred carefully into a sterile beaker containing 90 ml of PBS (1 : 10).

Culture into different fungal media

With the help of sterile inoculating loop the processed samples were inoculated into Sabouraud Dextrose Agar, Potato Dextrose agar and *Aspergillus* differential agar medium.

On Sabouraud Dextrose agar (SDA)

The processed samples were inoculated into SDA and incubated at 37^{o}C for 5-7 days. Greenish colonies were found.

On Potato Dextrose agar (PDA)

Colonies from SDA were sub-cultured into PDA and incubated at 37^{o}C for 5-7 days. On PDA, the colonies were yellow green with white to cream mycelia and yellow green edges and in some plates greenish colonies were seen.

On Aspergillus differential agar medium

Aflatoxigenic fungi in maize were determined by *Aspergillus* differential agar medium. Colonies from SDA and PDA were sub-cultured in *Aspergillus* differential agar medium. A bright orange colour on the reverse of the plates indicated a positive result.

Microscopic study

Micro-morphological characteristics of the pure culture colonies like conidia were observed as a wet mount in Lactophenol cotton blue stain for identification by the onidiospore appearance and arrangement (Thilagam *et al*., 2016).

Aflatoxin detection by Agra Strip total Aflatoxin Test (20 ppb Cut off)

Aflatoxigenic fungus was detected by Agra Strip total aflatoxin test 20 ppb cut-off (AgraStrip® Total Aflatoxin Qualitative Tests - Romer Labs) as described by Mohammad *et al.* (2019). The Agra Strip Total Aflatoxin Test is a one-step lateral flow immunochromatographic assay for total aflatoxin (B_1, B_2, G_1 and G_2) intended for use in grains and other commodities. It detects aflatoxin at 5 ppb or higher in grain samples by utilizing highly specific reactions between antibodies and aflatoxin (Delmulle *et al*., 2005; Xiulan *et al*., 2005; Stubblefield *et al.*, 1991). Antibody-particle complex is dissolved in assay diluent and mixed with sample extract. The mixed content is then wicked onto a membrane, which contains a test zone and a control zone. The test zone captures a free antibody-particle complex, allowing colour particles to concentrate and form a visible line. A positive sample with aflatoxin above the cut-off level will result in no visual line in the test zone. A negative sample will form a visible line in the test zone. The line will always be visible in the control zone.

Extraction procedure

A representative sample was ground using a Romer Series II® Mill, so that 75% will pass through a 20-mesh screen, then the subsample portion was thoroughly mixed. Ten gm of ground samples were taken into a clean jar with 20 mL of 50% ethanol extraction solution [50/50 (v/v) ethanol/water] and the jar was sealed and vortexed for one minute. The top layer of extract through which the sample was filtered by Whatman filter was collected.

Test procedure

All reagents and kit components were kept at room temperature (18 - 30°C) before use. Using a single channel pipette, 50 µL of assay diluent to each microwell was added. The coating conjugate was dissolved in the microwell by pipetting the content up and down 5 times. Fifty µL of sample extracts was added to each microwell, mixing the content in each well by pipetting it up and down three times. One test strip was put into one well. The test strip was allowed to develop colour for five minutes. The result was interpreted according to the manufacturer (AgraStrip® Total Aflatoxin Qualitative Tests - Romer Labs).

Result and Discussion

In culture media, greenish white colonies were found on SDA (Fig. 1), on PDA (Fig. 2) yellow green with white to cream mycelia and yellow green edges and in some plates greenish colonies were found. On Aspergillus differential agar medium (ADA) (Fig. 3), a bright orange colour on the reverse of the plates was found, which indicates aflatoxin production. By lactophenol cotton blue stain (LPCB) characteristic conidia were found under microscope (Fig. 4).

Aspergillus spp. was in 28 of 50 fresh samples (56%, Table 1) and 14 of 60 stored samples (23.2%, Table 2). Aflatoxin-producing fungus as well as aflatoxin were isolated in 14 of 50 fresh samples (28%, Table 3) and 6 of 60 stored samples (10%,

Table 4). Out of 110 maize samples, *Aspergillus* spp. was in 42 (38.2%) (Table 5). But Aflatoxin-producing fungus was in 20 (18.2%) samples (Table 5), similar to the findings of Rouhollah *et al.* (2012) and Mohamed *et al*. (2019).

Table 1: Prevalence of *Aspergillus* spp. in different areas

Area	No. of samples	Number of positive cases of *Aspergillus* spp.	Prevalence of *Aspergillus* spp. (%)	Chi-square	P-value
Fulbari	10	4	40	4.55	0.34
Kaharol	10	6	60		
Birol	10	8	80		
Birganj	10	6	60		
Sadar	10	4	40		
Total	50	28	56		

No significant (P>0.05) difference on the prevalence of *Aspergillus* spp.

