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Short communication

Seasonal variation of aflatoxin M_1 in raw milk from the Yangtze River Delta region of China

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ABSTRACT

The objective of this study was to evaluate the occurrence of aflatoxin M_1 (AFM_1) in raw milk samples from 18 dairy farms in the Yangtze River Delta region during four different seasons. A total of 72 tank milk samples was collected with 18 samples for each season. Milk AFM_1 was detected using LC-MS/MS. The AFM_1 was detected in 43 milk samples (59.7%) ranging in concentration from 10 to 420 ng/L. The concentration of AFM_1 in raw milk was significantly higher during the winter (123 ng/L) than during other seasons ($P < 0.05$). There was no significant difference between the spring (29.1 ng/L), summer (31.9 ng/L), and autumn (31.6 ng/L) ($P > 0.05$) seasons. This indicates that raw milk collected during the winter is at high risk for AFM_1 and that seasonal factors should be considered for the management of aflatoxins in both the feed and milk.

1. Introduction

Aflatoxins are secondary hepatotoxic metabolites that are produced primarily by *Aspergillus flavus, A. parasiticus, A. bombycis, A. ochraceoroseus, A. nomius, and A. pseudotamari* that contaminate plants and plant products (Bennet & Klich, 2003; Cheraghali et al., 2007; Iqbal, Paterson, Paterson, & Asi, 2010). The presence of aflatoxins in food and feed are of great concern worldwide because of their harmful mutagenic, teratogenic, carcinogenic and immunosuppressive effects (Kamkar, 2005; Oveisi, Jannat, Sadeghi, Hajimahmoodi, & Nikzad, 2006; Zinedine & Manes, 2009). Aflatoxins occur naturally in agricultural commodities and food. There are four main types of aflatoxin: B_1, B_2, G_1 and G_2. Aflatoxin M_1 (AFM_1), the monohydroxylated derivate of aflatoxin B_1 (AFB_1), is excreted into the milk of dairy cows that ingest AFB_1 contaminated feed and subsequently contaminates other dairy products (Allcroft, Roberts, & Butler, 1967). Because of the hepatotoxic and carcinogenic damage caused by aflatoxins, AFB_1 and AFM_1 are classified as class 1 human carcinogens (IARC, 1993; IARC, 2002).

Because of serious health concerns, the maximum residue level (MRL) for AFM_1 in milk and dairy products has been established in many countries to protect consumers. The international regulations for the MRL vary from 0 to 1.0 μg/kg depending on the country (Dashti et al., 2009; Stoloff, Van Egmond, & Parks, 1991). The European Commission (EC) has set an MRL of 50 ng/kg for AFM_1 in milk (EC, 2001). However, the MRL for both the USA (US FDA, 1996, p. 219) and China (MoH, 2011) is 500 ng/kg, which is 10-fold higher than the EC (2001).

The Yangtze River Delta (YRD) is a subtropical zone where temperature and moisture favor the growth of toxigenic *Aspergillus* (Mostrom & Jacobsen, 2011; Pan, Wang, Zeng, Xie, & Miao, 2011; Schindler, Palmer, & Eisenberg, 1967). The YRD is also an important raw milk production area where a total of 1.228 million tons of raw milk were produced in 2010 (*The fifth dairy industry, 2012*), with a major milk consumption district by 94.79 million of population in 2009 (Xu, 2012). However, little information is available regarding the seasonal occurrence of AFM_1 contamination of raw milk in the YRD, although some researchers have reported seasonal differences in AFM_1 contamination in milk in other areas of the world (Asi, Iqbal, Ariño, & Hussain, 2012; Assem, Mohamad, & Oula, 2011; Nemati, Mehran, Hamed, & Masoud, 2010).

In this study, the occurrence of AFM_1 was determined in raw milk samples from dairy farms in the YRD region during four different seasons.

