Review of Immunological Methods for the Quantification of Aflatoxins in Peanut and Other Foods

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ABSTRACT

Aflatoxin contamination is widespread in staple crops like peanut, maize, sorghum, pearl millet, chillies, pistachio, cassava etc., and compromises the safety of food and feed supplies. It is important to be able to detect and quantify aflatoxins in commodities to protect human and animal health. Many different methods, including antibody-based ones, are available for quantitative estimation of aflatoxins. However, most of these methods such as HPLC, HPTLC, and TLC are expensive and/or difficult to use in developed countries. Using the state-of-the-art facilities at ICRISAT, we developed polyclonal and monoclonal antibodies for the detection of total aflatoxins, aflatoxin B1 and M1 (secreted in milk). These were used to develop a simple and inexpensive competitive enzyme-linked immunosorbent assays (cELISA) that has lower detection limits (1.0 µg/kg) and cost (about $1 per sample) less than other available methods. More than 100 samples can be analyzed in a day. These tests have provided a unique opportunity for ICRISAT and its partners to conduct field studies to select resistant genotypes, identify high risk populations and determine the dietary sources to stimulate appropriate interventions to enhance the food and human health safety, trade and thereby farmers' income.

Key Words: Peanut, *Arachis hypogaea*, competitive ELISA, immunological methods.

Aflatoxins are the toxic secondary metabolites produced by *Aspergillus flavus* (Link) and *A. parasiticus* (Speare) contaminating staple crops like peanut (groundnut), maize, sorghum, pearl millet, chillies, pistachio, cassava, etc., and even found in milk from animals fed with the contaminated feed. Aflatoxin contamination effects quality of the produce, and food safety. It was reported that losses due to aflatoxin contamination in peanut was more than $26 million in the USA alone (Lamb and Sternitzke, 2001). Warm humid or drought conditions, insect/nematode pod damage in the field, over mature crops, rain at harvest, storing of improperly dried grains in the storage favor the proliferation of the fungi and subsequent aflatoxin production in peanut (Craufurd *et al*., 2006). Also, the existing technologies for peanut production, processing, and storage practices in most developing countries in the tropics and semi-arid tropics makes it difficult to totally eliminate aflatoxins, making them unavoidable contaminants. The problem of aflatoxin contamination is invisible and it is difficult to sort out the few contaminated grains from the commercial grain lots when it is present at low to moderate levels. In the absence of resistant varieties or technologies that eliminate the aflatoxin contamination in the food chain to mitigate hazardous effects on human and livestock health, it is essential to test the food products for aflatoxins before they are consumed (Waliyar *et al*., 2003). So, it is important to be able to detect and quantify aflatoxins in commodities to protect human and animal health.

Rationale for the development of immunological methods.

There are several chemical methods available for detection and estimation of aflatoxin, including high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and mini-column methods. However, most of these methods are expensive, laborious, time consuming, and require extensive sample cleanup (Dell *et al*., 1990; Goto and Manabe, 1989). There has been increase in demand for monitoring aflatoxins in developing countries in South-East Asia and sub-Saharan Africa (SSA), where high incidence of liver and other cancers prevail. To assess the risk posed by aflatoxin contamination through food and feeds, also to develop the resistant varieties or testing procedures to minimize aflatoxin, there is need for simple cost effective technologies for aflatoxin detection and estimation. The aflatoxin analysis by physiochemical methods such as HPLC, HPTLC, and TLC is expensive, laborious, requires extensive sample clean up, and is time consuming. This demand led researchers at ICRISAT to develop low cost technologies for aflatoxin estimation using immunological methods. Polyclonal antibodies were produced to aflatoxin B1

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Table 1. Cross reaction and minimal inhibition observed with aflatoxin B_1 (AFB1) monoclonal antibodies.