Table 2: Prevalence of *Aspergillus* spp. after storage

Storage time (months)	No. of samples	Number of positive cases of *Aspergillus* spp.	Prevalence of *Aspergillus* spp. (%)	Chi-square	P-value
4	14	0	0%	23.59	0.00***
8	12	0	0%		
12	14	2	14.3%		
16	10	6	60.0%		
20	10	6	60.0%		
Total	60	14	23.2%		

***Correlation is highly significant at the 0.01 level (2-tailed)

Table 3: Prevalence of aflatoxin-producing fungus in maize in different areas

Area	No. of samples	Number with Aflatoxin-producing fungus	Prevalence of aflatoxin-producing fungus (%)	Chi-square	P-value
Fulbari	10	2	20	2.38	0.67
Kaharol	10	2	20		
Birol	10	4	40		
Birganj	10	4	40		
Sadar	10	2	20		
Total	50	14	28		

Table 4: Prevalence of aflatoxin-producing fungus in maize after storage

Storage time (months)	No. of samples	No. with Aflatoxin-producing fungus	Prevalence of aflatoxin-producing fungus (%)	Chi-square	P-value
4	14	0	0	15.56	0.004**
8	12	0	0		
12	14	0	0		
16	10	2	20		
20	10	4	40		
Total	60	6	10		

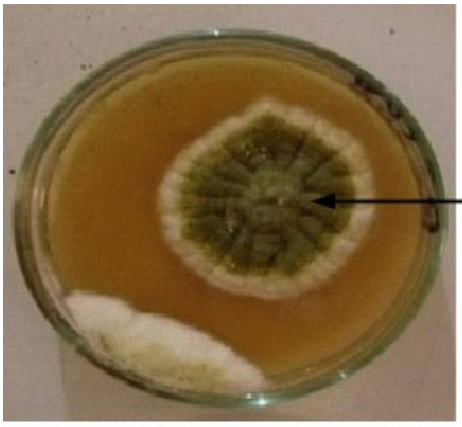

Fig. 1: Greenish white colonies in SDA

Fig. 2: Yellow green with white to cream mycelium on PDA

Fig. 3: Bright orange colour formation on reverse side of ADA

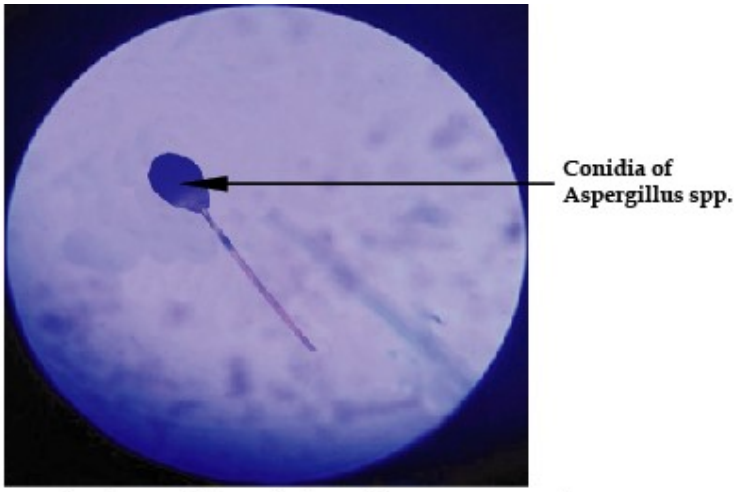

Fig. 4: Lactophenol cotton blue stain (LPCB) from SDA agar colony

Table 5: Screening of Aflatoxin by Agra Strip Total Aflatoxin Test (20 ppb Cut-off)

Sample No.	No of positive cases for *Aspergillus* spp.	No of positive cases (aflatoxin >20 ppb)	No of negative cases (aflatoxin <20 ppb)
1-10	3	2	8
11-20	4	2	8
21-30	3	2	8
31-40	5	3	7
41-50	4	2	8
51-60	2	1	9
61-70	6	3	7
71-80	3	1	9
81-90	5	2	8
91-100	4	1	9
101-110	3	1	9
Total = 110	42	20	90
Prevalence (%)	38.2	18.2	81.8

Prevalence of Aspergillus spp. (Table 2) and aflatoxin-producing fungus (Table 4) as well as aflatoxin increased significantly after storage ($P<0.01$) (Liu *et al*., 2006). Aflatoxin level was detected by Agra Strip total Aflatoxin test (20 ppb cut-off). Out of 110 samples, aflatoxin-producing fungus as well as aflatoxin was found in 20 samples with toxin levels more than 20 ppb (Table 5).

An effort was made to investigate the distribution of toxigenic fungi in the maize. It has been reported that grains and feeds are contaminated with toxigenic fungi and aflatoxin in Bangladesh (Begum *et al*., 2000, Dawlatana *et al*., 2002; Mohamed *et al*., 2019).

The Food and Drug Administration (FDA) has established action levels of 20 parts per billion (ppb) for grain and feed products. Mixing aflatoxin-contaminated grains with normal grains is not advisable.

Maize samples containing more than 20 ppb aflatoxin are presented (Table 5). Aflatoxin causes serious disease in man and animals. Early detection of toxins can allow preventive measures. Aflatoxin-contaminated maize in poultry feed is an alarming issue due to its accumulation in poultry that can be deleterious for human health. Improving storage facilities in this hot humid environment is essential for preventing spoilage and reducing the toxicity.

Conclusions

Grains stored for a long period are vulnerable to contamination with aflatoxin-producing fungi. Continuous surveillance is required to detect aflatoxin using macro and micro morphological fungal features. Good agricultural practice can reduce the risk of aflatoxin-producing fungus.

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