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Development and Application of an Indirect Competitive Enzyme-Linked Immunoassay for Aflatoxin M_1 in Milk and Milk-Based Confectionery

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High-titer rabbit polyclonal antibodies to aflatoxin M_1 (AFM1) were produced by utilizing AFM1−bovine serum albumin (BSA) conjugate as an immunogen. An indirect competitive enzyme-linked immunosorbent assay was standardized for estimating AFM1 in milk and milk products. To avoid the influence of interfering substances present in the milk samples, it was necessary to prepare AFM1 standards in methanol extracts of certified reference material (CRM) not containing detectable AFM1 (<0.05 ng/g). The reliability of the procedure was assessed by using CRM with AFM1 concentrations of <0.5 and 0.76 ng/g. Also, assays of milk samples mixed with AFM1 ranging in concentration between 0.5 and 50 ng/L gave recoveries of >93%. The relative cross-reactivity with aflatoxins (AF) and ochratoxin A, assessed as the amount of AFM1 necessary to cause 50% inhibition of binding, was 5% for AFB1 and much less for AFB2, AFG1, and AFG2; there was no reaction with ochratoxin A. AFM1 contamination was measured in retail milk and milk products collected from rural and periurban areas in Andhra Pradesh, India. Of 280 milk samples tested, 146 were found to contain <0.5 ng/mL of AFM1; in 80 samples it varied from 0.6 to 15 ng/mL, in 42 samples from 16 to 30 ng/mL, and in 12 samples from 31 to 48 ng/mL. Most of the milk samples that contained high AFM1 concentrations were obtained from periurban locations. The results revealed a significant exposure of humans to AFM1 levels in India and thus highlight the need for awareness of risk among milk producers and consumers.

KEYWORDS: Aflatoxin M_1; antibodies; ELISA; milk; milk-based confectionery

INTRODUCTION

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* on a variety of agricultural commodities (*1*, *2*). Aflatoxin M_1 (AFM1) is a major metabolite of aflatoxin B_1 found in milk of animals that have consumed feeds contaminated with aflatoxin B_1 (*3*). The toxic and carcinogenic effects of AFM1 have been convincingly demonstrated in laboratory animals (*4*−*6*), and therefore AFM1 is classified as a class 2B human carcinogen (*7*). According to the U.S. Food and Drug Administration, AFM1 in milk should not exceed 0.5 ng/mL (*4*, *8*). AFM1 is relatively stable during pasteurization, storage, and preparation of various dairy products (4, *9a*), and therefore AFM1 contamination poses a significant threat to human health, especially to children, who are the major consumers of milk. To minimize the occurrence of AFM1, it is essential to trace the sources of contamination with AFM1 by using assays that are rapid and cost-effective. Most established analytical methods for AFM1 detection involve thin-layer chromatography and high-performance liquid chromatography (*9b*, *10*). These techniques require extensive sample preparation and are expensive to perform. Therefore, a rapid and sensitive technique for routine assay of milk and other dairy products is necessary.

Enzyme-linked immunosorbent assay (ELISA)-based methods have been used to detect AFM1 in milk (*11*, *12*). In the past these methods have been shown to be quick, reliable, and cost-effective for the estimation of aflatoxin B1 and ochratoxin A (*13*−*15*). In this paper, we report the production of high-titer polyclonal antibodies against AFM1 and their use in an indirect competitive ELISA procedure for estimating AFM1. The method described for the preparation of samples is simple, and the protocol developed for the estimation is sensitive. To assess the extent of AFM1 contamination in milk and dairy products,

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a survey was conducted in rural and periurban areas located in the state of Andhra Pradesh, India.

MATERIALS AND METHODS

Materials. Aflatoxin M1, aflatoxin M1−bovine serum albumin (BSA) conjugate, Freund's complete adjuvant, Freund's incomplete adjuvant, goat anti-rabbit IgG−ALP conjugate, *p*-nitrophenyl phosphate, BSA, and Tween 20 were purchased from Sigma Chemical Co., St. Louis, MO. Microtiter plates (Maxi-sorp F96) were obtained from Nunc. All other inorganic chemicals and organic solvents were of reagent grade or chemically pure.

