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Research Article

DETECTION OF AFLATOXIN BASED ON RAPID SPOT TEST METHOD-A STUDY

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ABSTRACT
Mycotoxin contamination of various food stuffs and agricultural commodities is a major problem in tropics and subtropics, with special reference to Indian perspective where climate, agricultural and storage practices and conditions are conducive to fungal growth and toxin production. This work is to explain the hand out field effective and economical methods for detection of aflatoxins in the local conditions. Among 30 samples of feed ingredients all samples sorted in this work showed fungal contamination. Some samples were contaminated with *Aspergillus flavus* and rest with *A. nigri.* Results showed that isolates from sample 18 gave fluorescence within CMA test, while 9 isolates were positive to ammonia vapour test and 11 extracts were Dip-strip positive. All isolates exhibited reverse pigmentation on ADM, but showed fluorescence in 11 samples under UV radiation. 11 numbers of isolates reacted with Ehrlich's reagent and produce a blue violet ring on the filter paper during the first 6 min. Quality evaluation for those feed ingredients regarding to mould infestation and mycotoxins contaminations is necessary for the production of safe food. Qualitative detection of aflatoxin by field method and detection of aflatoxins and aflatoxigenic fungi by cultural and analytical method in easy, cost effective and rapid techniques is needed to validate a primary alarm of contamination. This is a pilot study which warrants long term prospective study to strengthen this view.

INTRODUCTION
Since last several decades food safety and consumer protection have gained immense importance. Hence, proof of authority, detection of adulteration and determination of residues and contaminants like drugs, pesticides, mycotoxins etc. are a focus of recent interest (Abo Dahab *et al.,* 2016).
Mycotoxin contamination of various food stuffs and agricultural commodities is a major problem in tropics and subtropics, with special reference to Indian perspective where climate, agricultural and storage practices and conditions are conducive to fungal growth and toxin production (Abo Dahab *et al,* 2016). The term mycotoxin is derived from "*Mykes*" a greek term for fungus and the latin word "*toxicum*" meaning poison. It is usually secondary metabolites formed mainly by saprophytic fungus growing on variety of food stuffs including animal feeds, which are differentiated structurally in diverse group of low molecular weight (MW<700) chemicals, so far identified 500 different types. Those types have a great diversity in different chemical, biological, physiochemical properties and they are generally thermally and chemically stable compounds (*Saber et al.,* 2016).
The number of mycotoxin known to exert toxic effects on human and animal health is constantly increasing and for this legislative provisions have also taken to control their presence in foods and feeds (*Saber et al.,* 2016).
Mycotoxins are produced by toxigenic fungi and moulds of the *Aspergillus, Penicilleum, Fusarium, Alterneria* and *Claviceps* genera under suitable micro environments like temperature and humidity conditions on different agricultural commodities in field or after harvest or during transportation or storage or even on milk causing significant health hazards for human and animal health and also cause economic losses (Saber *et al.,* 2016).
Aflatoxins and Ochratoxins A (OTA) are among many naturaly occurring toxic secondary metabolites by several specieses of *Aspergillus spp., Emericella astellata,* and *Peteromyces alliaceus* classified within the section Flavi of sub genus circumdati (Yazdani *et al.,* 2010).

Aflatoxins are not produced by other fungi and they are chemically difurano-coumarin derivatives produced via poliketide pathway (Yazdani *et al.*, 2010).

These afletoxins are potent carcinogenic, teratogenic, mutagenic, hepatotoxic, immunosuppressive, genotoxic, nephrotoxic and neurotoxic agents that cause significant damage to human and animal health by affecting many turget organs and systems, notably the liver, kidney, nervous system, endocrine system, immune system, muscle, skin, respiratory organs, digestive tract, genetal organs etc (Saber *et al.*, 2016). When present in food in sufficiently high levels, these fungal metabolites can have toxic effects that range from acute (e.g. diarrhoea, Vomition, Liver or Kidney detoriation), to chronic (e.g. Liver cancer, mutagenic and teratogenic and resulting symptoms range from skin irritation to immunosuppression, birth defects, neurotoxicity and death (Saber *et al.*, 2016). Among different aflatoxins the naturally occurring and well known ones are Aflatoxin B1, (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (Sudini *et al.*, 2015). Besides this, there is also Aflatoxin M1 (AFM1) which is classified as the most toxic in importance for human by several toxicologists (Rizwan *et al.*, 2014). Due to carcinogenic effects of AFB1, International Agency of Research on Cancer classified it as group-1 human carcinogen (Rizwan *et al.*, 2014) in human's liver and other types of mammals AFB1 is converted into AFM1. The presence of AFM1 can be detected in milk and its products within 12 to 24 hrs after the first exposure of AFB1. During the process of sterilization, pasturisation and varins dairy products preparation, the AFM1 remains stable (Rizwan *et al.*, 2014). AFM1 is produced when animals feed on AFB1 contaminated food, the gastrointestinal tract rapidly absorb it and convert it into metabolite AFM1 which appears in blood of animals within 15 minutes and then secreted in milk through mammary glands (Rizwan *et al.*, 2014).

