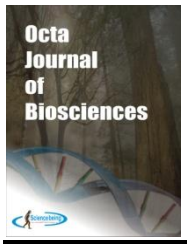
Octa Journal of Biosciences
International peer-reviewed journal
Octa. J. Biosci. Vol. 1(1):62-68

ISSN 2321 – 3663
May, 2013



Octa Journal of Biosciences

journal homepage: www.sciencebeingjournal.com

Scientific Planet Society

Research Article

Detection of Aflatoxin in Zea mays L. from Indian Markets by Competitive ELISA

Harish Chandra[1] Jyotsana Bahuguna[2] and Ajay Singh[3]

1. Department of Biotechnology, G. B. Pant Engineering College, Ghurdauri, Pauri, Uttarakhand, India
2. Department of Biotechnology, Indian Institute of Technology, Roorkee, Uttarakhand, India
3. Department of Chemistry, Uttaranchal Institute of Technology, Dehradun, Uttarakhand, India

Received Ist Dec. 2012 Revised 12th Feb. 2013 Accepted March18th 2013

*Corresponding Author, Dr. Harish Chandra, Assistant Professor, Department of Biotechnology, G.B.Pant Engineering College, Ghurdauri, Pauri, Garhwal-246194, Uttarakhand Email: hreesh5@gmail.com

Abstract: Aflatoxins are a family of related bisfuranocoumarin compounds produced by fungi *Aspergillus flavus* and *A. parasiticus*. It has been reported that out of the known strains of *A. flavus* and *A. parasiticus*, only about one-half produce toxins. In present study the occurrence of total aflatoxin contamination in Indian maize samples collected from local market of Lucknow city were investigated by competitive ELISA technique. The result showed that the fungal count ranges from 1.0×10^2 to 3.6×10^6 cfu/gm. However; no significant correlation could be established within fungal count and the aflatoxin level. Total aflatoxin content ranges from 9.0 to 250 ppb. However 7 samples do not have any trace of total aflatoxin level.

Key words: *Aspergillus flavus*; *A. parasiticus*; ELISA; total aflatoxin

INTRODUCTION

Aflatoxins are polyketide secondary metabolite produced by a very common food contaminating species *Aspergillus flavus* and *A. parasiticus*. There are 14 known aflatoxins, most of which are metabolites formed endogenously in animals administered by one major toxins, i.e., aflatoxin B_1, B_2, G_1 and G_2 (Fig 1). *A. flavus* produces B_1 and B_2 while *A. parasiticus* produces all of these four major toxins (Lillehoj, 1986). These toxins are usually found together with various foods and feeds in various proportions; however, aflatoxin B_1 is usually predominant and is the most toxic. Aflatoxins G_1 and G_2 are formed only by *A. parasiticus* (Klich and Pitt, 1988). When B_1 and B_2 are ingested by cattle, a portion of these aflatoxins is metabolized to M_1 and M_2 (Fig 1) which may be found in the dairy milk. The produced aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) are difuranocoumarin derivatives and potent hepatic

carcinogen causing cancer of liver in wide variety of animal species including human (Smith et al., 1994).

Aflatoxins are also known to cause aflatoxicosis as a result of consumption of contaminated agricultural products. All aflatoxin producing fungi may be classified taxonomically to Aspergillus section Flavi. *A. flavus* and *A.parasiticus* are the two main aflatoxin producers (Frisvad et al, 2006).

Many agricultural commodities such as maize, peanut are liable to infestation by aflatoxigenic molds and contamination with aflatoxin. Aflatoxin are worldwide important in public health, agriculture and economics. Aflatoxin have been found as contaminant in agricultural and food products especially in cereal and cereals product (Blesa et al., 2004; Pietri et al., 2004; Abbas et al., 2006) and animal feeds (Dalcero et al., 1998; Sassahara et al., 2005). They are both acutely and chronically toxic to animal including man, causing acute liver damage, liver cirrhosis, onset of tumors and teratogenic effects (Stoloff, 1977).