Antibody Production. The immunization schedule and methods of injection were essentially the same as those described previously (*15*). A New Zealand White inbred rabbit was injected subcutaneously with 100 μg of AFM1−BSA in 500 μL of sterile 0.01 M phosphate-buffered saline (PBS) emulsified with an equal volume of Freund's complete adjuvant. Subsequent immunizations were given with incomplete Freund's adjuvant. After four immunizations at weekly intervals followed by a booster after three weeks, the rabbit was bled at weekly intervals and the titer of the antiserum was determined by indirect competitive ELISA. Booster injections were given when a drop in the titer was noticed.

Monitoring Antibody Titers by Indirect ELISA. An indirect ELISA was used for determining antibody titers (*15*). Microtiter plate wells were coated with 50 ng/mL of AFM1−BSA in 0.2 M sodium carbonate buffer, pH 9.6 (150 μL/well) and incubated overnight in a refrigerator. Subsequent steps were performed at 37 °C for 1 h. After each step, the wells were washed four times with PBS-T. To minimize nonspecific binding, 200 μL of 0.2% BSA in PBS containing 0.05% Tween 20 (PBS−T BSA) was added. Antiserum was diluted in PBS−T BSA and held for 45 min at 37 °C. Antiserum dilutions in 50 μL volume were added to 100 μL of AFM1 at concentrations ranging from 10 to 0.01 ng/mL. One hundred and fifty microliters of goat anti-rabbit immunoglobulins (GAR IgG) conjugated to alkaline phosphatase was used at a 1:1000 dilution to detect rabbit antibodies attached to AFM1. After a 1 h incubation with substrate (*p*-nitrophenyl phosphate, 1 mg/mL) at room temperature, absorbance was recorded at 405 nm (A_{405}) with an ELISA plate reader (Titertek Multiskan).

Characterization of Antibodies. To assess the cross-reactivity of the antiserum, tests were made using aflatoxins (AF) AFB1, AFB2, AFG1, AFG2, and ochratoxin A. The protocol used was similar to that used for assessing antibody titers, except that a constant amount of antibody diluted 1:300000 (50 μL at an appropriate dilution) together with 100 μL of toxin was added to AFM1−BSA-coated wells. The optimum dilution of antibody required to obtain maximum sensitivity was determined by 50% displacement values of B/B_0, where B is the extinction for the sample containing AFM1 and B_0 is the extinction of the sample without toxin, derived from the slope of the calibration curves.

Source of Milk Samples. Two hundred and eighty samples were collected from villages surrounding the city of Hyderabad (periurban) and from the Ananthapur (rural) area of Andhra Pradesh, India. The samples included 216 raw milk samples, each of ~50 mL, collected from individual buffalos (116 samples from periurban and 100 from Ananthapur), 44 (250 mL each) of commercially available factory-sealed milk packets sold in Hyderabad, 10 samples of powdered milk marketed in sealed cans, and 10 samples of milk-based confectionery sold in retail markets in Ananthapur. Samples were either processed soon after collection or kept at 4 °C and analyzed within 3−4 days.

Preparation of Milk Samples for ELISA. Samples (usually 15 mL) were centrifuged at ambient temperature for 10 min at ~2000*g*. An equal volume of methanol was added, and the mixture was shaken on a rotary shaker for 30 min at 250 rpm and then filtered through Whatman No. 41 filter paper. For samples of powdered milk [including certified reference material (CRM)] and confectionery, 10 g was suspended in 100 mL of distilled water, heated to ~50 °C, homogenized in a Waring blender, and then processed as for liquid milk.

Indirect Competitive ELISA Procedure. The protocol was similar to that for determining antibody specificity except that AFM1 standards in 100 μL, ranging from 10 ng/mL to 1 pg/mL, were prepared in AFM1-free milk sample extract (CRM milk samples with AFM1 concentration of <0.05 ng/g). Methanol extracted and filtered test milk samples were diluted to 1:10 in PBS−T BSA. A 100 μL aliquot of each sample was added to a well containing 50 μL of antiserum diluted to 1:300000. Standard curves were obtained by plotting log values of AFM1 standards against optical density at A_{405}. Concentration of AFM1 in the sample extract was determined from the standard curves and expressed in nanograms per milliliter using the following formula: AFM1 concentration (ng/mL) in sample extract × dilution with buffer × extraction solvent volume used (mL) ÷ sample volume (mL). To test the recovery of AFM1 from spiked milk samples, AFM1 standards were added to obtain concentrations ranging from 0.25 to 50 ng/mL in 10 mL milk samples known not to contain detectable AFM1 and then extracted and assayed as above.

