Food Control 20 (2009) 1080–1085

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Detection of aflatoxin M1 in milk products from China by ELISA using monoclonal antibodies

Shi Chun Pei [a,*], Yuan Yuan Zhang [a], Sergei A. Eremin [b], Won Jong Lee [c]

[a] Food College, The HLJ August First Land Reclamation University, Daqing 163319, China
[b] Faculty of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119992, Russia
[c] Department of Food Science, Kangnung National University, Gangneung, Gangwon 210-702, Republic of Korea

ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 5 February 2009
Accepted 10 February 2009

Keywords:
Aflatoxin M1
ELISA
Milk
Contamination
China

ABSTRACT

A rapid and sensitive indirect competitive enzyme-linked immunosorbent assay (ELISA) method using monoclonal antibody for measuring aflatoxin M1 (AFM1) in milk and milk products has been described. One monoclonal antibody was isolated and characterized after fusion of myeloma cells with spleen cells isolated from BALB/c mice that had been immunized with AFM1 carboxymethyl oxime conjugated with bovine serum albumin (BSA). Cross-reactivities of the anti-AFM1 monoclonal antibody clone were 100, 13.9, 6.7 and <1% against AFM1, aflatoxin B1 (AFB1), aflatoxin G1 (AFG1) and deoxynivalenol (DON), respectively. Assays of milk samples mixed with AFM1 ranging in concentration from 0.1 to 3.2 ng/ml gave mean ELISA recovery of 98%. The limit of detection concentration of AFM1 was 0.04 ng/ml. AFM1 contamination was measured in 12 samples of raw milk, 15 samples of powdered milk, 104 samples of liquid milk and four cheese samples collected from different supermarkets in Northeast of China. Of 135 milk samples tested, 55 (41%) samples contained AFM1 at levels that ranged from 0.32–0.50 ng/ml, 24 (18%) samples contained 0.16–0.32 ng/ml, and 18 (13%) samples contained 0–0.16 ng/ml; in 38 (28%) samples AFM1 was not detected. The results indicate that the necessary precaution will have to be taken to minimize the AFM1 contamination in milk and milk products from Northeast of China.

1. Introduction

Aflatoxins (AFs) are extremely toxic substances produced by certain species of *Aspergillus*, especially *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* that contaminate plants and its products. *A. flavus* produces only B aflatoxins, while the others produce both B and G aflatoxins (Creepy, 2002; Tekinsen & Tekinsen, 2005). Aflatoxins M1 (AFM1) is the hydroxylated metabolites of aflatoxin B1 (AFB1) and may be found in the milk of animals that are fed with AFB1 contaminated feeds (Galvano et al., 2001). AFs are both acutely and chronically toxic for animals and humans, and can cause dangerous illnesses including acute liver damage, liver cirrhosis, tumor induction and are also teratogen (Deshpande, 2002). AFB1 is the most potent hepatocarcinogen known in mammals, and the risk assessment of which is very well known (Virdis, Corgiolu, Scarano, Pilo, & De Santis, 2008). Although the toxicity of AFM1 is less than that of its parent compound, AFM1 is known to be hepatotoxic and carcinogenic (Lee, Kwak, Ahn, & Jeon, 2009). Therefore its toxicity, initially classified by WHO–International Agency for Research on Cancer (IARC) as a Group 2B human carcinogen (IARC, 1993), has now been classified as Group 1 carcinogen (IARC, 2002).

When lactating animals are fed with feedstuffs containing AFB1, this metabolite can be converted to AFM1. Thus, AFM1 concentration in milk and milk products depends on the levels of exposure and the amount of AFB1 ingested (Cathey, Huang, Sarr, Clement, & Phillips, 1994). The forming of AFM1 occurs in liver and it is secreted into milk in the mammary gland of dairy cows. AFB1 once ingested by mammals is quickly absorbed from the gastro-intestinal tract and appears as the metabolite AFM1 in blood after just 15 min (Moschini, Masoero, Gallo, & Diaz, 2007). In China, 284 samples of corn, peanut, rice, walnut and pine nut from markets of Chongqing, Fujian, Guangdong, Guangxi, Hubei, Jiangsu, Shanghai and Zhejiang provinces were analyzed for AFB1, and of samples, 62.2% corn, 40.0% peanut, 19.1% rice, 43.8% walnut and 16.7% pine nut were contaminated with AFB1 (Wang & Liu, 2006). The data clearly show the need for such AFB1 risk assessment in China, and that AFM1 contamination in milk and milk products should be monitored.

Milk and milk products are a good source of many nutrients such as protein and calcium for humans, especially for children. However, milk and milk products are the most potent source of aflatoxin among foods. AFM1 is relatively stable during heat treatments like pasteurization of milk and milk-based products

* Corresponding author. Tel.: +86 0459 6819238; fax: +86 0459 6819235.
E-mail address: peisc2002cn@yahoo.com.cn (S.C. Pei).