Corn (*Zea Mays* L.) is one of the major cereals crops of global importance, and has always been an important commodity to be traded overseas as food, feed and an industrial grain crop in several countries including India. Unfortunately, it is also vulnerable to the growth of aflatoxigenic fungi, resulting into subsequent aflatoxin production which causes major yield and economic losses (Oyebangi and Efiuvwevvvere, 1999). Aflatoxin causes mortality and reduce productivity in farm animal and are also detrimental to human as high concentration have been associated with liver cancer. *A.flavus* may parasitically colonizes silks and invade mature corn kernel in the field producing aflatoxins (Payne et al., 1988). Aflatoxigenic fungi may infect the crop prior to the harvesting period and remain as such while storage and finally appear in corn products. Aflatoxin contaminated groundnut cake contributed to the death of more than 200,000 broiler chicken in 1994. A poultry farm in Chitradurgh, Karnataka State (India), lost more than 2000 chicken as a result of feeding them with aflatoxin contaminated maize meal. In addition to maize and groundnut, many commodities including spices (Jelinek et al., 1989; Vasanthi and Bhat, 1998) are contaminated by aflatoxin. In India, human disease outbreak attributable to consumption of aflatoxin contaminated maize have been described by Krishnamachari et al (1975).

The aim of the present study is to investigate the presence of aflatoxin in maize available in Lucknow the capital city of Uttar Pradesh (India) using ELISA with immune-affinity column clean-up process to ascertain the vulnerability of the local population to get exposed to this toxin.

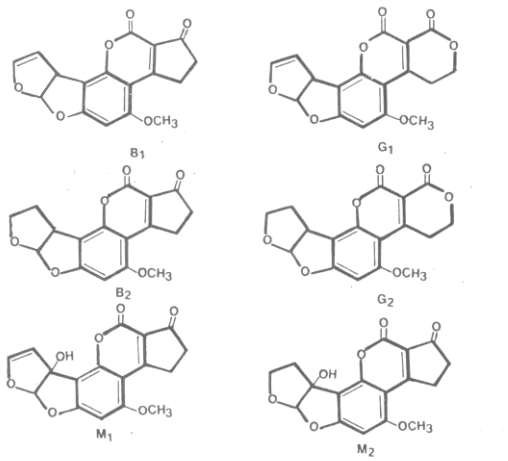


Fig.1 Structures of aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2.

MATERIALS AND METHOD

Sampling

Sample of maize grains (n=32) was collected from local market of Lucknow, India. All samples were stored in sealed plastic bags and kept at room temperature in dark and dry conditions. 100 gm sample of maize was ground and are immediately analyzed for total aflatoxin.

Viable plate count of mycoflora

Fungal count in the grains of maize was done by using pour plate method as described in IS: Protocol (IS 5403: 1999) in which 11 gm of finely ground sample were aseptically transferred to 99 mL of pre sterilized 0.85% of normal saline solution under laminar air flow (Manufacturer details) making further dilutions by transferring 1 mL of sample from to the tubes containing 9 mL of 0.85% normal saline repeated till dilution of 10^{-6} level was achieved. 1 ml of final diluted sample was poured aseptically to their respective plate followed by the addition of 25 mL of pre sterilized and cooled to room temperature PDA media (HiMedia India) plates with chloramphenicol supplement (HiMedia India). After solidifying, plates were incubated in inverted position at 28 ± 1 ^{o}C for 5 days. Same dilution was also plated on ADM (Aspergillus Differential Medium). Total fungal count was calculated after five days of incubation ensuring the growth of fungal mycelia.

