

ORIGINAL RESEARCH ARTICLE

Detection and Quantitation of Aflatoxin for the Diagnosis of *Aspergillus flavus*

Geeta Rajbhandari Shrestha^{1*} and Amin Udhin Mridha²¹Amrit Campus, Tribhuvan University, Kathmandu, Nepal.²University of Chittagong, Chittagong, Bangladesh

Abstract

Aflatoxins are the potent mycotoxins produced by *Aspergillus flavus*, which is hepatotoxic causing hepatocellular carcinoma. *A. flavus* produces sufficient amount of Aflatoxin B₁ under favourable environments. Inhalation of spores and use of Aflatoxin B₁, contaminated food by *Aspergillus* spp., could transfuse the toxins in the blood streams. The presence of these toxins in body fluid can be detected by immunological assays and which provides an effective technique for the diagnosis of the disease caused by *A. flavus*. Aflatoxins producing strain of *A. flavus* were screened in Aflatoxin Producing Medium. Production of Aflatoxin B₁ by *A. flavus* was studied in different parameters such as incubation periods, temperatures, pH variations, sucrose concentration in Yeast Extract Sucrose medium and different natural media such as par-boiled rice, corn and groundnuts. The detection of toxins was done by TLC using silica gel (Merk) coated plates and confirmative test was done by Association of Official Analytical Chemists (AOAC) method. Presence and quantization was done by Enzyme Linked Immunosorbent Assay (ELISA) technique. Highest amount of Aflatoxin B₁ was reported 68.56 ng/ml by ELISA in synthetic medium (Yeast Extract Sucrose) with 2% sucrose, pH 5.5, on 14th days of incubation, at 28±1°C (p-value 0.05). Similarly, highest amount was recorded in groundnuts (121.20ng/g) by ELISA and (500ng/kg) by TLC methods. ELISA is one of the most efficient methods used for detection and diagnosis of human diseases cause due to exposure of Aflatoxin B₁ and *A. flavus*.

Key words: Aflatoxin, *Aspergillus flavus*, ELISA, AOAC, TLC

***Correspondence Author:**

Email: ga.gi.cha@hotmail.com

Introduction

Mycotoxins are secondary metabolites produce by different fungi under certain environmental conditions. Mycotoxins cause acute kidney failure (ochratoxin), damage of central nervous system (tremogenic mycotoxin) and damage the upper respiratory tract. Aflatoxins are the most potent and naturally occurring mycotoxins produce by *Aspergillus flavus* and *A. parasiticus*. Many factors affect the growth of fungi and contamination aflatoxins on foods and feeds. Different factors affecting Aflatoxins contamination include the climate of the region, the genotype of the crop planted, soil type, minimum and maximum daily temperatures, and daily net evaporation. Contamination of toxins can occur at any time of growth of plant, pre and post harvesting periods and storage conditions [1].

It was found that high doses (>6000mg) exposure of aflatoxins may cause acute toxicity with lethal effect and prolonged exposure to small doses is carcinogenic [2]. Mainly Aflatoxin B₁ (AftB₁) is the potent carcinogenic toxins to some animals and humans [3], which is one of the leading cause of cancer deaths worldwide [4]. When animals are

exposed to through the contaminated feeds, the toxin is converted into aflatoxinM₁ and contaminate in their milk. It is one of the sources of contamination aflatoxins of dairy products [5]. The exposure of aflatoxins causes many diseases such as hepatocellular carcinoma (HCC), impaired growth, immune suppression etc. Exposure of aflatoxins and the risks of toxic doses are more prevalent in the poor nations worldwide in both urban-rural areas, however more strongly in the rural populations [6]. These diseases are more common in most of the developing countries. It was estimated that 550,000–600,000 new HCC cases worldwide each year, of which about 25,200–155,000 were due to aflatoxins exposure. Most of the people from developing countries such as sub-Saharan Africa, Southeast Asia, and China including Nepal are suffering from HCC. Their prevalence accounted largely due to aflatoxins contamination in food [7]. It is essential to control contamination of food and feeds for minimization of outbreak due to aflatoxins. The presence of AftB₁ biomarker reflects the formation of the reactive metabolite and the level of DNA damage in the livers. Enzyme Linked Immunosorbent Assay (ELISA) is one of the diagnostic test, depends upon

protein content, is used to determine aflatoxins qualitatively and quantitatively in the samples [8]. ELISA and a monoclonal antibody against AFB₁, AFB₁ bound to albumin can be used to detect aflatoxins in urine and blood samples. The presence of aflatoxins residues adducts, and metabolites are assayed directly in tissues, fluids and excreta [9] and analyzed. The method is easy and inexpensive that developed with the necessary reliability, accuracy, and sensitivity to bring immunoassay technology.