0956-7135/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodcont.2009.02.004

or processing into cheese (Bakirci, 2001; Galvano, Galofaro, & Galvano, 1996; Stoloff, 1989). AFM1 intake, even at low concentrations, causes a significant risk to human health, especially to children who are the major consumers of milk (Rastogi, Dwivedi, Khanna, & Das, 2004; Thirumala-Devi et al., 2002). Due to serious health concerns, many countries have regulations to control the levels of AFM1 in milk and dairy products. The European Commission prescribes that the maximum level of AFM1 in raw milk, heat-treated milk and milk for the manufacture of milk-based-products should not exceed 50 ng/kg (Commission Regulation (EC), 2006b). However, US' and China's regulations on levels of AFM1 in milk is limited at 500 ng/kg. In Austria and Switzerland the maximum level is even lower at 10 ng/kg for infant food commodities (FAO, 1997).

Current aflatoxin detection methods include thin-layer chromatography (TLC) (Sassahara, Pontes Netto, & Yanaka, 2005), liquid chromatography/electrospray-tandem mass spectrometry (Cavaliere, Foglia, Pastorini, Samperi, & Laganà, 2006), high-performance liquid chromatography (HPLC) (Hu, Zheng, Zhang, & He, 2006). 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. Over the last 20 years, the importance and application of immunoassays, especially ELISA, has grown significantly. ELISA is not only suitable tool for quick and sensitive analysis with high sample throughput (Lee, Wang, Allan, & Kennedy, 2004), but also cost-effective, fast, and requires only a small sample volume for analysis (Sherry, 1997). In this paper, we report the production of high affinity monoclonal antibodies against AFM1 and use a rapid and sensitive indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) to estimate AFM1. This study was also carried out to evaluate the occurrence of milk and milk products contamination with AFM1 in Northeast of China by ELISA using monoclonal antibodies.

2. Materials and methods

2.1. Materials

Aflatoxin M1 carboxymethyl oxime conjugated with bovine serum albumin (BSA), Freund's complete adjuvant, Freund's incomplete adjuvant, aflatoxin B1, deoxynivalenol, goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP), hypoxanthine, dimethyl sulphoxide, polyethyleneglycol 4000 (PEG 4000), bovine serum albumin, o-phenylenediamine, Tween-20 were purchased from Sigma Chemical (St. Louis, MO, USA). St. Dulbecco's Modified eagle medium (DMEM) and RPMI medium 1640 were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Fetal Bovine Serum (FBS) was purchased from PAA cell culture company (Pasching, Austria). Mice (BALB/c) were purchased from Vital River Laboratory Animal, Inc. (Beijing, CN). All other inorganic chemicals and organic solvents were of reagent grade or chemically pure.

2.2. Production of monoclonal antibody

The immunization schedule and methods of injection were essentially the same as those described by Holtzapple, Carlin, Rose, Kubena, and Stanker (1996). Six 8-week-old female BALB/c mice were injected subcutaneously with 50 μg/ml of AFM1-BSA in 50 μl of sterile 0.01 M phosphate-buffered saline (PBS) emulsified with an equal volume of Freund's complete adjuvant. Subsequent immunizations (boosted) were given with incomplete Freund's adjuvant. Blood samples were removed from the tail at intervals after each boost and assayed by indirect ELISA for antibody titer. After the mice were given a final booster 3 days, the mouse with the highest serum titers was killed. The spleen was removed aseptically and splenocytes were fused with cells of the SP2/0-Ag14 murine myeloma line at a ratio of 1:10 in the presence of PEG 4000 (Devi et al., 1999). The fusion cell were diluted with fresh HAT medium and distributed into each well with feeder cell of five Costar plates. After 10 days, hybridoma supernatants were analyzed by indirect ELISA for the presence of antibodies against AFM1. Culture supernatants from the cells that gave an absorption value of over 3 were transferred to 24-well microculture plates in DMEM containing 20% FBS. Supernatants from 24-well culture plates were tested again and only those clones that maintained absorption values over 3 in ELISA tests were chosen for further selection. Cell suspensions from each well of the 24-well- cultured plate were diluted to give approximately one cell per well and distributed into a 96-well culture plate. Those that contained a single hybridoma in each well were retained to obtain antibody for further characterization (Devi et al., 1999). Abundant monoclonal antibody ascites were obtained from big female BALB/c mice, and the ascites was purified by zincic acid–ammonium sulfate method.