AFM1 is considered to be carcinogenic and hepatotoxic for humans and various others species (Rizwan *et al.*, 2014).

Ochratoxin A was discovered as the metabolite of *Aspergillus ochraceus* in 1965 which is nephro-carcinogenic and teratogenic mycotoxin detected in several food products produced by two genera of fungi: *Aspergillus spp* & *Penicillium* spp (Yazdani *et al.*, 2010).

It has been estimated that due to contaminated foods, throughout the world in progressing countries, more than 500 million people have risk of chronic exposure to aflatoxins (Rizwan *et al.*, 2014).

Due to aflatoxins, the hepatocellular carcinoma is the primary disease (Liver cancer of HCC) according to WHO, globally hepatocellular carcinoma is the leading cause of cancer death (Rizwan *et al.*, 2014).

Due to hepatocellular carcinoma, each year 550,000 – 600,000 new cases occur out of which 83% deaths occur in East Asia and sub Saharan Africa. Since, milk and its derivatives are consumed daily and more over that they are of primary importance in the diet of children, most countries have set up maximum permissible levels of AFB1 in feed and for AFM1 in milk. The accumulation of small amounts of aflatoxins from a variety of foods would eventually reach a toxic theresh hold level in the body (Yazdani *et al.*, 2010).

In view of hazardous nature of aflatoxins which gained global significance due to its deleterious effect on human and animal health and as well as its importance in international trade, regulatory measures on its content in food ranging from 0.05 ppb (0.05 µgm/kg as per europian union); to 0.5 ppb (0.5 µgm/kg as per united states food and drug administration) have been introduced in various countries all over the world (Rizwan *et al.*, 2014; Sashidhar, 1993).

The government of India has fixed the tolerance limit of 30 ppb for agricultural commodities under the prevention of food adulteration act (Sashidhar, 1993).

A great success has been achieved through a number of survey and monitoring programs in several countries attempting to reduce mycotoxigenic fungi and mycotoxins a food using plant products like extracts and oils from cloves, arise and star- arise seeds, basil, cinnamomum, marigold spearmint, garlic, onion, thime, cassia and sweet-basil which have been reported to inhibit toxigenic and food born molds (Saber *et al.*, 2016).

However, in India and in many developing countries of the world, meaningful strategies for implementing legislation is limited by non availability of simple, rapid, cost effective and reliable analytical methods for

screening and detecting aflatoxins which could be easily performed either under field conditions or in the laboratory.

This article tries to explain the hand out field effective and economical methods for detection of aflatoxins in the local conditions.

MATERIALS AND METHODS

Collection of Samples

Agricultural commodities were classified as low-and-high fat samples. Fifteen numbers of low fat samples, i.e., maize was collected from different firm houses surrounding Kalyani in the quantity of 200 gm each. Again high fat commodities i.e., groundnuts were collected from different firm houses of the same area in the quantity of 200 gm each. This collection was done over 4 months.

Dip Strip Test for Aflatoxin Production Ability

100 gms of each representative samples was ground to fine powder with portable grinder and after that "dip strip method" for detection of aflatoxin was followed as screening test of aflatoxin under field condition following the methods described by Sashidhar (1993). In this case dip strips are made off polyester film (20 x 20 cm, 200 µm thick) and are coated with 250 µm thick silica gel (TLC grade) with soluble starch as binder and activated at 110^{O}C for 1hr. After activation, strips were cut to 40 mm x 120 mm long and used as dip strip for aflatoxin analysis. Aflatoxin extract was extracted by the stated method from which 5µL was spotted on to a dip strip and developed in chloroform: acetone (95 : 5) solvent system for 12 to 15 minutes in a developing chamber. The aflatoxins in sample were visualized under UV-lamp.