Materials and Methods

Aspergillus flavus was isolated from the atmosphere of Kathmandu by gravity plate method. Lyophilized Aflatoxins producing strains of *A. flavus* was obtained from United States Department of Agriculture and used as the reference to compare the Aflatoxin B₁ production with that isolated from air. Aflatoxins standard was purchased from Sigma Co. The different strains of *A. flavus* producing aflatoxins were screened on Aflatoxin Producing Medium (APM) as described by Donald et al. (1981) [10]. Extraction of Aflatoxin B₁ was carried out as the method described by Abarca et al, (1988) and Chu (1987) [11, 12]. Detection of Aflatoxin B₁ was done by Thin Layer Chromatography (TLC) as the method described by using pre-coated silica gel plates (Merck) [13]. The confirmation test was done by Association of Official Analytical Chemists (AOAC) method [14]. Quantization of AftB₁ in different samples were carried out by ELISA in various parameters such as incubation periods (7th, 9th, 11th, 13th, and 15th days), pH variations (4.5, 5.5 and 6.5), temperatures (24^o, 28^o and 32^oC), and concentrations of sucrose (1.5%, 2% and 2.5%) in Yeast Extract Sucrose medium (YES) and different media like; Synthetic Low Salt medium (SLS), Coconut medium (CM), natural media such as par-boiled rice, corn and groundnut described by Davis et al.1966 [15]. For the study of production of Aflatoxin B₁, three replicas were maintained in each parameter. The experimental results from this study were analyzed by SPSS 16.0 [10, 12].

Results

The highest amounts of AftB₁ (68.56 ng/ml) was recorded in YES medium with 5.5 pH, 2% sucrose and after 14 days of incubation at 28 ±1°C (Figure 1A). The various temperatures have significant effect on the production of AftB₁ (Figure 2B). However, sucrose concentrations different and pH have no significant the effect, since p-values are more than 0.050 (Figure 2A and Figure 2B).

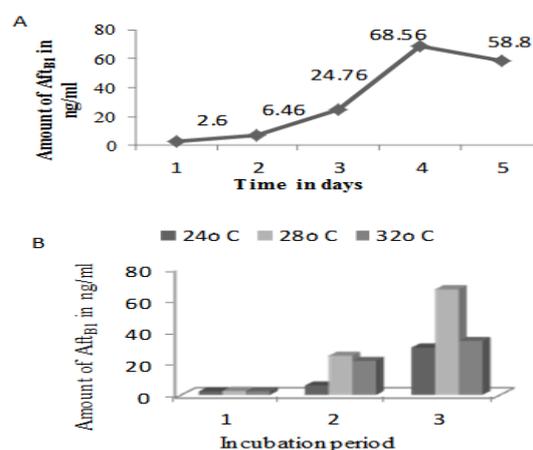


Figure 1: Effect of A. Incubation periods B. Temperature on production of Aflatoxin B₁

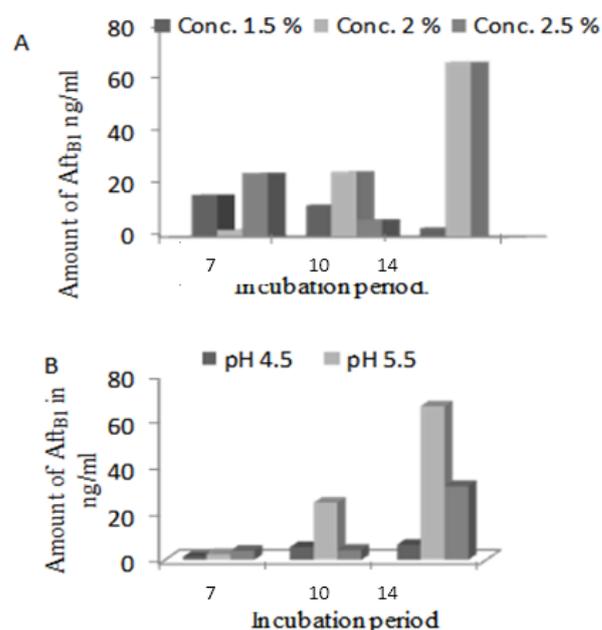


Figure 2: Effect of A. Sucrose concentration and B. pH in YES medium on the production of Aflatoxin B₁

Spearman's rho test showed that there is no significant linear relation between two variables- incubation period (time in days) and production of AftB₁ (ng/mL) as the p-value is more than 5% level of significance (Table 1).