2.3. Monitoring antibody titers by ELISA

An indirect ELISA was used for determining antibody titers. Nunc microwell plates (GIBCO) were coated with 1.5 μg/ml of AFM1-BSA (100 μl per well) in 0.2 M sodium carbonate buffer, pH 9.6 and incubated at 4 °C overnight (Thirumala-Devi et al., 2002). Plates were washed four times after each step of incubation with PBS-T. To minimize nonspecific binding, 250 μl 0.4% PBSM (4 g defatted milk powder in 100 ml 0.01 M PBS) was added, and held for 45 min at 37 °C. Then 100 μl hybridoma cell culture supernatant or antiserum dilution (diluted with 4% PBSM) was added to each well and incubated for 1 h at 37 °C. The titers of hybridoma cell culture supernatant or antibody were determined by the addition of 100 μl anti-mouse IgG-horseradish peroxidase (HRP) conjugate diluted 1:5000 with 4% PBSM. The microwell plates were held for 45 min at 37 °C, and then subsequently, 100 μl of OPD (0.004 g o-phenylenediamine in the 4.8 ml citric acid and 5.2 ml Na_2HPO_4) substrate was added to each well and incubated for 15 min at 37 °C. The reaction was stopped by 50 μl of 2 M H_2SO_4 and the absorbance was measured at 492 nm in VersaMax microplate reader (Molecular Devices Corporation, CA, USA).

2.4. Characterization of antibodies

Determination of isotype: Commercially available SBA Clonotyping™System/HRP (Cat. No. 5300-05) kits from Southern Biotech were used to determine the isotypes of the MAbs produced by hybridoma cell lines.

Cross-reactivity: To assess the cross-reactivity of the MAb, tests were made using AFM1, AFB1, AFG1 and DON. The protocol used was similar to that used for assessing antibody titers, except that the toxins analogs were present. After the initial PBSM incubation, there was a stepwise addition of 50 μl of MAb diluted 1:8000, then 50 μl of each toxin standard (all toxin standards were prepared fresh on the day of the assay with different combinations from 0.08 ng/ml to 10 ng/ml). 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 sample containing toxins and B_0 is the extinction of the sample without toxins, derived from the slope of the calibration curves.

2.5. Sample preparations

From March 2008 to May 2008, a total of 12 raw milk samples were collected from three farms, and 15 powered milk samples,

104 milk products and four cheeses were randomly collected from supermarkets in Heilongjiang Province. Samples were either analyzed right away or stored at 4 °C no longer than 3–4 days. AFM1 is water soluble (Deshpande, 2002), so the powder based samples (10 g) were suspended in 100 ml of warm deionized water. Subsequently, these samples as well as liquid milk samples were centrifuged at 3500 g for 10 min at 4 °C. The upper creamy layer was removed and samples were further diluted 20 times (v/v) with deionized water. Two grams of cheese were added to 16 ml methanol and stirred for 30 min at 250 rpm. The suspension was filtered (Millipore, 0.45 μm) and filtrate was centrifuged at 2700 g for 15 min at 15 °C, and the upper phase was removed and an aqueous-methanol layer (100 μl) was added to 0.01 M PBS (900 μl, dilution 1:10). The AFM1 content was analytically determined on 100 μl of this solution using the same technique as in milk.

2.6. AFM1analysis in samples by indirect competitive ELISA

The quantitative analysis of AFM1 in samples was performed by indirect competitive ELISA. The protocol was similar to that for determining antibody specificity except that AFM1 standards concentration ranging from 0.04 ng/ml to 5 ng/ml were prepared in AFM1-free milk samples extract (certified reference material(CRM), ERMI-BD282, aflatoxin M1, zero level). Fifty microliter aliquot of each sample was added to a well containing 50 μl of purified ascites antibodies which was diluted 1:8000. Standard curves were obtained by plotting of AFM1 standard against optical density at A_{492}. Concentration of AFM1 in the milk samples extract was determined per milliliter using the following formula:

AFM1 concentration(ng/ml)in sample extract

÷ milk sample dilution factor.

The concentration of AFM1 in cheese extract was determined per kilogram using the following formula:

AFM1 concentration(ng/ml)in sample extract

× 90(dilution factor) ÷ 2(g) × 1000.

For the recovery test of AFM1 from spiked milk samples, AFM1 standards were added in 10 ml milk samples known not to contain detectable AFM1 to obtain concentrations ranging from 0.01 to 3.2 ng/ml, and then extracted and assayed as described above.

3. Results and discussion

3.1. Isolation and characterization of MAb against AFM1

Hybridoma production: The fusion efficiency (number of wells showing cell multiplication in each well of the 96-well plates) was 75.8%. Hybridoma supernatants were analyzed by indirect ELISA for the presence of antibodies against AFM1. Of all the wells tested, 23 wells gave a signal that was at least 3.0 O.D. units (at 492 nm) greater than background (background signal approximately 0.045). Out of the 23 wells, six positive wells have been chosen to be subcloned for three cycles by limiting dilution. For each cloning procedure, the cells were cultured in HT medium on a layer of macrophage feeder cells, and final clones were obtained from wells that had been seeded with 1cell/well. Finally, we selected one stable hybridoma 3B1, which was the most sensitive and further used in detection AFM1 in raw milk and milk products.