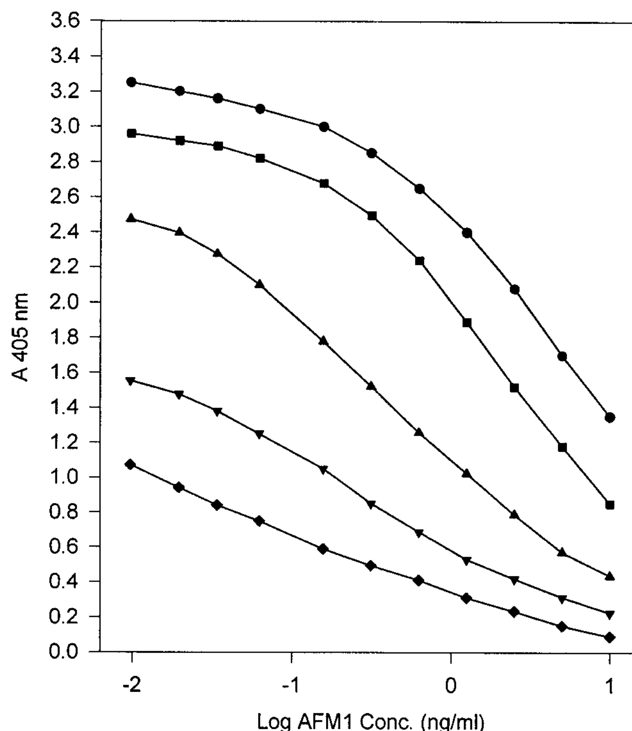

Figure 1. Standard curves for AFM1 by indirect competitive ELISA at different dilutions of antibody: 1:20000 (●); 1:40000 (■); 1:80000 (▲); 1:160000 (▼); 1:320000 (◆).

RESULTS

Production of Antibody. Antibodies were detected 6 weeks after initial immunization, and their titer increased after booster injection. The antibody titer was 1:320000 after 10 weeks of initiation of immunization.

Optimum AFM1—BSA and Antibody Dilution for ELISA. Antiserum at a dilution of 1:300000 gave optimum results. AFM1−BSA conjugate concentration of 50 ng/mL was found to be optimum for coating the plates. The curves for AFM1 standards at concentrations ranging from 0.01 to 10 ng/mL were found to be approximately linear (**Figure 1**). Utilizing these curves, 50% inhibition of binding of AFM1 was estimated to occur at 0.5 ng/mL.

Cross-Reactivity of Antiserum with Aflatoxins and Ochratoxin A. The results as shown in **Figure 2** demonstrated that the antibodies cross-reacted with AFB1 (at 5% level), cross-reacted weakly with AFB2, AFG1, and AFG2, and did not react with ochratoxin A.

Processing of Milk Samples for ELISA. Methanol-extracted and filtered milk samples could not be used directly in ELISA

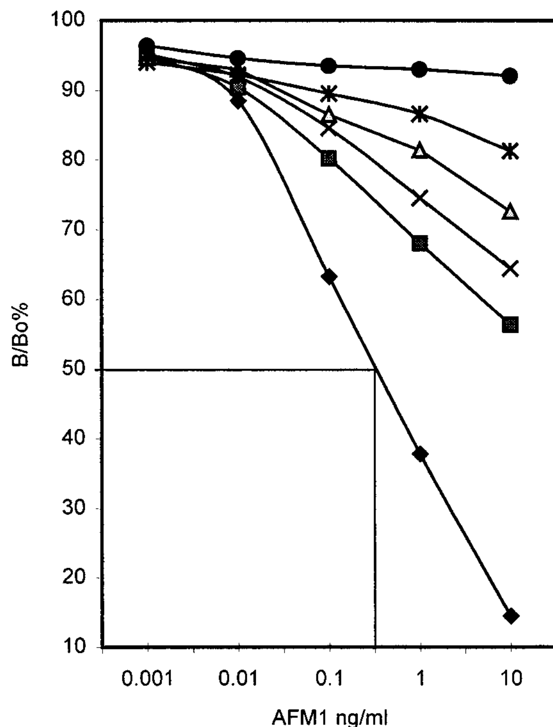

Figure 2. Cross-reactivity of AFM1 antiserum (◆) to AFB1 (■), AFB2 (×), AFG1 (▲), AFG2 (∗), and ochratoxin A (●).