AFB1 clone identity	Cross reaction				Minimal inhibition		
	B1	B2	G1	G2	B1	B2	G1
	%	%	%	%	ng/mL	ng/mL	ng/mL
10D5 1A11	100	2	12	<1	0.001	–	–
5D8 2B1	100	–	110	–	1	–	1
13D1-1D9	100	2	100	<1	0.01	–	0.01
5F2-1E8	100	12	100	3	0.1	–	0.1
3G7-1B8	100	22	100	<1	0.1	1	0.1
11C8-1A8	100	20	66	1	0.01	–	0.01
3F7-1B9	100	15	60	<1	<0.01	–	<0.01
5H4-1B1	100	13	72	1	<0.01	–	<0.01
6G12-2B3	100	7	50	<1	<0.01	–	<0.01
6E12-1E5	100	60	75	5	0.1	0.1	0.1

*Concentration of aflatoxin (ng/ml) required for 1st significant inhibition of binding of antibody to AFB1-BSA solid phase.

(AFB1) and M1 (AFM1), and by using advances in hybridoma technology, monoclonal antibodies to aflatoxins also were produced for the detection of total aflatoxins (Devi *et al*., 1999; Thirumala-Devi *et al.,* 2002). Ten hybridoma cell lines were selected that produce monoclonal antibodies with a range of specificities among the four major aflatoxins (Table 1). The monoclonal and polyclonal antibodies were used to develop a simple, sensitive, specific and inexpensive competitive enzyme-linked immunosorbent assay (cELISA) that has lower detection limits (1.0 µg/kg) and cost (about $1 per sample) less than other available methods. More than 100 samples can be analyzed in a day and the results obtained are comparable with HPLC analyses. The cELISA assay is simple, easy to perform, requires minimum laboratory facilities, and most of the chemicals are available locally in developing countries. Moreover sample extraction for ELISA test is simple and quick involving single step methanol extraction. Consequently, many types of immunoassays, including radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), as well as several novel immunochemical screening tests were developed.

Competitive ELISAs.

The distinguishing feature of the competitive assay format is that the combination of an unknown amount of analyte introduced from the sample and the reference analyte compete for binding to a limited number of antibody binding site. This assay can be performed with either the analyte or the antibody adsorbed to the solid phase. Two types of cELISAs have been developed for the analysis of aflatoxins, and both types are heterogeneous assays which produce the uniform results. Direct ELISA involves the use of an aflatoxin-enzyme conjugate, whereas indirect ELISA involves protein-aflatoxin conjugate and a second antibody to which the enzyme has been conjugated (Ramakrishna and Mehan, 1993).

Indirect competitive ELISA.

In the indirect competitive ELISA, commercially available AFB1-BSA conjugate was coated on to the wells of a microtiter plate. Later, aflatoxin standards, as well as samples with specific rabbit antibodies, were added to the plate before incubating it at 37 C for 1 hr. After washing the plate to determine the amount of antibody bound in the wells, goat anti-rabbit IgG labeled with alkaline phosphatase was added followed by addition of p-nitrophenyl phosphate substrate. Thus, toxin in the sample or standard and toxin bound on the well surface competes for the binding site on the specific antibody in the solution (Waliyar *et al.*, 2005a). Aflatoxin recovery studies showed that mean recoveries of AFB1 from the peanut samples spiked with 1–250 µg/kg of toxin ranged from 67 to 112% (Table 2). Also, there was good correlation be-

Table 2. Recovery of aflatoxin B_1 (AFB1) by indirect competitive ELISA from groundnut samples spiked with different concentrations of toxin.

AFB_1 added	AFB_1 recovered	Recovery
µg/kg	µg/kg ± SE* (CV%)	%
1	0.67 ± 0.09 (13.23)	66.67
5	3.83 ± 0.34 (8.83)	76.67
10	11.23 ± 0.71 (6.34)	112.33
20	19.87 ± 1.17 (5.89)	99.39
30	27.70 ± 1.39 (5.01)	102.47
50	51.23 ± 0.95 (1.86	102.47
75	66.63 ± 3.28 (4.93)	88.84
100	101.17 ± 5.67 (5.61)	101.17
150	135.49 ± 8.80 (6.50)	90.79
200	173.03 ± 7.01 (4.05)	86.56
250	225.03 ± 11.38 (5.06)	90.1
Mean		92.5 (6.16)

*SE = Standard error; CV = Coefficient of variation.