Antibody characterization: The antibody used was IgG_{2a} antibody with λ light chain. This information was important for determination of toxin. Preliminary experiments were performed to assess the optimal coating of antigen to antibodies ratio. Aflatoxin M1-BSA and anti-aflatoxin M1 antibody concentration were optimized by comparing dose-response curves obtained using different combinations of antigen (5 μg/ml, 2.5 μg/ml, 1.3 μg/ml, 0.7 μg/ml, 0.4 μg/ml, 0.2 μg/ml, 0.1 μg/ml) and antibodies dilutions (1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1:64000 v/v), according to an optimized experimental design (Magliulo et al., 2005). The optimized coating antigen concentration and antibody dilution were 0.7 μg/ml and 1:8000 (v/v).

It was reported that AFB1 was the most biologically potent hepatocarcinogen, and AFG1 was also considerably carcinogenic, but AFB2 and AFG2 possessed much lower activity (Wong & Hsieh, 1976). In this study, the cross-reactivities of AFM1, AFB1, AFG1, and DON were determined by CI-ELISA. Fig. 1 shows the CI-ELISA results and demonstrates that the concentrations of AFM1, AFB1, and AFG1 necessary to cause 50% inhibition of binding (IC_{50}) of the antibody were found to be 0.62, 4.46, and 9.25 ng/ml, respectively. Cross-reactivities (%) were calculated using the following formula: $IC_{50AFM1} \div IC_{50\ \ reactant} \times 100\%$, cross-reactivities of the anti-AFM1 monoclonal antibody were 100, 13.9, 6.7 and <0.1% against AFM1, AFB1, AFG1 and DON, respectively. Woychik, Hinsdill, and Chu (1984) prepared four MAbs in their experiment. Two of antibodies, AMW-1 and AMW-4 were found to be specific for AFM1. The specificities of antibody (AMW-1) which had higher affinity to AFM1 were determined by direct competitive ELISA. The concentrations of AFM1, AFB1, and AFG1 necessary to cause 50% inhibition of binding of the antibody AMW-1 were found to be 25, 260, and 260 ng/ml, respectively. There are three reports of the production of monoclonal antibodies against AFM1 in China (Jiang et al., 2007; Li, Han, Zhang, & Luo, 2000; Pan & Pang, 2005). The antibodies that were prepared by Li et al. (2000) and Jiang et al. (2007) were shown to be specific to AFM1 with limited cross-reaction to AFB1, AFB2, AFG1, and AFG2. Jiang et al. (2007) developed an indirect competitive ELISA method for the detection of AFM1 in the laboratory, and the limit of detection (LOD) was 0.07 ng/ml. However, they didn't use the antibodies to develop immunoassays for detection of AFM1 levels in the milk products. The main aim of generating monoclonal antibodies was to obtain antibodies with appropriate antigen affinity, cross-reactivity and sensitivity.

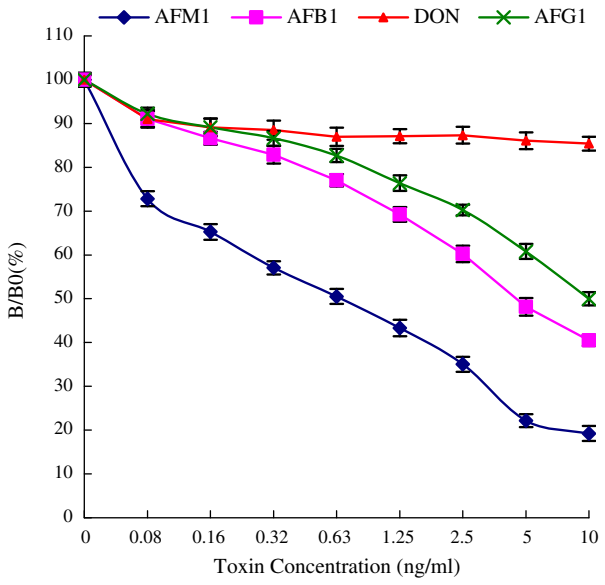

Fig. 1. Cross-reactivity of AFM1 antibodies with AFM1, AFB1, AFG1 and DON in indirect ELISA. Data represent the mean of three replications ± SD. Graph plotted as a percentage binding (B/B_0) against mass of toxin per milliliter (ng/ml). Cross-reactivity of the 3B1 anti-AFM1 MAb was 100, 13.9, 6.7 and <0.1% against AFM1, AFB1, AFG1 and DON, respectively.