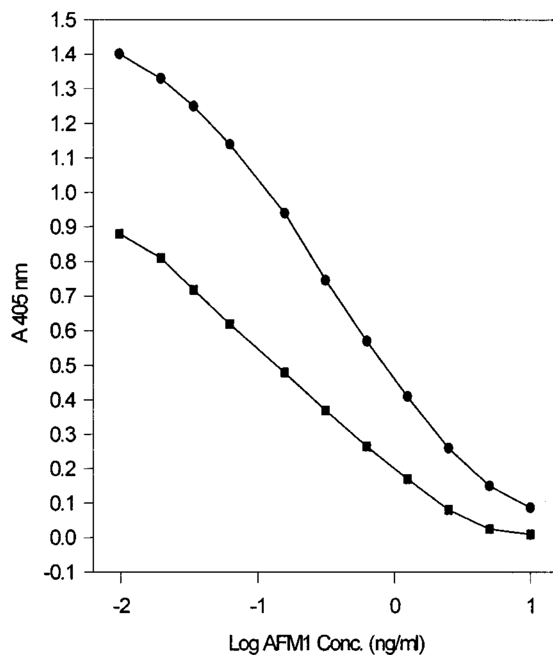

Figure 3. Effect of milk sample extracts on the standard curve of AFM1 by indirect competitive ELISA. Standard curves were prepared for AFM1 standards, by diluting either in BSA PBS–T (●) or in BSA PBS–T containing milk sample extracts (■).

as a result of interference from substances present in milk (**Figure 3**). However, preparation of milk samples in PBS−T BSA containing AFM1-free milk (diluted 1:10) prevented nonspecific reaction.

Recovery of AFM1 from Spiked and CRM Milk Samples. To test the accuracy of AFM1 estimations, toxin concentrations ranging from 0.01 to 50 ng/mL were added to the milk samples and assayed by ELISA. Milk samples known not to contain AFM1 were compared with samples deliberately contaminated with known amounts of AFM1. It is apparent from the results presented in **Table 1** that the procedure gave accurate results for AFM1 concentrations exceeding 0.25 ng/mL.

Table 1. Recovery of AFM1 from Artificially Contaminated Milk Samples As Determined by ELISA

no.	concn of AFM1 used for spiking samples[b] (ng/mL)	estimated concn of AFM1[a] (ng/mL)	% recoveries of AFM1 in spiked samples
1	0.25	0.26 ± 0.1	104 ± 7.8
2	0.5	0.47 ± 0.1	94 ± 7.0
3	1	0.97 ± 0.1	97 ± 7.5
4	5	4.53 ± 0.4	93 ± 8.3
5	10	9.43 ± 0.8	94 ± 7.8
6	25	27.4 ± 1.4	108 ± 7.4
7	50	48.1 ± 2.2	95 ± 3.1
8	CRM < 0.05	0.07 ± 0.2	140 ± 9.7
9	CRM = 0.76	0.79 ± 0.1	97 ± 2.2

[a] Each sample was spiked with a known concentration of AFM1, extracted in 70% methanol, and assayed. Data represent the mean of three replications ± SD. [b] Determined by the following formula: detected AFM1 (ng/mL) divided by the concentration of AFM1 used for spiking and multiplied by 100. Values are means ± SD.

Occurrence of AFM1. The incidence and levels of AFM1 in samples are summarized in **Table 2**. Analysis of three replicates of 280 milk samples showed that 134 samples (48%) contained AFM1 at levels ranging from 0.6 to 48 ng/mL. It was observed that the contamination of AFM1 was greater (93%) in the samples obtained from periurban areas than those from rural areas (2%). It is noteworthy that 50% incidence was observed in the powdered milk samples intended for infants and 30% in milk-based confectionery, although the numbers of samples tested were insufficient to obtain an accurate picture of the incidence of contamination.

DISCUSSION

India is currently the largest milk producer in the world. It is consumed as liquid milk and in milk-based confectioneries, which are made from condensed milk. It is apparent from a recent study that the utilization of milk in infant food formulations has increased substantially in recent years (*16*). Therefore, the quality of milk products has, and will have increasingly, a profound influence on the health of people in various age groups.

Contamination of milk with AFM1 and its ill effects on humans are well documented (*7*, *8*, *17*). Risks (especially its carcinogenic effects) due to the consumption of AFM1-contaminated milk have been assessed in some countries (*8*), but there are no reports on the distribution of AFM1 in milk produced by small-scale farmers or by large diaries in Andhra Pradesh. Testing of milk for AFM1 is not practiced by the milk industry in India. We attribute this to the lack of rapid and cost-effective technologies for AFM1 estimation.