2. Materials and methods

2.1. Sample collection

This study occurred from November 2011 to September 2012. A total of 72 raw milk samples was collected from 18 dairy farms in

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four major cities, Shanghai ($n = 6$), Hangzhou ($n = 4$), Nanjing ($n = 4$) and Jinhua ($n = 4$), including the main farms of raw milk production in the YRD. These dairy farms with the capacity of 15–40 tons of raw milk production per day were using three types of collection system: pipeline milking, parallel milking and rotary milking. All the raw milk was pumped into milk-holding tanks with refrigeration system. Approximately 600 mL of all-day milk was collected from the bulk-tanks of each dairy farm, and bronopol (Broad Spectrum Microtabs II, D and F Control Systems Inc., Dublin, CA) was added to the samples as a preservative. The milk samples were stored at −20 °C until analysis using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

2.2. Chemicals and supplies

HPLC grade methanol and acetonitrile were purchased from Alfa Aesar (Ward Hill, MA). The AFM_1 standard was obtained from Sigma–Aldrich (A6428, Sigma Chemical Company, St Louis, MO). A stock solution of AFM_1 was made from the AFM_1 standard dissolved in trichloromethane, which was then diluted with a blank matrix solution, a standard milk that is negative for AFM_1, for the preparation of different concentrations of working standard solutions. All stock and working standard solutions were stored in brown vials at −20 °C. Other inorganic chemicals and organic solvents were of analytical reagent grade and were obtained from Guangzhou Chemical Regent Factory (Guangdong, China).

2.3. Analytical procedure

Milk AFM_1 levels were determined using the official method from the Ministry of Health P. R. China (MoH, 2010). Briefly, 50 mL well-distributed milk sample was placed in a lidded centrifuge tube and heated to 35–37 °C in a water bath. The sample was centrifuged for 15 min at 4208 × g, and the separated supernatant was passed through an AFLAPREPM immunoaffinity cleanup column (R-Biopharm Rhone Ltd., Glasgow, Scotland) coupled with a vacuum manifold at a flow rate of 2–3 mL/min. The column was washed with 4 mL acetonitrile for more than 60 s, and the AFM_1 was then eluted from the columns into 10 mL tubes. The eluate was evaporated to approximate dryness under a gentle stream of nitrogen at 30 °C. The resultant residue was dissolved up to 1 mL acetonitrile/water (v/v, 1:9). Then, 10 μL of this solution was injected into the LC-MS/MS apparatus and analyzed at the optimized conditions.

The LC-MS/MS system consisted of an Agilent 1200 Series rapid resolution liquid chromatograph (Palo Alto, CA) coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray ionization source. The LC column was a CAPCELL PAK MGIII-C18 (150 mm × 2.1 mm i.d., 1.8 μm) column (Shiseido, Tokyo, Japan). The mobile phase was 0.1% formic acid/acetonitrile/methanol (v/v/v, 56/22/22) at a flow rate of 0.3 mL/min. Quantitative analysis was performed using the multiple reaction monitoring mode. The AFM_1 was identified in the positive ion mode. The AFM_1