Isolation and Purification of Fungi

Czapek's agar medium was used for isolation and purification of fungi.

Coconut Based Medium Test

Coconut milk agar medium (CMAM) was prepared following standard method (Abo Dahab *et al.,* 2016). The plates were inoculated with pure culture and incubated at 28^{O}C. The presence or absence of fluorescence ring in the agar surrounding the colonies under UV light (365nm) after 7 days incubation was noted and the result were recorded as positive or negative.

Ammania Vapour Test

Aspergillus isolates were grown on yeast extract sucrose agar (YES ager) and the culture plates were incubated at 28^{O}C for 7 days (Abo Dahab *et al.,* 2016). After 7 days the plates were inverted over 2 ml of 29% NH_4OH reagent. After 10 minutes, undersides of aflatoxigenic isolates turned into plink to red color.

Ehrlich's Aldehtde Test (EAT)

The Ehrlich's reagent was prepared by 1% p-dimethyl amino benzaldehyde in 75 ml of elhanol and 25 ml of conc. HCL. 5 mm agar plug was cut off from the centre of *A. flavus* colony grown on YES Agar at 28^{O}C for 7 days. The agar plug was placed with the mycelial side on a filter paper sodden by Ehrlich's Reagent. The agar plug was removed and a drop of Ehrlich's reagent was applied on the filter paper. The reaction was visible after 2-6 minutes with the production of blue – violet ring on the filter paper (Jefremova *et al.,* 2016).

Aspergillus Differential Medium (ADM)

Aspergillus isolates were subjected to ADM for culture following the process described by Abo Dahab *et al.,* (2016). 3% (W/V) methylated Beta-Cyclo dextrine steitised using a syringe filter of 0.2 µm (Whatman GD/x 13mm) were added to autoclaved ADM in a petridish. The presence or absence of fleuroscence ring in the agar surrounding the colonies observed under UV light was scored as positive or negative.

RESULTS AND DISCUSSION

Result

Among 30 samples, all samples showed fungal contamination. Some samples were contaminated with *Aspargillus flavus* and rest with *A. nigri.*

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Results showed that isolates from sample 18 give fluorescence within CMA test, while 9 isolates are positive to ammonia vapour test and 11 extracts are Dip-strip positive. All isolates exhibited reverse pigmentation on ADM, but showed fluorescence in 11 samples under UV radiation. 11 numbers of isolates reacted with Ehrlich's reagent and produce a blue violet ring on the filter paper during the first 6 min. (Table 1).

Table: 1: Results of Screening for AF-Producing Strains by Dip-Strip Method, CMAM, Ammonia Vapour, ADM Methods

No. of Isolate	CMAM	Ammonium. Vapour	ADM	Ehrlich's Test	Dip-Strip
1.	-ve	-ve	+ve	-ve	-ve
2.	-ve	-ve	+ve	-ve	-ve
3.	+ve	-ve	+ve	-ve	-ve
4.	+ve	-ve	+ve	-ve	-ve
5.	+ve	-ve	+ve	-ve	-ve
6.	+ve	-ve	+ve	-ve	-ve
7.	+ve	-ve	+ve	+ve	+ve
8.	+ve	-ve	+ve	-ve	-ve
9.	+ve	-ve	+ve	+ve	+ve
10.	+ve	-ve	+ve	-ve	-ve
11.	+ve	-ve	+ve	-ve	-ve
12.	-ve	+ve	+ve	-ve	-ve
13.	-ve	+ve	+ve	-ve	-ve
14.	-ve	+ve	+ve	-ve	-ve
15.	-ve	+ve	+ve	-ve	-ve
16.	-ve	+ve	+ve	-ve	-ve
17.	+ve	-ve	+ve	+ve	+ve
18.	+ve	-ve	+ve	+ve	+ve
19.	+ve	-ve	+ve	+ve	+ve
20.	-ve	+ve	+ve	-ve	-ve
21.	-ve	+ve	+ve	-ve	-ve
22.	-ve	+ve	+ve	-ve	-ve
23.	-ve	+ve	+ve	-ve	-ve
24.	-ve	+ve	+ve	-ve	-ve
25.	+ve	-ve	+ve	+ve	+ve
26.	+ve	-ve	+ve	+ve	+ve
27.	+ve	-ve	+ve	+ve	+ve
28.	+ve	-ve	+ve	+ve	+ve
29.	+ve	-ve	+ve	+ve	+ve
30.	+ve	-ve	+ve	+ve	+ve