Sample preparation and Immunoaffinity Clean up

5g of finely ground maize was extracted with 25mL of 70% aqueous methanol using a laboratory homogenizer and filtered through a filter paper (Whatman no.1). 100µL of each filtrate were diluted with 600µL of dilution buffer and 50µL of diluted sample employed to immunoaffinity column (R-Biopharm Ag, Darmstadt, Germany) for cleaning up of the samples. The basis of washing of samples involved antigen-antibody reaction. The column containing gel suspension to which monoclonal antibodies were attached covalently. The antibodies are specific for the Aflatoxin B_1, B_2, G_1, G_2 and M_1. Total aflatoxin content finally eluted with 0.5mL of HPLC grade methanol and analyzed through ELISA microplate Reader Model 680 (Bio-Rad India)

Quantification of Total aflatoxin content

Quantitative analysis of total aflatoxin was performed by competitive ELISA using RIDASCREEN total aflatoxin kit, Darmstadt, Germany. 50µL of standard solution of aflatoxin and cleaned eluted sample were added to the wells of microtiter plate in replicates. 50µL of peroxidase enzyme conjugate and 50µL of mouse monoclonal anti-aflatoxin antibodies were added to each well of microtiter plate and incubated at room temperature in the dark for 30 minutes. After washing thoroughly with 250 µL distilled water three times, 50µL of urea peroxidase (substrate) and 50µL of tetramethylbenzidine (chromogen) were added to each well to mix thoroughly and was further incubated for 30 minute at room temperature in the dark. Reaction was stopped by adding 100µL 1M sulphuric acid (stop reagent) and the absorbance was measured at 450nm using ELISA microplate reader Model 680 (Bio-Rad India). A calibration curve was drawn using a wide range of total aflatoxin standards with concentration of 0 ppt to 4050 ppt. (Fig.2).

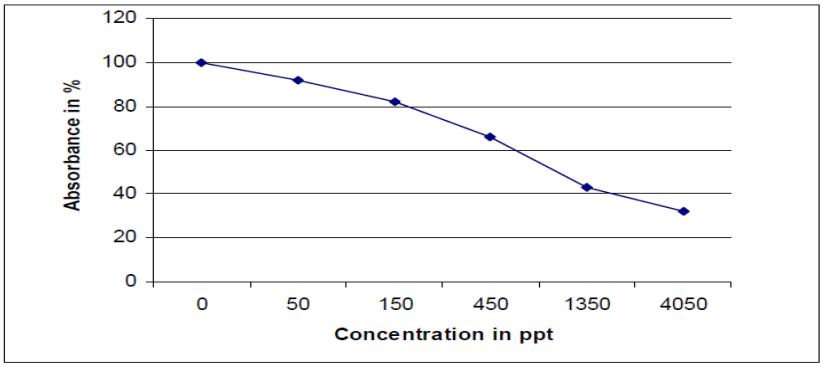


Fig.2 Standard curve of different concentration of Total Aflatoxin

RESULTS AND DISCUSSION

The Food and Drug Administration (FDA) has established an" Action Level" of 20 ppb for aflatoxin in corn in interstate commerce (Table 1). In India the regulatory level are set at 30 ppb for all foods. This is the action at which federal agencies may take action including seizure of the corn or prohibition of its sale. Elevators do not accept corn with 20 ppb or more of aflatoxin unless they have a known alternative use. Aflatoxins are very potent compounds that cause variety of human and animal health problems. On rare occasions, livestock can die from ingesting aflatoxin contaminating feed. Most commonly aflatoxin reduces the feed efficiency and reproductivity of livestock. It can suppress the immune system of animals, leading to more frequent occurrence of infectious diseases. In the United State alone, the economic loss from mycotoxin is estimated to be $932 million (CAST, 2003).

