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Qualitative and quantitative detection of aflatoxin B_1 in poultry sera by enzyme-linked immunosorbent assay

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Abstract. An indirect competitive inhibition type enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of aflatoxin B_1, in poultry sera. Preincubation of aflatoxin B_1, samples with the antibody prior to competition yielded better results in terms of higher sensitivity. After competition, amount of antibody bound to solid phase was measured by incubation with anti-rabbit immunoglobulins coupled with horse raddish peroxidase. Intensity of colour decreased as the amount of free aflatoxin B_1, increased. Final detection of aflatoxin B_1, was made by (i) visual comparison with standard aflatoxin B_1 using dot-ELISA (qualitative) and (ii) by plate-ELISA, where optical density was measured at 492 nm (quantitative). Plate-ELISA was more sensitive than dot-ELISA, with sensitivity limits being 100 fg and 1 pg per 10 µl, respectively. However, due to ease and speed of performance, dot-ELISA has greater potential as a test for the diagnosis of mycotoxicosis at the field level.

Keywords. Aflatoxins; ELISA; anti-aflatoxin antibodies; poultry sera.

1. Introduction

Aflatoxins are a family of closely related secondary metabolites produced by fungi viz., *Aspergillus flavus* and *A. parasiticus*. These are highly toxic and carcinogenic compounds. Among these, aflatoxin B_1 (AFB_1) is one of the most potential environmental carcinogen, with toxic effects on humans through its direct consumption in food products or as metabolic residues in animal tissues. Due to highly immunosuppressive and carcinogenic nature of AFB_1, even low level of contamination is important. Thus, there is a need to limit their concentration in feeds. Therefore, suitable analytical methods for detection and quantitation must be available for effective food/feed safety programmes.

Various analytical methods viz., TLC, GLC, HPLC etc., are available for its detection in feed/biological fluids. However, these methods though sensitive are cumbersome and need an extensive clean up of the samples and require expensive instruments.

However, we need simple, rapid, sensitive and specific tests, which do not require elaborate clean processing of feeds in order to screen large number of samples. Immunoassays due to a combination of high sensitivity and specificity ensure minimal sample preparation and hence contribute to high rates of sample analysis. A number of immunoassays have been described for their detection in food/feed stuffs (Biermann and Terplan 1980; Ueno *et al* 1983; Chu 1984; Morgan *et al* 1986; Park *et al* 1989; Fukal 1990).

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Aflatoxin contamination of food is one of the major sources of morbidity. Analysis of food samples provides only an indirect evidence of aflatoxin ingestion, whereas, direct evidence can only be obtained by analysis of body fluids as already reviewed (Dorner and Cole 1989). Monitoring of their concentration in body fluids requires the determination of even trace amounts because of their potent biological activity. Also, their measurement in fluids would give a direct measurement of exposure. Therefore, the present study was aimed at developing an immunoassay for AFB_1, detection in poultry sera from the birds fed on the toxin supplemented diet.

2. Materials and methods

2.1 *Chemicals*

AFB_1-BSA, BSA, Freund's adjuvant-incomplete, complete (CFA, IFA), Diamino benzidine dihydrochioride (DAB) and *o*-phenylene diamine dihydrochioride (OPD) were procured from Sigma Chemical Co., St. Louis, USA. Goat, anti-rabbit Igs-HRP was procurred from Dakopats, Denmark.

2.2 *Production of antibodies*

Antibodies against AFB_1 were raised in rabbits by immunization with sub-cutaneous injection of 400 μg of AFB_1–BSA, followed by an intramuscular booster (400 μg) after 15 days and freed from anti-BSA antibodies in the same manner described by Kapur *et al* (1996).

2.3 *Competitive inhibition ELISA for AFB_1 detection*

Qualitative detection of AFB_1 by competitive inhibition dot-ELISA was carried out as per figure 1. In step 3(a) antiserum and standard AFB_1 were preincubated for 1 h before being allowed to react with solid phase bound AFB_1. For quantitation, plate-ELISA was performed as per figure 1, with a few modifications i.e., optimum antigen concentration (obtained in checkerboard titration) used was 1 μg/well and the substrate used was OPD, instead of DAB.

