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Detection of aflatoxins in animal feeds

R.Suganthi*, J.Fathima Benazir, M.Padmini Pooja, C.Swathee, R.Namitha, K.Sudharsan Balaji
School of Biotechnology, Dr. G.R.Damodaran College of Science, Coimbatore-641014, Tamilnadu, (INDIA)
Tel : +91 422 2572719
E-mail : sugantham2000@gmail.com
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ABSTRACT

Aflatoxins are toxic and carcinogenic metabolites produced by species of *Aspergillus*, but *Aspergillus flavus* and *Aspergillus parasiticus* are of most concern. Aflatoxins compose a family of toxic compounds- B1 (most toxic), B2, M1, M2, G1 and G2. The present paper reports the differentiating between aflatoxin producing *Aspergillus flavus* and the non-producers. The detection of aflatoxin concentration in animal feeds was done by two methods, Thin Layer Chromatography (TLC) and Enzyme Linked Immuno Sorbant Assay (ELISA). Results of range 1 -38 ppb were obtained establishment of mycotoxin analytical laboratories in animal feed manufacturing units.

KEYWORDS

Aflatoxin;
Aspergillus flavus;
Animal feed;
Thin layer chromatography;
ELISA.

INTRODUCTION

Mycotoxins are poisonous chemical compounds produced by certain fungi. There are many such compounds, but only a few of them are regularly found in food and animal feedstuffs such as grains and seeds. Nevertheless, those that do occur in food have great significance in the health of humans and livestock. Since fungi produce them, mycotoxins are associated with diseased or mouldy crops, although the visible mould contamination can be superficial. The effects of some food-borne mycotoxins are acute, symptoms of severe illness appearing very quickly. Other mycotoxins occurring in food have longer-term chronic or cumulative effects on health, including the induction of cancers and immune deficiency.

Mould and mycotoxin contamination of food is serious although usually neglected. According to Food and Agriculture Organization, moulds contaminate 25% of the world crop. Fungal invasion of agricultural commodities is common in the fields (*Fusarium spp.*, *Aspergillus spp*. and *Penicillium spp*.) with considerable seasonal variations. Mycotoxins are usually found in mixed form. The production of mycotoxins does not correlate directly with the growth of moulds, and while fungistatic and fungicidal compounds may affect the mould invasion, this does not necessarily entail the drop in the level of mycotoxins.

There are five mycotoxins, or groups of mycotoxins, that occur quite often in food: deoxynivalenol/ nivalenol; zearalenone; ochratoxin; fumonisins; and aflatoxins. T-2 toxin is also found in a variety of grains but its occurrence, to date, is less frequent than the preceding five mycotoxins. The food-borne mycotoxins likely to be of greatest significance for human health in tropical developing countries are the fumonisins and aflatoxins.

Aflatoxins were first identified in the 1960s and

compose a family of toxic compounds. Aflatoxin B1 is the most carcinogenic and best studied of the compounds. The toxic effects include acute hepatitis, immunosuppression, and hepatocellular carcinoma. In humans, the risks associated with aflatoxin consumption are well documented, and the International Agency for Research on Cancer (IARC) has designated aflatoxin as a human liver carcinogen.

Aflatoxicosis is poisoning that result from ingestion of aflatoxins in contaminated food or feed. Aflatoxin poisoning is reported from all parts of world in almost all domestic and non domestic animals like cattle, horses, rabbits, and other non human primates. Aflatoxicoses is also reported in humans in many parts of the world.

Diet is the major way through which humans as well as animals are exposed to aflatoxins. Apart from this, exposure to aflatoxin can be through ingestion of contaminated milk containing Aflatoxin M1 (metabolite of AFB1). Occupational exposure to aflatoxins in agricultural workers, people working in oil mills, and granaries has been reported[1].

After wide experimentation on many animal species like rats, rainbow trout's, aflatoxin especially aflatoxin B1 is confirmed as a potential carcinogen (IARC 1993). Metabolism plays a major role in deciding the degree of toxicity[2]. After ingestion, aflatoxin is metabolized by cytochrome p450 group of enzymes in the liver, where it is converted to many metabolic products like aflatoxicol, aflatoxin Q1, aflatoxin P1, and aflatoxin M1, depending on the genetic predisposition of the species. Along with the above another metabolite called aflatoxin 8, 9 epoxide is also formed. The amount of this metabolite decides the species susceptibility as this can induce mutations by intercalating in to DNA, by forming an adduct with guanine moiety in the DNA. This intercalation of Epoxide causes a G to T transversion at codon 249 in p53 gene in liver, which may lead to hepatic carcinoma. This was observed in most of the experimental models, and it is presumed that this is the major reason for aflatoxin carcinogenicity[3]. Moreover species susceptibility to aflatoxin mainly depends on its liver detoxification systems, genetic make up, age and other nutritional factors[4].