Table1- FDA Regulatory Levels for Total Aflatoxins in Livestock Feeds and Human Food

Commodity	Concentration ppb
All products, except milk, designated for humans	20
Corn for immature animals and dairy cattle	20
Corn and peanut products for breeding beef cattle, swine, and mature poultry	100
Corn and peanut products for finishing swine	200
Corn and peanut products for finishing beef cattle	300
Cottonseed meal (as a feed ingredient)	300
All other feed stuffs	20
Milk	0.5*

Food and Drug Administration (FDA) Compliance Policy Guides 7120.26, 7106.10, 7126.33 (revised 1994); *=Aflatoxin M_1

A.flavus invades and infects developing seed of maize in the field before harvest, and mature seeds during harvest, and in storage. Preharvest contamination with aflatoxin is aggravated by drought stress and elevated temperature during seed maturation. Damage by insect is another important predisposing factor by providing injury sites through which A. flavus can invade seeds in the stores and in the field. Maize and groundnuts continue to be the major source of aflatoxin, particularly in India (Sinha, 1990). Sinha (1990), had done survey for three consecutive years in some state of Bihar and revealed heavy infestations of mycotoxin-producing fungi with different maize samples. Aflatoxin-producing fungi had the highest frequency of occurrence in all the cases and aflatoxins were the most common mycotoxins elaborated by these fungi. Maize samples of the Kharif crop had a greater incidence of aflatoxins (47%) than the samples of Rabi crop (17%). Stored maize grains also had a high incidence of aflatoxins (43%). Most of the contaminated samples contained aflatoxins at levels above 20 micrograms/kg.

Result of maize samples analysed for total aflatoxin and fungal count are presented in Table1. Viable count of mycoflora in maize sample and poultry feed sample are in the range of 1.0 x 10^{2}—3.6 x 10^{6} cfu/gm. Currently, a viable plate count method for detecting mould contamination is used to determine the mycological quality of foods and agricultural commodities (Liewen and Bullerman, 1992). A high mould count indicates the possibility of aflatoxin contamination, but not a confirmatory test for the presence of aflatoxin contamination. Same dilution was also plated in ADM for the isolation of *A. flavus* which was merely used for the identification or detection of *A. flavus*. ADM (Aspergillus differential media) is a selective medium for *A. flavus* group of fungi which develop characteristic reverse orange color after 42 hr incubation at 30°C (Pitt et al., 1983). On ADM out of 32 samples, 18 samples are found positive for *A. flavus*. Aflatoxin contamination in maize was determined by using competitive ELISA prior to Immunoaffinity column clean up and found that out of 32 sample of maize tested 25 samples were found positive for total aflatoxin and out of 25 sample, 19 sample was exceeding the maximum limit set by FDA (Table1). The immunoaffinity cleanup procedure in particular, boosts not only the performance, the

owing to the provision very clean extract (absence of interfering substance but also the case of aflatoxin determination (Stroka et al., 2002). Highest total aflatoxin level was recorded in M-1 sample i.e. 250 ppb and lowest in sample M-5, M-10 and M-25 i.e. 9 ppb. However 7 samples does not have any trace of aflatoxin contamination. Detection of mould using ELISA and viable plate count, the result shows positive correlation, the ELISA reading correlate the plate count and aflatoxin levels however no absolute pattern,Similar type of finding were also reported by Yong and Cousin (2001).

Table 2- Total aflatoxin and fungal count in Maize sample

Sample	Fungal count cfu/gm	Total Aflatoxin (in ppb)	Growth on ADM* agar
M-1	2.2×10^6	250 .0	+
M-2	1.7×10^2	ND	-
M-3	2.5×10^6	144.0	+
M-4	4.2×10^4	126.0	+
M-5	3.0×10^5	9.00	+
M-6	4.2×10^5	126.0	+
M-7	5.7×10^5	162.0	+
M-8	3.2×10^3	80.0	+
M-9	2.9×10^3	24.5	-
M-10	2.3×10^2	9.0	-
M-11	3.0×10^3	12.5	-
M-12	2.8×10^4	34.0	+
M-13	1.2×10^3	ND	+
M-14	3.2×10^4	45	-
M-15	5.0×10^5	83	+
M-16	1.2×10^2	ND	-
M-17	4.0×10^3	15	+
M-18	3.6×10^6	164	+
M-19	2.8×10^4	72	+
M-20	1.0×10^2	ND	-
M-21	3.1×10^6	62.0	+
M-22	5.3×10^5	80.0	+
M-23	3.4×10^5	34.8	-
M-24	8.2×10^3	22.0	-
M-25	2.1×10^2	9.0	-
M-26	6.0×10^2	ND	-
M-27	2.9×10^2	ND	-
M-28	3.5×10^2	ND	-
M-29	6.0×10^4	40	+
M-30	3.2×10^4	28	+
M-31	3.6×10^5	18	+
M-32	2.8×10^6	54	-