In this paper, one monoclonal antibody that had high sensitivity and affinity has been obtained.

The cross-reactivities of antibody against AFB1 and AFG1 were 13.9% and 6.7%, respectively, and these mycotoxins will have negligible effect on detection of AFM1 of milk products. There was a linear relationship between the amount of AFM1 in milk and AFB1 in feed consumed by the animals (Dragacci, Gleizes, Fremi, & Candlish, 1995). About 0.3–6.2% of AFB1 in animal feed is transformed to AFM1 in milk (Unusan, 2006), and it is mainly affected by milk yield and stage of lactation (Masoero et al., 2007; Van Eijkeren, Bakker, & Zeilmaker, 2006). AFM1 can be detected in milk 12–24 h after the first ingestion of AFB1 (Sadeghi et al., 2009).

3.2. Recovery of AFM1 from spiked milk samples

Milk samples that do not contain AFM1 were compared with samples deliberately contaminated with known amounts of AFM1 (Table 1). ELISA recovery value of AFM1 from the milk samples was between 80% and 128%, and the average recovery value was 98% for the range of 0.1–3.2 ng/ml, which was consistent to the recommended recovery (70–110%) of Commission Regulation (EC), 2006a) about mycotoxins in foodstuffs and was in accord with most analytical techniques, including immunoaffinity column clean-up coupled with HPLC. (Kim et al. 2000; Lee et al., 2009; Rodriguez Velasco, Calonge Delso, & Ordonez Escudero, 2003). The results showed that the monoclonal antibody had high sensitivity to AFM1. Thus, the MAb that was obtained in this experiment is applicable for routine screening of AFM1 in milk and other milk products.

The standard curve for AFM1 detection by indirect competitive ELISA is depicted in Fig. 2. The absorption is inversely proportional to the AFM1 concentration in the samples. The calibration curve was found virtually linear in the 0.04–5 ng/ml range. Intra-day ($n = 6$) and inter-day (6 different days) variation values at a fortification level of 0.04–5 ng/ml were 2.3–4.6% and 3.2–17.2%, respectively. The limit of detection concentration of AFM1 was 0.04 ng/ml.

3.3. Occurrence of AFM1

The occurrence and the distribution of AFM1 concentration obtained are presented in Table 2. Analysis of three replicates of 135 samples showed that 55 (41%) samples contained 0.32–50 ng/ml, 24 (18%) samples contained 0.16–0.32 ng/ml, 18 (13%) samples contained 0–0.16 ng/ml, and in 38 (28%) samples AFM1 was not detected. All of the samples were below the Chinese limit, while 72% (97 out of 135) milk products samples were found to be higher than the maximum acceptable limits (50 ng/kg) of the EU. Contamination with AFM1 is a serious problem for public health. It seems

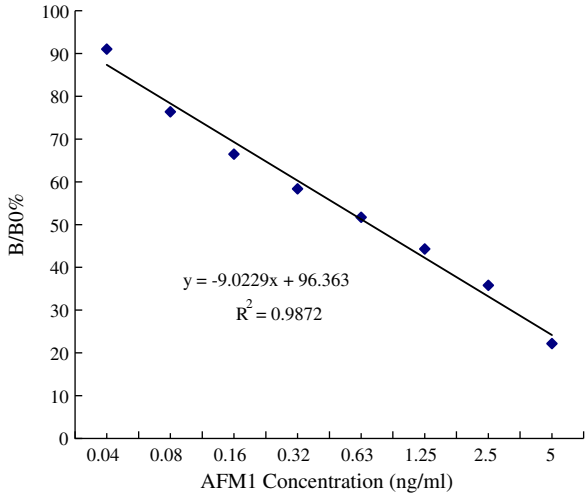

Fig. 2. Standard curve of AFM1 by indirect competitive ELISA.

that the present status of this mycotoxin in Northeast of China is still legal since the contamination of all the samples with AFM1 did not exceed the China legal limit of 0.5 μg/kg. However, the danger of AFM1 contamination to humans, especially children cannot be overlooked. Therefore, there is a need to routinely monitor AFM1 as a food quality control measure for human health.

Results showed that 100% ($n = 12$) of raw milk samples were contaminated with AFM1. The high concentration rate is most likely due to inappropriate feeding of animals located in urban and semi-urban areas. There are various reasons, such as low availability of green fodder and excessive use of concentrated feed, cottonseed cake, corn, soybean, threshed wheat straw, paddy straw, and wheat bran. All these commodities are vulnerable to attack of moulds and there is a high possibility of presence of AFB1 in these commodities (Dutton & Kinsey, 1996; Hussain, Anwar, Munawar, & Asi, 2008; Sassahara et al., 2005). AFM1 was detected in all of the powdered milk samples ($n = 15$) and all cheese ($n = 4$). The concentration of AFM1 in 67% (10 out of 15) powered milk samples and all cheese were from 0.16 to 0.32 ng/ml. The results revealed that 63% (66 out of 104) milk products (liquid milk) including madzoon, pure milk, and milk beverage were detected with AFM1 (Table 2). AFM1 concentration in 40% (42 out of 104) milk products samples were from 0.32 to <0.5 ng/ml, and 6% (6 out of 104) were from 0.16 to 0.32 ng/ml, and 17% (18 out of 104) were from 0 to 0.16 ng/ml, and in 37% (38 out of 104) of liquid milk samples, AFM1 was not detected.