Several methods of aflatoxin analysis of grain exist, each with different levels of precision, relative costs and ease of application. These include the black light, mini-column, ELISA (serological), and chromatography techniques.

Grain infected with *Aspergillus flavus* produces a compound called kojic acid, which characteristically produces a greenish-yellow fluorescence when examined in dark room under long-wave ultraviolet light (black light). Best results are obtained on cracked or ground kernels. The aflatoxin does not fluoresce; it is the indirectly related kojic acid that fluoreces. Thus the black light test is an indirect, presumptive test that does not directly detect aflatoxin. Black light positive samples usually contain some aflatoxin, but this method cannot quantify the aflatoxin levels and can give false positive responses. Due to these deficiencies, the black light test, it not recommended for aflatoxin screening. However, if it is used and a grain sample is found positive with the black light test, it is recommended that a representative sample of this lot to be tested using a more determinative sample of this lot be tested using a more determinative test such as a commercial antibody test (ELISA) or the mini-column test .

The main objective of our work is to detect the concentration of aflatoxin from animal feeds by ELISA and TLC.

MATERIALS AND METHODS

Animal feed samples were collected and stored in airtight packets and numbered. Moulds were isolated by serially diluting feed samples and spread plating on Czapek Dox Agar (CDA) or Potato Dextrose Agar (PDA) medium. PDA is recommended for the isolation and enumeration of yeasts and moulds from dairy and other food products. CDA is employed to screen toxigenic fungi. *A.flavus* was picked up from the culture plates and streaked on slants to maintain. A smear of every pure culture was stained with lacto phenol cotton blue to study the typical morphological features. The cultures were tested for the enzymes Amylase and Protease by plating on Starch Agar and Casein Agar medium. *A.flavus* was spread plated. A line streak of *Lactobacillus plantarum* at the centre of the plate was done to screen for the antifungal activity of lactic acid bacteria[5].

UV Photography

A.flavus patched plates, incubated for 3 days at 28ºc were placed upside down, irradiated with UV light and photographed. The color of the colonies was recorded to distinguish between aflatoxin producers and non-producers. Aflatoxin producers absorbed UV light and appeared grey to black, while the non-producers reflected UV light and appeared white[6].

Thin layer chromatography

Thin layer chromatography is routinely used and is a highly recommended method for aflatoxin quantification. Toxin is extracted from ground feed samples using acetone and chloroform and evaporated to dryness. The final extract is sufficiently clean for uni-dimensional TLC. The final extract is diluted with 100-500µl chloroform. Precoated silica gel 'G' plates were used and developed in unsaturated tanks, using chloroform: acetone (90:10 v/v). The developed plate was viewed under UV. The fluorescence of the spots was compared to that of the standards for quantification[7]. Aflatoxin content (ppb) = (S× C ×D / Y × W) ×100 where S - Volume of standard matching with sample in fluorescence, C - Concentration of standard, D -Dilution, Y - Volume of sample spotted and W - Effective weight.

Enzyme linked immuno sorbant assay

ELISA is the most sensitive method to detect aflatoxin concentration. The basis of the test is antigen-antibody reaction. The wells in the micro titer strips are coated with capture antibodies directed against anti-aflatoxin antibodies. Standards or the sample solutions, aflatoxin-enzyme conjugates and anti-aflatoxin antibodies are added. Free and enzyme conjugated aflatoxin compete for the aflatoxin antibody binding sites (competitive enzyme immunoassay). At the same time, the immobilized capture antibodies also bind the aflatoxin-antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen to a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photo metrically at 450nm; the absorption is inversely proportional to the aflatoxin concentration in the sample.