Chulze (2010) concluded that some mycotoxins can contaminate maize before harvest and there level can be increase during storage, because agro-ecosystem is a complex and interrelated between biotic and abiotic factors in stored maize. Zinedine et al. (2007) investigated the fifty eight samples of cereals which is used for the human consumption (20 corn flour, 17 wheat flour) and poultry feed (n=21) purchased from market of Rabat in Morocco by HPLC with immunoaffinity column (IAC) cleanup and Flourometric determination. The incidence of Aflatoxin in corn, wheat flour and poultry feed was about 80, 17.6 and 66.6% respectively. Contamination of 10% sample of corn was higher than that of limit set

by EU regulation for Aflatoxin B1 and Total Aflatoxin. Reddy et al. (2002) surveyed the occurrence of aflatoxin in maize and found 41 out of 95 samples i.e. 43.16% are found contaminated with aflatoxin (ranges from 10 to > 100 ppb). Various surveys conducted reveal that Indian feeds have been found to contain toxic metabolite products which are produced by certain strains of mold Aspergillus sp. or by other mold species.

Conclusion

From above study we have found that high level of aflatoxin in maize indicates consumers risk for exposure to high levels of aflatoxin. Aflatoxin contamination of market peanut, therefore, is an important public health concern. Precautions should be taken for proper storage of maize in order to prevent microbiological and chemical hazards.