The samples used in this experiment do not represent the entire milk samples from Northeast areas of China because they were mainly based on the industrial dairy farms and supermarkets. We have no information on the quality of the fodder given to these animals that produced milk. The fodder may be contaminated due to the unpredictable climatic and environmental conditions and the inability of certain agricultural systems. Thus, cows' feed samples from various cowsheds must be routinely evaluated for aflatoxin and those with excessive contaminations should be discarded to keep the dairy cow's feeds away from fungal contamination as much as possible. High incidences and levels of AFM1 contaminations were also found in Indonesia, Philippines and Thailand (Henry et al., 2001; Tajkarimi et al., 2008). Exact data are needed to demonstrate AFB1 accumulation in fodder samples. Comparison of AFB1 data with AFM1 levels measured in the corresponding milk samples would also be a valuable approach to find the real source of aflatoxin accumulation.

The quality of milk and milk products has a profound influence on the health of infants and young children, because the consump-

Table 1
Recovery of AFM1 from artificially contaminated Milk samples as determined by competitive ELISA.

No.	Conc. of AFM1 used for spiking samples (ng/ml)	Estimated conc. of AFM1[a] (ng/ml)	Recoveries % of AFM1 in spiked samples[b]
1	0.1	0.10 ± 0.01	101.7 ± 10.4
2	0.2	0.25 ± 0.02	128.3 ± 11.5
3	0.4	0.32 ± 0.02	80.0 ± 5.0
4	0.8	0.67 ± 0.14	83.3 ± 17.1
5	1.6	1.31 ± 0.19	81.9 ± 12.3
6	3.2	2.81 ± 0.28	87.9 ± 8.8

[a] 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.

[b] Each sample was spiked with a known concentration of AFM1, and assayed. Data represent the mean of three replications ± SD.

Table 2
Incidence and range of AFM1 in milk samples as determined by indirect competitive ELISA.

Type of milk sample	Total no. of samples	No. of samples with AFM1 contents in the ranges (ng/ml)				
		ND[a]	>0–0.16	0.16–0.32	0.32–0.5	>0.5
Raw milk	12	0	0	4 (33%)	8 (67%)	0
Powdered milk (g of dry milk/mL of solution)	15	0	0	10 (67%)	5 (33%)	0
Milk products (liquid milk)[b]	104	38 (37%)[c]	18 (17%)	6 (6%)	42 (40%)	0
Cheese	4	0	0	4 (100%)	0	0
Total	135	38 (28%)	18 (13%)	24 (18%)	55 (41%)	0

[a] ND: not detected.
[b] Liquid milk including madzoon, pure milk, and milk beverage.
[c] Parenthesis indicates percent of total samples.

tion of milk and milk products by them is very high. Contamination of milk with AFM1 and its negative effects on humans are well documented (Rothschild, 1992). Its risk, especially carcinogenic effect due to the consumption of milk contaminated by AFM1, has already been assessed in some countries. Liu (2006) collected 168 samples from Tianjin, Shanghai which are municipalities directly under the Central Government, and Shijiazhuang which is the capital of Hebei province. All the samples were detected by competitive ELISA, and his results showed the AFM1 concentrations of two samples were between 0.25 ppb and 0.5 ppb, others were less than 0.25 ppb. However, there are no reports on the contamination of AFM1 in milk in Northeast of China yet, which could be attributed to the lack of rapid and cost-effective technologies for AFM1 estimation. Thus, we have investigated AFM1 levels from China showing AFM1 contamination of milk products from Northeast area of China. The presence of AFM1 in milk and milk products from Northeast of China seems to still exist for public health concern and indicate that AFM1 concentration should be continuously monitored.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (No. 30771799), from the Department of Education Item of Heilongjiang Province (No. 1151hz025), from Study Abroad Returned Personnel Fund of Heilongjiang Province (No. Lc05c11), and from China Postdoctoral Science Foundation (20070410882).