RESULTS AND DISCUSSION

Animal feed samples were collected from different areas where different climatic and storage conditions persisted. The samples were stored in airtight packets and were given code numbers, serially diluted and spread plated. Mixed colonies appeared. Aspergillus *flavus* colonies were picked up and maintained in CDA/PDA slants. *Aspergillus flavus* was stained using lacto phenol cotton blue for observing the morphological features hyphae bearing the conidiophores for confirmation of the strain. The isolated strains were then subjected to biochemical assays. The cultures showed zone of clearance on starch agar media on staining with iodine and on casein agar medium, thus showing positive results for amylase and caseinase. *Aspergillus flavus* was plated and Lactobacillus was streaked on the same plate. Inhibition zone was observed, thus suggesting that the growth of *Aspergillus flavus* was inhibited by *Lactobacillus*.

Aspergillus flavus patched plates were viewed under UV after 2 days of incubation. Strains producing Aflatoxin appeared grey to black. The Aflatoxin content of the feed samples was quantified by performing Romers method of thin layer chromatography. Samples showed Aflatoxin concentration of the range 4ppb-38ppb. Three samples showed highest Aflatoxin concentration of 16ppb, 17ppb, and 38ppb respectively. Among the recent techniques Aflatoxin concentration was quantified by ELISA, which gave more precise results of the range 1ppb-22ppb. One sample showed highest Aflatoxin concentration of 22ppb.

The majority of *Aspergillus* species isolated on CDA medium were identified as *Aspergillus flavus* of which 80% of the samples were screened for aflatoxin production. All the *Apergillus flavus* isolated were not found to be aflatoxin producers. The results were similar to the report of Arrus et al., 2005, where all *Apergillus flavus* are not capable of producing aflatoxin. Similar results were reported by Diener and Davis in 1966.

Lactobacillus has been reported to be involved in antimicrobial activities but few reports of antifungal activity have been published. The present work clearly shows the antifungal effects of *Lactobacillus* species on aflatoxigenic fungus isolated. Antifungal activities by a *Lb. casei* strain that inhibited both the growth and the

aflatoxins production of *A. parasiticus* has been reported[5]. Indeed, Vanne et al., 2000 showed that the growth of toxigenic storage fungi could be restricted by LAB *in vitro*. Karunaratne and coworkers (1990) reported a *Lb.plantarum* that was able to inhibit the growth of *A.flavus* but felt the effect was due to a combination of acidity and microbial competition. This conclusion was further clarified through use of the same inocula strains by Gourama and Bullerman (1995). From the results, it could be safely concluded that the action of the suspensions of lactic acid bacteria used in this work is broad all being active against more than one *Aspergillus* sp. similar other workers have reported result. Further work as to identify as well as characterize the active ingredient involved in the *in vitro* inhibition of the toxigenic *Aspergillus* strains by LAB species needs to be carried out as to prescribe the necessary procedure for its use in the preservation of various foods and feed ingredients.

According to Cole in 1986 and Moss in 1995, there is a diverse range of analytical methods for mycotoxins detection in foods, animal feeds and raw materials. In many countries in the world some form of thin layer chromatography (TLC) still is the method of choice because of economic constraint. Therefore an attempt was made to detect the concentration of aflatoxin using the simplest, economic, and rapid method the TLC. The maximum concentration of aflatoxin detected ranges from 17-38 ppb (17μg/kg- 38 μg/kg) by TLC. The aflatoxin concentration detected was of medium aflatoxin content (5-50 ppb) in 200μl chloroform. The aflatoxin concentration detected by the most sensitive ELISA ranges from 3-22 ppb (3μg/kg - 22μg/kg).

Aflatoxin B1 and its metabolite aflatoxicol can be detected in eggs and edible tissue from hens given feed contaminated with aflatoxin B1 at a level of 8 ppm. Kan et al, 1989 concluded that aflatoxin B1 level in feed of about 100 ppm which is much higher than the tolerance of 20 ppb do not impair performance of broilers and laying hens.

CONCLUSION

The aflatoxin content of the feed samples was effectively quantified by TLC and ELISA methods which gave results of range 1ppb-38ppb. In conclusion this study has shown serious risk for public health since all age groups consume milk world wide. For this reason milk and milk products should be free of aflatoxin contamination. Hence it is extremely important to check aflatoxin levels in cattle feeds regularly, particularly important, storage conditions of feeds must be strictly controlled. Aflatoxin being a potent carcinogen it is necessary to check safety levels in feeds before it is administered to animals. Hence, establishment of mycotoxin analytical laboratories in animal feed manufacturing units become a prime importance.

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