Table 3. Comparison of analysis of aflatoxin B_1 (AFB1) concentration in naturally contaminated chilli samples by indirect competitive-ELISA and HPLC methods.

Sample grades	AFB_1 (µg/kg) ± SE (CV%)		t-test
	ELISA (n=27)	HPLC (n=18)	
Chilli grade 1	2.07 ± 0.22 (10.58)	1.95 ± 0.15 (7.69)	0.38
Chilli grade 1	2.02 ± 0.31 (8.87)	2.98 ± 0.28 (6.71)	−3.48*
Chilli grade 1	21.20 ± 1.53 (7.22)	19.88 ± 1.12 (5.63)	0.62
Chilli grade 2	40.70 ± 3.26 (8.02)	37.28 ± 2.15 (4.08)	0.78
Chilli grade 2	77.82 ± 6.51 (8.37)	70.01 ± 4.09 (5.76)	0.76
Chilli grade 2	99.93 ± 7.55 (7.56)	92.87 ± 4.23 (4.54)	0.69
Chilli grade 3	140.10 ± 6.65 (4.74)	129.06 ± 8.06 (6.39)	1.05
Chilli grade 3	188.86 ± 10.73 (5.69)	204.85 ± 9.45 (4.61)	−1.03
Chilli grade 3	283.00 ± 14.84 (5.24)	242.38 ± 11.62 (4.79)	1.93
Mean of sub-sample CV (%)	(7.36)	(5.57)	

*S = signifiant at P = 0.05.

tween ELISA and HPLC estimations (Table 3) in eight of the nine chilli samples tested in which the toxin ranged from 2–283 µg/kg (Reddy *et al.*, 2001). Similarly, AFM1 recoveries ranged from 94–100% in artificially contaminated milk samples in the indirect competitive ELISA (Table 4). Indirect competitive ELISA requires less antiserum than direct competitive ELISA and does not require preparation of a toxin-enzyme conjugate, but it takes 1 hr of extra analytical time. To shorten the assay time for indirect ELISA, modifications can also be made by conjugating the enzyme to the antibody, which is then used in ELISA instead of using a second antibody-enzyme conjugate. A number of studies have been carried out to investigate the efficacy of direct and indirect immunoassays by comparing them with HPLC or TLC and there was good correlation among the methods (Ramakrishna and Mehan, 1993; Reddy *et al.*, 2001).

Table 4. Recovery of aflatoxin M1 (AFM1) from artificially contaminated milk samples as determined by ELISA.

No.	Concentration of AFM1 used for spiking samples[a]	Concentration of AFM1 estimated[b]	Recovery of AFM1 in spiked samples
	ng/mL		%
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 mean of three replications ± SD. CRM = Certified reference milk sample.

[b]Determined by the formula, Detected AFM1 (ng/mL) divided by the concentration of AFM1 used for spiking and multiplied by 100. Values are Means ± SD.

Direct competitive ELISA.

In the direct competitive assay, specific antibodies are first coated on to a high quality high binding microtiter plate. Then the sample solution or various concentrations of standard toxin are generally incubated simultaneously with the enzyme conjugate in the ELISA plate. After appropriate washings, the amount of enzyme bound to the plate is determined by incubation with suitable substrate (p-Nitrophenyl phosphate for the alkaline phosphatase enzyme). At ICRISAT, a different kind of conjugate was prepared using commercially available AFB1-BSA which was conjugated to enzyme alkaline phosphatase or penicillinase or horse radish peroxidase and used in the direct ELISA instead of AFB1-enzyme conjugate (Anjaiah *et al.*, 1989; Waliyar *et al.*, 2005a). Moreover, this procedure is simple and the conjugate is stable at 4 C for more than a year. In the direct ELISA, toxin in the sample and toxin-enzyme conjugate compete for the antibody binding coated on the solid plate surface.