References

Bakirci, I. (2001). A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey. *Food Control, 12*, 47–51.
Cathey, C. G., Huang, A. G., Sarr, A. B., Clement, B. A., & Phillips, T. D. (1994). Development and evaluation of a minicolumn assay for the detection of aflatoxin M1 in milk. *Journal of Dairy Science, 77*, 1223–1231.
Cavaliere, C., Foglia, P., Pastorini, E., Samperi, R., & Laganà, A. (2006). Liquid chromatography/tandem mass spectrometric confirmatory method for determining aflatoxin M1 in cow milk comparison between electrospray and atmospheric pressure photoionization sources. *Journal of Chromatography A, 1101*, 69–78.
Commission Regulation (EC) (2006a). No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of European Union*, L 70, 12–34.
Commission Regulation (EC) (2006b). No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of European Union*, L 364, 5–24.
Creepy, E. E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127*, 19–28.
Deshpande, S. S. (2002). Fungal toxins. In S. S. Deshpande (Ed.), *Handbook of food toxicology* (pp. 387–456). New York: Marcel Decker.
Devi, K. T., Mayo, M. A., Reddy, K. L. N., Delfosse, P., Deddy, G., Reddy, S. V., et al. (1999). Production and characterization of monoclonal antibodies for aflatoxin B1. *Letters in Applied Microbiology, 29*, 284–288.
Dragacci, S., Gleizes, E., Fremi, J. M., & Candlish, A. A. G. (1995). Use of immunoassaffinity chromatography as a purification step for the determination of aflatoxin M1 in cheeses. *Food Additives and Contaminants, 12*(1), 59–65.
Dutton, F. A., & Kinsey, A. (1996). A note on the occurrence of mycotoxins in cereals and animal feedstuffs in Kwazulu Natal, South Africa, 1984–1993. *South African Journal of Animal Sciences, 26*(2), 53–57.
Food and Agriculture Organization (1997). Worldwide regulations for mycotoxins 1995. *A compendium FAO food and nutrition paper 64*, Rome.
Galvano, F., Galofaro, V., & Galvano, G. (1996). Occurrence of stability of aflatoxin M1 in milk and milk products: A worldwide review. *Journal of Food Protection, 59*, 1079–1090.
Galvano, F., Galofaro, V., Ritieni, A., Bognanno, M., De Angelis, A., & Galvano, G. (2001). Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy: Second year of observation. *Food Additives and Contaminants, 18*(7), 644–646.
Henry, S. H., Whitaker, T. B., Rabbani, I., Bowers, J., Park, D., Price, W. (2001). Aflatoxin M1, Chemical safety information from inter government organizations. <www.inchem.org/documents/jecfa/jecmono/v47je02.htm>.
Holtzapple, C. K., Carlin, R. J., Rose, B. G., Kubena, L. F., & Stanker, L. H. (1996). Characterization of monoclonal antibodies to aflatoxin M1 and molecular modeling studies of related aflatoxins. *Molecular Immunology, 33*, 939–946.
Hu, Y. Y., Zheng, P., Zhang, Z. X., & He, Y. Z. (2006). Determination of aflatoxins in high-pigment content samples by matrix solid-phase dispersion and high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry, 54*, 4126–4130.
Hussain, I., Anwar, J., Munawar, M. A., & Asi, M. R. (2008). Variation of levels of aflatoxin M1 in raw milk from different localities in the central areas of Punjab, Pakistan. *Food Control, 19*, 1126–1129.
IARC (1993). *Some naturally occurring substances: Food items and constituents heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risk to humans* (Vol. 56). International Agency for Research on Cancer. pp. 489–451.
IARC (2002). *Some mycotoxins, naphtalene and styrene. IARC monographs on the evaluation of carcinogenic risk to humans* (Vol. 82). International Agency for Research on Cancer. pp. 171–300.
Jiang, T., Yu, Q., Li, M., Liu, Z., Zheng, J., Huang, B., et al. (2007). Development of monoclonal antibody against aflatoxin M1 and immunoassay for aflatoxin M1. *Chinese Journal of Public Health, 23*(1), 43–45.
Kim, E. K., Shon, D. H., Ryu, D., Park, J. W., Hwang, H. J., & Kim, Y. B. (2000). Occurrence of aflatoxin M [sub 1] in Korean dairy products determined by ELISA and HPLC. *Food Additives and Contaminants, 17*(1), 59–64.
Lee, J. E., Kwak, B. M., Ahn, J. H., & Jeon, T. H. (2009). Occurrence of aflatoxin M1 in raw milk in South Korea using an immunoaffinity column and liquid chromatography. *Food Control, 20*, 136–138.
Lee, N. A., Wang, S., Allan, R. D., & Kennedy, I. R. (2004). A rapid aflatoxin B1 ELISA: Development and validation with reduced matrix effects for peanuts, corn, pistachio, and soybeans. *Journal of Agricultural and Food Chemistry, 52*, 2746–2755.
Li, J. Y., Han, C. H., Zhang, J., & Luo, X. Y. (2000). Establishment of monoclonal antibodies against aflatoxin M1. *Journal of Hygiene Research, 29*(1), 59–61.
Liu, R. (2006). Producing of aflatoxin M1 kits and raw dairy safety alert of aflatoxin M1. *The Master's Thesis of Tianjin University of Commerce, China*.
Magliulo, M., Mirasoli, M., Simoni, P., Lelli, R., Portanti, O., & Roda, A. (2005). Development and validation of an ultrasensitive chemiluminescent enzyme immunoassay for aflatoxin M1 in milk. *Journal of Agricultural and Food Chemistry, 53*, 3300–3305.
Masoero, F., Gallo, A., Moschini, M., Piva, G., & Diaz, D. (2007). Carryover of aflatoxin from feed to milk on dairy cows with low or high somatic cell counts. *Animal, 1*, 1344–1350.
Moschini, M., Masoero, F., Gallo, A., & Diaz, D. (2007). Mucosal absorption of aflatoxin B1 in lactating dairy cows. *Animal Science, 6*(Suppl. 1), 324–326.
Pan, W., & Pang, G. (2005). Screening of monoclonal antibodies against aflatoxin M1. *Food Science and Technology, 5*, 6–8.
Rastogi, S., Dwivedi, P. D., Khanna, S. K., & Das, M. (2004). Detection of aflatoxin M1contamination in milk and infant milk products from Indian markets by ELISA. *Food Control, 15*, 287–290.
Rodriguez Velasco, M. L., Calonge Delso, M. M., & Ordonez Escudero, D. (2003). ELISA and HPLC determination of the occurrence of aflatoxin M1 in raw cow's milk food. *Food Additives and Contaminants, 20*(3), 276–280.
Rothschild, L. J. (1992). IRAC class 1 human carcinogen. *Journal of Agricultural and Food Chemistry, 34*, 62–66.