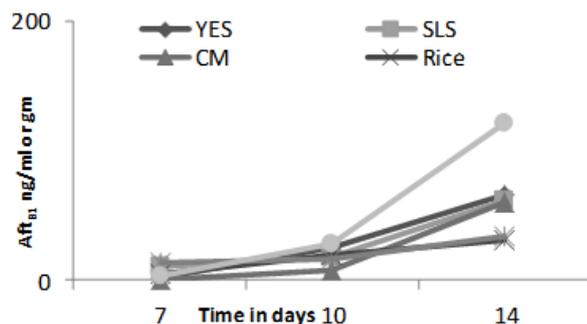
Table 1: Analysis of Aflatoxin B₁ produced by *A.flavus*; TLC and ELISA method in various incubation periods.

Note: ++ denotes for intensity of fluorescence

| No. | Incubation periods* | Aflatoxin B ₁ | |
|-----|---------------------|--------------------------|-------------|
| | | TLC | ELISA ng/ml |
| 1 | 7 | + | 2.6 |
| 2 | 9 | ++ | 6.46 |
| 3 | 11 | +++ | 24.76 |
| 4 | 13 | +++ | 68.56 |
| 5 | 15 | +++ | 58.8 |

Kruskal-Wallis test showed that there is no significant effect of a particular medium on the production of AftB₁ (the p-value of 0.998 is more than 5% level of significance). Moreover, each of six medium has equal effect on it. However, the highest

16 B (121.20ng/g) of aflatoxin B₁ was obtained in natural medium; groundnuts.



Note: YES = Yeast extract medium, SLS= Synthetic low salt medium, CM= Coconut medium

Figure 3: Effect of different media on the production of Aflatoxin B₁ in different incubation periods at 28 ±1°C.

Discussion

Aflatoxins produced by food borne *Aspergillus flavus* and *A. parasiticus* cause several human diseases such as Hepatocellular carcinoma (HCC), or liver cancer. Contamination of Aflatoxins can occur at any stage of food production from pre-harvest to storage [16]. Its contamination is determined by the most commonly used method; thin layer chromatography (TLC). The thin layer chromatography method was used for screening and quantification. Aflatoxins production is low during early stages of growth that increases with the decline of growth and the maximum production was recorded after the stationary phase of growth, which soon decline in its production (Table 1 and Figure 1). Similarly, West et al. (1973) reported that the aflatoxin level falls after reaching a maximum, which might be due to non-specific chemical mechanism for degradation of the toxin [17]. Smith and Moss (1985) also reported that very low aflatoxins was produced during the phase of vigorous growth in a laboratory and when some nutritional factor runs out and limits growth toxins biosynthesis occurs rapidly [18]. Factors that affect aflatoxins contamination include the climate of the region, the genotype of the crop planted, soil type, minimum and maximum daily temperatures, and daily net evaporation. Wilson and Payne (1994) and Fandohan et al. (2005) reported that many factors affect the growth of *Aspergillus* fungi and the level of aflatoxins production in food [16, 19]. It is essential to reduce Aflatoxins contamination of food by providing unsuitable condition to produce toxins. AftB₁ production is also promoted by stress or damage to the crop due to drought prior to harvest, insect activity, poor timing of harvest, heavy rains at harvest and post-harvest, and inadequate drying of the crop before storage [20]. Humidity, temperature,

and aeration during drying and storage are also important factors for aflatoxins contamination.

The most effective method of detection and quantification of aflatoxins contamination in foods and feeds is ELISA by which a very low amount of it can be detected [12]. In this study the highest amounts of AftB₁ (68.56 ng/mL) was recorded in YES medium with 5.5 pH, 2% sucrose and after 14 days of incubation at 28 ±1°C. The study on the effect of different parameters on Aflatoxin B₁ production showed that temperature has significant effect (P= 0.050). However, different pH and sucrose concentrations have no significant effect, since P= > 0.050. This method can be employed for the detection and quantification of aflatoxins exposure of human and causes of hepatocellular carcinoma due to *Aspergillus flavus* from Urine, blood samples. Groopman et al (1994) showed that aflatoxin metabolites in urine reflect recent exposure (i.e. 2-3 days) whereas the measurement of aflatoxins albumin adducts in blood reflects exposure over a longer period (i.e. 2-3 months) [21]. Qian et al (1994) studies showed correlation of aflatoxins intakes to biomarker levels and to disease [22].

Conclusions

The spores of *A. flavus* under the favourable environmental conditions produce sufficient amount of AftB₁ that effect on human health. AftB₁ had been detected and quantified by TLC and ELISA. Aflatoxin B₁ could use as a marker for the diagnosis of various diseases caused by *Aspergillus* species, especially *A. flavus*. More research is needed to determine aflatoxins levels in biological specimens that are associated with adverse health effects.

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