In both the cELISAs, after addition of the substrate, the resulting color development is then measured spectrophotometrically at 405 nm using an ELISA reader to record the optical density values for standards as well as unknown samples. To calculate the aflatoxin content in the unknown samples, a regression curve using AFB1 standard's optical density (OD) values was drawn, and based on the regression equation the AFB1 in the samples was determined (Fig. 1). Because the antibody and toxin-enzyme concentrations are constant, the color intensity as a function of enzyme reaction is

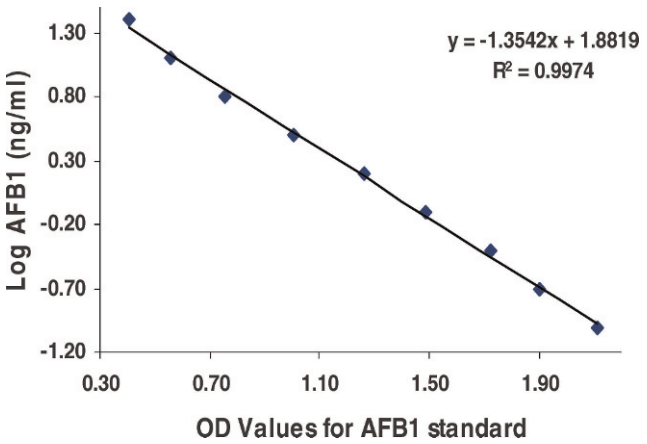

Fig. 1. Linear regression curve and equation for aflatoxin standard in cELISA.

inversely proportional to the toxin concentration in the testing samples or toxin standards. Less color indicates more toxin while more color indicates less/no toxin. In general, cELISA are approximately 10–100x more sensitive than radio immunoassay (RIA) when purified toxins are used. As little as 5 pg of pure aflatoxin can be measured. Since a clean-up step is generally not necessary, many samples can be analyzed within a relatively short period. The sensitivity of the cELISAs for the analysis of aflatoxins in foods and feeds is generally in the range of 0.1–250 μg/kg. Due to the use of better antibody and toxin-enzyme conjugates, the time required to run the cELISA has improved considerably. Thus, the entire ELISA procedure can be completed within 2 hr (Chu, 1995; Lee *et al*., 2004). The sensitivity of the ELISA is improved when the clean-up treatment is included in the assay protocols. Many mycotoxin detection kits based on immunoassays are available commercially; however, they are very expensive and some times non-specific reactions develop probably due to storage problems.

The application of mycotoxin immunoassays is not limited to foods and feeds; it has been used as a sensitive approach for monitoring of mycotoxins in body fluids and tissues and organs of humans and animals that have been exposed to the mycotoxins. Currently we are developing immunoassays to detect the aflatoxin in human and animal blood. This will not only be helpful in detecting aflatoxin in the human blood but also for monitoring aflatoxicosis in human beings and livestock (Anitha *et al.*, 2007).

Matrix interference.

One of the common challenges of immunoassay for food analysis is matrix interference causing false positives. Because there is a high probability of the presence of structurally related compounds in the sample that may react with the antibody, the sample matrix should be tested before the assay. This occurs when (a) the enzyme activity is inhibited by the presence of interferences in the sample extracts and/or, (b) the interaction between the antigen (AFB1) and the antibody is hindered in an immunoassay (Lee *et al.*, 2004). The matrix interferences with the chilli sample extract were observed and were resolved by diluting the toxin standards in the toxin free extracts of chilli (Reddy *et al.*, 2001). Moreover, these matrix interferences can be reduced by a number of methods such as dilution of sample extractor or removal of interferences by sample clean up procedures. The degree of matrix interference varied with different food samples, so individual validation and optimization of the extraction and estimation protocol would be necessary for each commodity sample type. In most of the immunoassays, sample cleanup is not necessary. Samples taken after extraction from solid matrix could be used directly in the assay after appropriate dilution in the assay buffer. Nevertheless, the sensitivity increased after appropriate clean-up treatment (Waliyar *et al*., 2005a).

Radioimmunoassay (RIA).