Discussion

Dip strip test show positivity depending on the fluorescence under long wave UV lamp in presence of aflatoxin in the samples qualitatively which may be used as a simple kit which is cost effective and reliable for monitoring aflatoxin in food and feed because polyester dip strips afford easy handling rather than TLC plates.

Most common fungi in cultured sample were *Aspergillus flavus* & *A. niger* respectively.

Cultural and analytical methods were used to analyze the potential aflatoxin production by strains isolated from representative samples to find whether there is any relation between qualitative kit based method and cultural & analytical methods, if any.

The fleuroscence methods are based on detection of Afs produced into agar medium (Abo Dahab *et al.*, 2016).
Coconut milk agar medium (CMAM) is generally used for rapid detection of aflatoxins production by *Aspergillus spp.* (Abo Dahab *et al.*, 2016).
Cultivation on CMAM is a primary tool to validate aflatoxin production (Abo Dahab *et al.*, 2016).
Further a blue fleuroscence surrounding aflatoxigenic colonies UV light is seen on the reverse of the plates (Abo Dahab *et al.*, 2016).
However, due to frequent false negative test CMAM is an unreliable method for detecting the toxigenic potential of *Aspergillus flavus* and *A. parasiticus* (Abo Dahab *et al.*, 2016).
Literature showed that YES medium was comparatively inducive for aflatoxin production rather than Czapek's aflatoxin producing ability (APA) media and different coconut agar medium (Abo Dahab *et al.*, 2016). In contrast some literatures showed high AFB1 production in Czapek's agar over APA and CMAM (Abo Dahab *et al.*, 2016).
By exposing the aflatoxigenic colonies to NH_4OH vapours will result in quick color change of the reverse side from brownish or yellow color to pale red (Abo Dahab *et al.*, 2016).
Versicolorin, a precurssor of AFB1 produced by *A. flavus* is the compound that produces purple red color in alkaline conditions (Abo Dahab *et al.*, 2016).
Literature showed that there are some other compounds that can be responsible for this color change (Abo Dahab *et al.*, 2016).
It has been identified that these compounds are anthraquinone derivatives associated with aflatoxin biosynthetic pathway in *Aspergillus spp.*, as the biosynthetic intermediates like norsolorinic acid, averantin, averufin, Versicolorin-A, nidurufin, hemiacetal for AFB1. The explanation for the color change is that anthraquinone compounds behave as pH indicator dyes which will change color (Abo Dahab *et al.*, 2016).
Ehrlich's test was used for detection of cyclopiazonic acid produced by *A. flavus*. The cyclopiazonic acid produced by *A. flavus* reacted with Ehrlich's reagent and produced a blue violet ring on the filter paper during first 6 minutes. In the next minutes, the blue violet ring quickly disappeared. This filter paper method was described by Jefremova *et al.*, (2016).
The positive colored reaction is caused by the indole secondary metabolites produced by moulds reacting with Ehrlich's reagent (Jefremova *et al.*, 2016).
The results of this test are obtained very fast but they are only approximate.
The development of yellowish orange color in the reverse of the colonies grown on aspergillus differentiation agar (ADA) was a result of reaction of ferric ions from ferric citrate with aspergellic acid molecules (Abo Dahab *et al.*, 2016).
This result was taken as a positive reaction for the production of aflatoxin.
Litrature shows that fleuroscence ring in this media under UV in presence of methyl-beta-cyclodextrine also shows positive case (Abo Dahab *et al.*, 2016).

Conclusion

Maize and groundnut is an important and significant food resources for human and animals. Quality evaluation for those feed ingredients regarding to mould infestation and mycotoxins contaminations is necessary for the production of safe food.
Qualitative detection of aflatoxin by field method and detection of aflatoxins and aflatoxigenic fungi by cultural and analytical method in easy, cost effective and rapid techniques is needed to validate a primary alarm of contamination. Though, this is a pilot study which warrants long term prospective study to strengthen this view.

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