REFERENCES

1. Abbas, H.K., Cartwright, R.D., Xie, W., Shier, W.T. (2006); Aflatoxin and Fumonisin contamination of corn (maize, Zea mays) hybrids in Arkansas. *Crop Prot*., 25:1-9.
2. Blesa, J., Soriano, J.M., Molto, J.C., Manes, J. (2004); Limited survey for the presence of aflatoxins in foods from local market and supermarkets in Valencia, Spain. *Food Add.Cont*., 21:165-171.
3. CAST. 2003. Mycotoxins: Risks in Plant, Animal, and Human Systems. Report 139. Ames, IA: Council for Agricultural Science and Technology.
4. Chulze, S.N. (2010); Strategies to reduce mycotoxins levels in maize during storage: a review. *Food Add. Cont*., 27: 651–657.
5. Dalcero, A., Magnoli, C., Luna, M., Ancasi, G., Reynoso, M.M., Chiacchiera, S., Miazzo, R., Palacio, G (1998); Mycoflora and naturally occurring mycotoxins in poultry feeds in Argentina. *Mycopathologia*; 141:37-43.
6. Frisvad, J.C., Neilsen, K.F., Samson, R.A (2006); Recommendations concerning the chronic problem of misidentification of mycotoxigenic fungi associated with foods and feeds. In: Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U, Editor. Advances in food mycology. New York: Springer, pp 33-46.
7. IS-5403: 1999. Methods for Yeast and mould count of foodstuffs and poultry and animal feed. Bureau of Indian Standards, New Delhi.
8. Jelinek, C.F. Pohland, A.E., Wood, G (1989); Worldwide occurrence of mycotoxins in food and feeds. *J. Assoc. Off. Analyt Chem Inter*., 72:223-230.
9. Klich, M.A. Pitt, J.I (1988); Differentiation of Aspergillus flavus from Aspergillus parasiticus and closely related species. *Trans. Br. Mycol. Soc*., 91: 99-108.
10. Krishnamachari, K.A.V.R., Bhatt, R.V., Nagarajan, V., Tilak, T.B.G (1975); Hepatitis due to Aflatoxicosis- an outbreak in Western India. *Lancet*.,1061-1062.
11. Liewen, M.B. and Bullerman, L.B (1992); Toxigenic fungi and fungal toxins In: Vanderzant, C., Splittstoesser, D.F. (Eds), Compendium of methods for the microbiological examination of Foods. 3rdEdn. American Public Health Association, Washington, DC. 811-819.
12. Lillehoj (1986); The aflatoxin in maize problem the historical Prospectives. In: Aflatoxin in maize. A proceeding of the workshop. Mexico. 13 – 27.
13. Oyebangi, A.O. and Efiuvwevwere, B.J.O (1999); Growth of spoilage mold and aflatoxin B1 production in naturally contaminated or artificially inoculated maize as influenced by moisture content under ambient tropical condition. *Inter Biodeter Biod*., 44:209-217.
14. Payne, G.A., Hagler, W.M.Jr., Adkins, C.R (1988); Aflatoxin accumulation in inoculated ears of field-grown maize. *Plant Disease*., 72:422-428.
15. Pietri, A., Bertuzzi, T., Pallaroni, L., Piva, J. (2004); Occurrence of mycotoxins and ergosterol in maize harvested over 5 year in Northern Italy. *Food Add. and Cont.*, 21:479-487.
16. Pitt, J.I., Hocking, A.D., Glenn, D.R (1983); An improved medium for the detection of *Aspergillus flavus* and *Aspergillus parasiticus*. *J Appl Bacteriol*., 54:109-114.
17. Reddy, D.V, R., Thirumala-Devi, K., Reddy, S.V., Waliyar, F., Mayo, M.A., Rama Devi, K., Ortiz, R. & Lenne, I.M (2002); Estimation of aflatoxin level in selected food and feeds in India. Hanak, E., Boutrif, E., Fabre, P., Pineiro, M (Ed.). Food Safety Management in developing countries. Proceeding of the International Workshop, CIRAD-FAO, 11-13 December 2000, Montpellier, France.
18. Sassahara, M., Pontes Netto, D., Yanaka, E.K. (2005); Aflatoxin occurrence in food stuff supplied to dairy cattle and aflatoxin M1 in raw milk in the North of Parma state. *Food Chem Toxicol*., 43:981-984.
19. Sinha, K.K (1990); Incidence of Mycotoxin in maize grains in Bihar State, India. *Food Add. Cont*., 7:55-61.
20. Smith, J.E., Lewis, C.W., Anderson, J.G., Solomons, G.I (1994); Mycotoxin in human

nutrition and health. In: Report EUR 16048 EN, European Commission, Directorate-General XII, Brussels, Belgium.

21. Stoloff, L (1977). Aflatoxin- An overview: In: J.V. Rodricks, C.W. Hesseltine & M.A.Mehlman (Ed.). Mycotoxin in Human and Animal Health. Pathotox Publisher. Park forest South III. 7-28.
22. Stroka, J. and Anklam, E (2002); New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. *Trends Anal Chem*., 21(2): 90-95.
23. Vasanthi, S. and Bhat, R.V (1998); Mycotoxin in foods-Occurrence, health and economic significance and food control measures. *Indian J Med. Res*., 108:212-224.
24. Yong, R.K. and Cousin, M.A. (2001); Detection of moulds producing aflatoxins in maize and peanuts by an immunoassay. Inter. J. Food Microbiol.65:27-38.
25. Zinedine, A., Juan, C., Soriano, J.M., Molto, J.C., Idrissi, L. and Manes, J (2007). Limited survey for the occurrence of aflatoxins in cereals and poultry feeds from Rabat, Morocco. *Intern J Food Microbiol*., 115: 124-127.