Analytical methods have been successfully applied for AFM1 estimation in milk (*9a,b*, *18*, *19*). However, immunological methods for the estimation of various aflatoxins have been shown to be cost-effective (*12*) and therefore preferred for surveillance. We have found only two reports of the production of polyclonal antibodies for AFM1 (*11*, *12*). The antibodies were shown to be specific to AFM1 with limited cross-reaction to AFB1. The concentrations causing 50% inhibition of binding to the antibody by AFM1 and AFB1 were reported to be 0.65 and 100 ng/mL (*11*) and 8.5 and 15.0 ng/mL (*12*), respectively. The same antibodies produced by Harder and Chu (*11*) were shown to give higher sensitivity in a direct competitive ELISA (*20*, *21*). Antibodies produced in the current work show 50%

Table 2. Incidence and Range of AFM1 in Milk Samples As Determined by Indirect Competitive ELISA

sample type[a]	total no. of samples	AFM1 contamination					
		% samples > 0.5 ng/mL	no of samples with AFM1 (ng/mL) contents in the ranges of				
			0–0.5	0.6–15	16–30	31–45	48
raw milk (periurban)	116	93	8	59	37	11	1
raw milk (rural)	100	2	98	2	0	0	0
milk packets	44	36	28	11	5	0	0
powdered milk (g of dry milk/mL of solution)	10	50	5	5	0	0	0
milk products (g of dry milk/mL of solution)	10	30	7	3	0	0	0

[a] See text for details.

inhibition of binding at 0.5 ng/mL for AFM1 and >100 ng/mL for AFB1. Thus, they are highly specific and sensitive. These antibodies also have very high titers (1:300000), which are much greater than those of previous antibodies (cf. 1:300) (*12*) and can therefore be used at high dilution in assays. Thus, the protocols developed for the production of high-titered antibodies for aflatoxin B1 and ochratoxin A (*13*, *15*) could be applied for the production of high-titered polyclonal antibodies for AFM1. A problem in the use of the antibodies was the methanol extracts of milk, which caused nonspecific reaction in an indirect competitive ELISA. This was minimized by preparing AFM1 standards in certified AFM1-free milk. Results of AFM1 analysis were shown to be accurate by comparisons at regular intervals with European Union certified samples of known AFM1 concentrations. The reliability of the procedure was also assessed by a comparison with milk samples mixed with different AFM1 concentrations.

This is, to our knowledge, the first report from India showing AFM1 contamination of milk samples from periurban areas of a rapidly developing metropolis, the city of Hyderabad. Ananthapur was chosen to represent a rural area because >30% of small-scale farmers there produce milk and also groundnut haulms are used as fodder.

The major feed ingredients for cattle in periurban areas of the city of Hyderabad are cotton cake, groundnut cake, rice bran, and straw. We have analyzed some of the ingredients for aflatoxin content. The majority of cotton and groundnut cakes are contaminated with aflatoxin at levels exceeding 500 ng/g, and the highest was 3000 ng/g in one sample. They may be contributing to the high levels of AFM1. High incidence of aflatoxins in various ingredients of cattle feeds has been reported from India (*22*−*24*). This can be attributed to the prevalence of such optimum climatic factors as temperature and humidity for mold growth. In villages where AFM1 levels were found to be low, the animals are allowed to graze and the main feed ingredient was rice bran. However, more detailed study is needed to understand the various factors that contribute to high versus low AFM1 contamination. In rural areas such as Ananthapur, the majority of the farmers allow their animals to graze. Rice straw and food wastes are the main feed supplements. Interestingly, at the two rural locations where AFM1 was found to exceed 0.5 ng/mL, groundnut haulms containing small pods were used as the major supplement. Nevertheless, systematic studies are essential to ascertain the reason for AFM1 contamination.

Frequent contamination of AFM1 in milk and dairy products has led to the assessment of risk due to liver cancer (*8*). Our data clearly show the need for such a risk assessment in India. The results also highlight the importance of surveillance, particularly in periurban areas, for AFM1 contamination in milk and milk-based confectionery. The AFM1 estimation methods described and illustrated in this paper could form the basis of a low-cost risk assessment procedure. Ultimately such surveillance procedures must be linked to technical, policy, and institutional interventions that will lead to the reduction of AFM1 in milk production systems in India.

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