Finally ELISA was tested for the performance in sera samples of poultry birds for AFB_1 detection. For this purpose, 50 chicken sera samples obtained from the Department of Veterinary Pathology, from the birds fed on a normal diet and diet supplemented with 1·25 ppm of standard AFB_1 were screened both by plate- and dot-ELISA

3. Results

3.1 *Competitive inhibition dot-ELISA for qualitative detection of AFB_1 in sera samples*

When solid phase bound and standard amounts of free AFB_1 (5 μg-1 fg) were simultaneously allowed to compete for antibodies, i.e., without preincubation, a Sensitivity of 500 pg was obtained. The assay was 100% sensitive as it showed positivity in all sera samples collected from birds fed on a controlled diet containing known amounts of

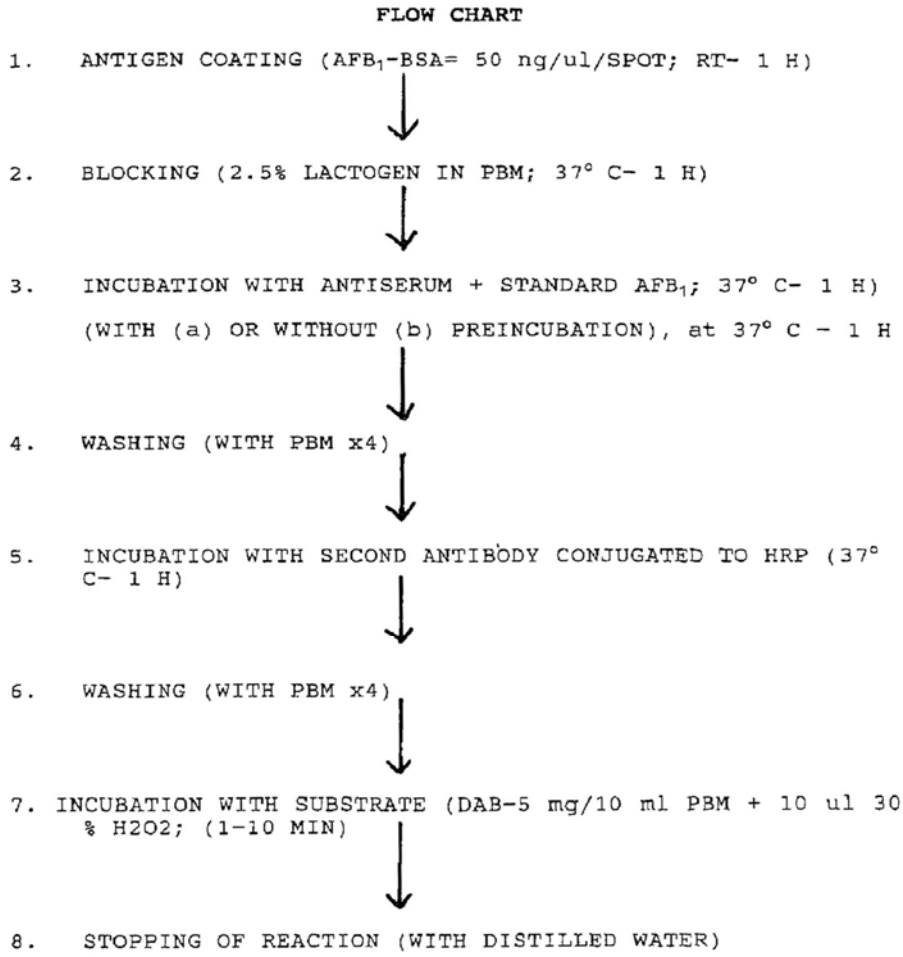

Figure 1. Indirect competitive inhibition dot-ELISA protocol for detection of AFB_1, in poultry sera.

standard free AFB_1. Their positivity with dot-ELISA was confirmed by standard method of Romer (1975) followed by TLC (Cocker *et al* 1984). A few false positives were observed in sera collected from birds fed on a toxin free diet. Significant improvement of the assay was noted after incorporation of an additional step of preincubation of free standard AFB_1 (10 μg-100 fg) with antibody prior to competition. Here a sensitivity of as low as 1 pg was obtained. No false positives were observed. The assay was 100% specific, though the sensitivity was 95% (figure 2).