The principle of aflatoxin quantification is similar to cELISA. However, an enzyme label, radio isotope-labeled antibodies or aflatoxin standards are used as reporter molecules. The specific activity of the radioactive marker plays an important role in determining the sensitivity of the assay. Although ^{14}C, ^{3}H, and ^{125}I labeled mycotoxins have been used in various RIAs, ^{3}H labeled toxins were most commonly used. However, ^{125}I labeled toxins have been shown to provide the highest sensitivity (Chu, 1995). The RIA procedure involves incubation of specific antibody simultaneously with a solution of unknown sample or known standard and a constant amount of labeled toxin. After separation of free and bound toxin, the radioactivity in those fractions is determined. The toxin concentration of the unknown sample is determined by comparing the results to the standard curve which is established by plotting the ratio of radio activities in the bound fraction and free fraction vs. log concentration of unlabeled standard toxin.

The RIAs have been used for analysis of aflatoxins in corn, wheat, peanut, milk, serum and eggs with a minimum detection limit ranging from 0.25–0.5 ng in each assay when titrated mycotoxins are used as the marker. However, because of sample matrix interference, the lower limit of mycotoxin detection in food or feed samples is about 2–5 μg/kg. The sensitivity of the RIA can be improved by a sample clean-up

procedure after extraction. Although RIA provides sensitive and accurate mycotoxin analysis, use of radioactive legend poses difficulties in safe disposal. Thus, it has been primarily used in the laboratories permitted to use radioisotopes. As a consequence, it mainly remained as a research tool in well established laboratories.

Antibody based immuno-screening tests.

By shortening the incubation time and adjusting antibody and toxin-enzyme conjugate concentrations in the direct or modified indirect competitive ELISA system, it is possible to do a quick screening test at certain toxin levels. Based on the principle of antigen-antibody interactions such as in ELISA, several other immuno-screening tests with sensitivity similar to ELISA have been developed. In this, the antibody is immobilized on a paper disk or other affinity membranes, which is used directly as a strip or mounted either on a plastic card or a cup. The reaction is carried out on the wetted membrane disk. Upon completion, the absence of color (or decrease in color that is generally blue) at the sample spot indicates the presence of toxin in the sample; and the entire test can be completed in less than 1 hr.

Another test is the immunoaffinity method, which is applicable to mycotoxins such as aflatoxins that have fluorescence. In this assay, aflatoxin extracted from the sample is first diluted with buffer at pH 7.0 and subjected to disposable affinity column containing anti-aflatoxin antibody coupled with Sepharose gel. Samples such as milk and urine can be applied to the column directly after adjusting the pH and dilution. After washing, aflatoxin is removed from the column with the methanol, subjected to treatment with iodine/bromine solution, and fluorescence is determined. The affinity column serves as a specific clean-up and concentration tool for further analysis by HPLC method (Chu, 1995).

Conclusions

The cELISA tests has provided a unique opportunity for ICRISAT and its partners to select breeding populations possessing resistance to aflatoxin contamination, and to evaluate food, feed and related commodities for aflatoxin contamination. This is contributing to stimulate appropriate interventions to enhance the food and human health safety and enhance trade and farmers' incomes. ICRISAT helped in establishing 17 aflatoxin monitoring laboratories in India, Mozambique, Kenya, Malawi and Mali that use our cELISA technologies. Training was provided to the local personnel to manage the facilities. The diagnostic reagents are widely distributed to partners in Asia and Sub-Sahara Africa. These laboratories are contributing to the quality certification of the farmers produce and enhancing the competitiveness of the produce in domestic and international markets. For instance, National Small Farmer Association of Malawi (NASFAM) and ICRISAT have established collaboration for testing the peanut produced for aflatoxin content. Based on the level of contamination, NASFAM graded peanut lots into permissible (<4 μg kg^{-1} or <20 μg kg^{-1}) and non-permissible (>20 μg kg^{-1}). Graded peanut lots found favorable market for regional and global export, benefiting the farmers (Waliyar *et al*., 2005b). Thus, the aflatoxin testing lab at Malawi contributed to the revival of peanut exports to Europe and South Africa. ICRISAT is planning to increase aflatoxin testing facilities to strengthen the local capacities for aflatoxin monitoring in Sub-Sahara Africa and Asia.

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