Sadeghi, N., Oveisi, M. R., Jannat, B., Hajimahmoodi, M., Bonyani, H., & Jannat, F. (2009). Incidence of aflatoxin M1 in human breast milk in Tehran, Iran. *Food Control, 20*, 75–78.

Sassahara, M., Pontes Netto, D., & Yanaka, E. K. (2005). Aflatoxin occurrence in foodstuff supplied to dairy cattle and aflatoxin M1 in raw milk in the North of Paranaá state. *Food and Chemical Toxicology, 43*(6), 981–984.

Sherry, J. P. (1997). Environmental immunoassays and other bioanalytical methods: Overview and update. *Chemosphere, 34*, 1011–1025.

Stoloff, L. (1989). Aflatoxin M1 in perspective. *Food Protection, 43*, 226–230.

Tajkarimi, M., Aliabadi-Sh, F., Nejad, A. S., Poursoltani, H., Motallebi, A. A., & Mahdavi, H. (2008). Aflatoxin M1 contamination in winter and summer milk in 14 states in Iran. *Food Control, 19*, 1033–1036.

Tekinsen, K. K., & Tekinsen, O. C. (2005). Aflatoxin M1 in white pickle and van otlu (herb) cheeses consumed in southeastern in Turkey. *Food Control, 16*(7), 565–568.

Thirumala-Devi, K., Mayo, M. A., Hall, A. J., Craufurd, P. Q., Wheeler, T. R., Waliyar, F., et al. (2002). Development and application of an indirect competitive enzyme-linked immunoassay fro aflatoxin M1 in milk and milk-based confectionery. *Journal of Agricultural and Food Chemistry, 50*, 933–937.

Unusan, N. (2006). Occurrence of aflatoxin M1 in UHT milk in Turkey. *Food and Chemical Toxicology, 44*, 1897–1900.

Van Eijkeren, J. C. H., Bakker, M. I., & Zeilmaker, M. J. (2006). A simple steady-state model for carry-over of aflatoxins from feed to cow's milk. *Food Additives and Contaminants, 23*, 833–838.

Virdis, S., Corgiolu, G., Scarano, C., Pilo, A. L., & De Santis, E. P. L. (2008). Occurrence of aflatoxin M1, in tank bulk goat milk and ripened goat cheese. *Food Control, 19*, 44–49.

Wang, J., & Liu, X. M. (2006). Surveillance on contamination of total aflatoxins in corn, peanut, rice, walnut and pine nut in several areas in China. *Chinese Journal of Preventive Medicine, 40*(1), 33–37.

Wong, J. J., & Hsieh, D. P. H. (1976). Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. *The Proceedings of the National Academy of Science USA, 73*(7), 2241–2244.

Woychik, N. A., Hinsdill, R. D., & Chu, F. S. (1984). Production and characterization of monoclonal antibodies against aflatoxin M1. *Applied and Environmental Microbiology, 48*, 1096–1099.