With the affinity purified anti-AFB_1 antiserum, the assay employing standard free AFB_1, had a sensitivity of 100 pg and BSA did not interfere in the competition assay.

All these assays were repeated 4-5 times and consistent results were obtained in each assay. There was no shift in the positivity or negativity of the samples tested and hence, this assay can be safely employed for routine detection of AFB_1 in the sera samples.

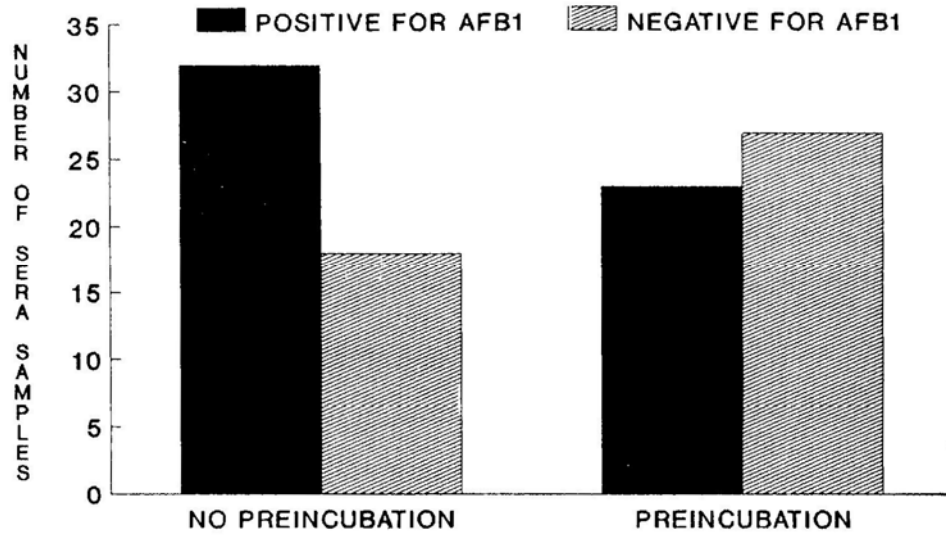

Figure 2. Competative inhibition dot-ELISA for detection of AFB_1 in polutary sera.

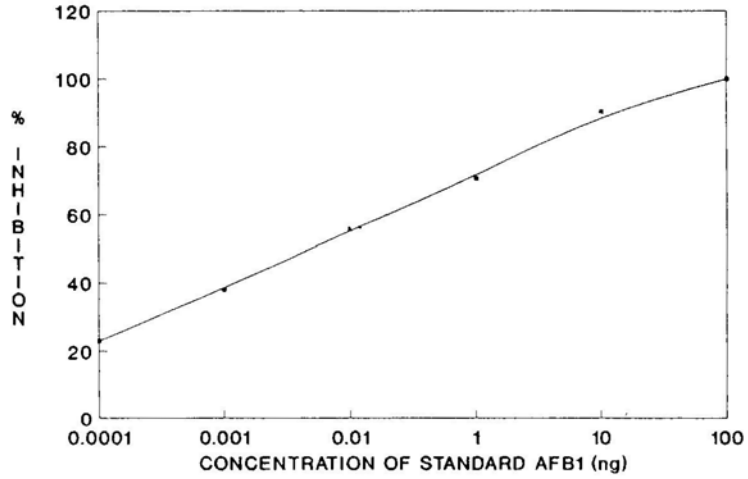

Figure 3. Standard curve for AFB_1, detection (from competitive inhibition plate ELISA).

In all the above assays, at a concentration of 100 ng or above, no colour developed thereby indicating 100% inhibition. This was further supported by our results on plate-ELISA (figure 3). Hence, the assay was only qualitative at concentrations above 100 ng and partially quantitative below it. Further, the samples when tested at different volumes of 10, 20 and 40 μl,showed no variation in the quantitation.

3.2 *Competitive inhibition plate-ELISA for quantitation of AFB_1*

For quantitation, a standard Curve was prepared by incorporating known amounts of standard AFB_1 in the assay (1 fg-10 μg). Per cent binding inhibition recorded

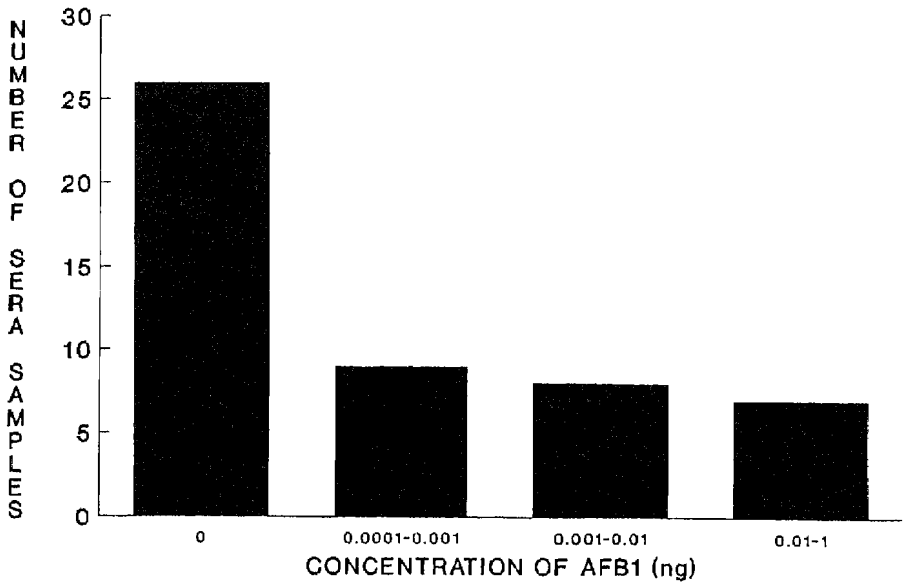

Figure 4. Competitive inhibition plate-ELISA for AFB, detection in poultry sera.

different concentrations of standard AFB_1 is shown in figure 3. The assay was repeated 3 times and no significant difference in the respective percentage inhibition with the various concentrations of standard AFB_1 was observed (data not shown). This variation did not affect the quantitation of AFB_1. On the basis of this inhibition, AFB_1 was quantitated in 50 chicken sera samples, the results of which are given in figure 4. Range of AFB_1 detected was 100 fg to 1 ng.

4. Discussion

AFB_1 is a highly immunosuppressive and carcinogenic secondary metabolite of fungi produced by *A. flavus* and *A. parasiticus*, found in a wide range of agricultural commodities. Conventional methods used for its detection viz., TLC, GLC, HPLC, etc., have limitations in terms of sensitivity, ease and duration time of test. Therefore, there is a need to develop a highly sensitive, specific, simple and nonradioactive tests. Recently enzyme immunoassays have become established as routine procedures in many developing countries (Morgan *et al* 1986; Park *et al* 1989; Wilkinson *et al* 1988).

Chu and Ueno (1977) for the first time developed ELISA for AFB_1 detection with a sensitivity of 0·2-2 ng/0·5 ml sample. In the present study, in dot-ELISA, a sensitivity was improved from 500 pg to 1 pg by including an additional step of preincubation, as also reported earlier (Shashidhar and Rao 1988). Sensitivity limit obtained in dot-ELISA in the present study (1 pg) is much higher than the 20 ng limit reported by Singh and Jang (1987). In plate ELISA, a sensitivity of 100 fg was obtained as also reported by Morgan *et al* (1986), but higher than the one achieved by Biermann and Terplan (1980). As far as we are aware, this is the first report of quantitation of AFB_1 in poultry sera.

This paper has described ELISA for detection and quantitation of AFB_1 in poultry sera. Dot-ELISA is intended for screening samples at the field level for on-site

monitoring of feed samples also. This is because the serum concentration of aflatoxin reflects the level of toxin found in food and subsequent consumption of this contaminated food. Also, the toxin ingested regularly, does not disappear rapidly, levels remaining significantly high due to release of toxin from tissue stores. Hence, it would be worthwhile to test this assay for AFB_1 detection in the tissues also.

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