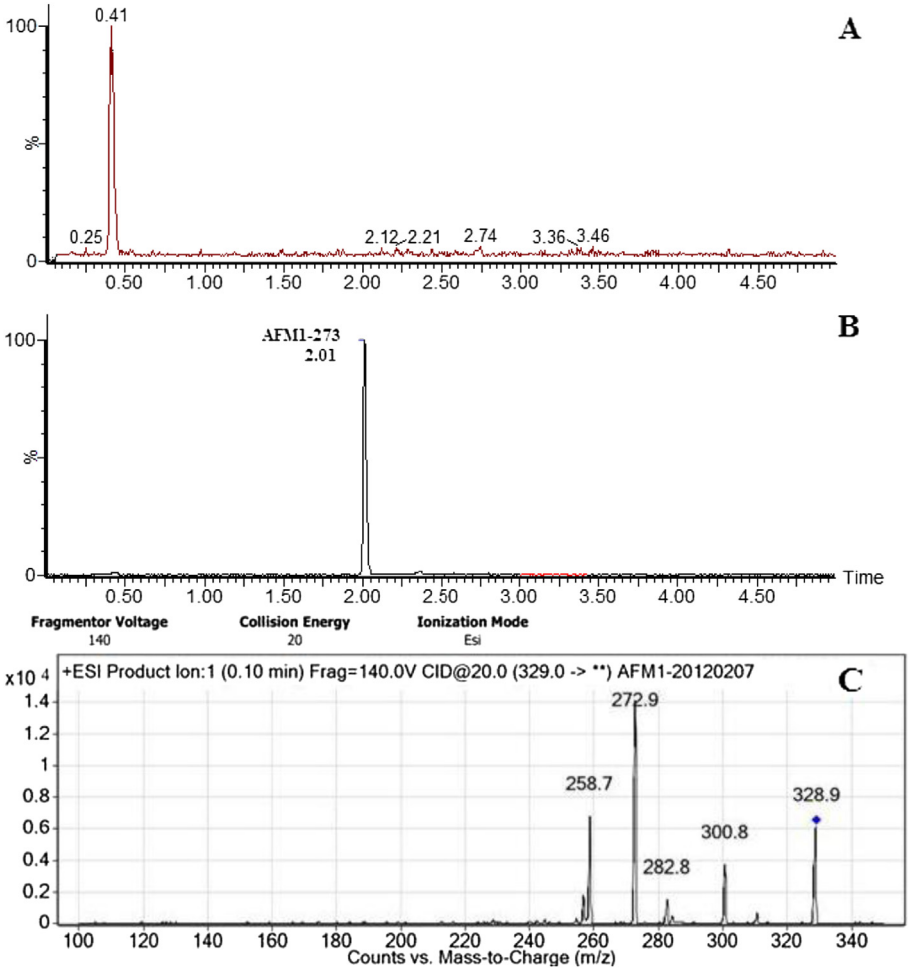

Fig. 1. Chromatograms of an AFM_1 negative sample (A) and a sample naturally containing AFM_1 (B). CID spectrum of AFM_1 (C).

Table 1
Occurrence of aflatoxin M_1 (AFM_1) in raw milk samples during different seasons.

Season	Positive[a] (%)	AFM_1 concentration (ng/L)			
		Minimum	Maximum	Median	Mean ± SD
Spring	14 (77.8)	11	98	24.5	29.1 ± 22.6[b]
Summer	8 (44.4)	11	82	17.0	31.9 ± 26.7[b]
Autumn	5 (27.8)	16	76	22.0	31.6 ± 25.3[b]
Winter	16 (88.9)	10	420	97.5	123.6 ± 101.0[c]
Total	43 (59.7)	10	420	31.0	65.0 ± 77.8

[a] Positive samples are those samples in which the AFM_1 concentration in the raw milk exceeded the quantitation limit of 10 ng/L. The values in parentheses indicate the percentage of the positive samples in the total.
[b,c] The values followed by different letters in the same column are significantly different ($P < 0.05$).

Table 2
Distribution of aflatoxin M_1 (AFM_1) in raw milk samples during different seasons.

Season	Samples over EC limit[a] (%)	Range of AFM_1 concentration (ng/L)				
		<10	10–50	50–100	100–250	250–500
Spring	1 (5.6)	4	13	1	0	0
Summer	2 (11.1)	10	6	2	0	0
Autumn	1 (5.6)	13	4	1	0	0
Winter	13 (72.2)	2	3	4	7	2
Total	17 (23.6)	29	26	8	7	2

[a] The EC legal limit of AFM_1 in milk is 50 ng/L (EC, 2001). The values in parentheses indicate the percentage of samples.

dominant precursor ion was obtained from the electrospray ionization source at a cone voltage of *m/z* 329, and the product ions for quantification and confirmation were obtained at *m/z* 273 and *m/z* 259. The limit of quantification was based on the minimum amount required to produce a signal-to-noise ratio of 10. The limit of quantitation was set at 10 ng/L AFM_1.

Prior to analysis of the samples, the analytical procedure was validated on the basis of recoveries and the relative standard deviation values. The results showed that the recoveries of AFM_1 ranged from 90.7 to 97.8%, and the relative standard deviation values ranged from 2.2 to 4.6%, which met the performance criteria of the Commission Regulation EC No. 401/2006 (EC, 2006). Chromatograms of an AFM_1 negative sample and an AFM_1 positive sample (82 ng/L), including the CID spectra of AFM_1, are presented in Fig. 1.

2.4. Statistical analysis

Differences in the concentration of AFM_1 in the milk samples from the four different seasons were statistically analyzed by a one-way analysis of variance (ANOVA) using the SPSS version 19.0 software (SPSS, 2010). The level of confidence required for significance was set at $P \leq 0.05$.

3. Results and discussion

The average concentration of milk AFM_1 during the winter (123 ng/L) was significantly higher than other seasons ($P < 0.05$). There was no significant difference ($P > 0.05$) between the spring (29.1 ng/L), summer (31.9 ng/L), and autumn (31.6 ng/L) seasons (Table 1). The highest incidence and highest concentration of milk AFM_1 were found during the winter and were 3.2 and 5.5 times higher than the autumn, respectively (88.9% vs. 27.8%; 420 ng/L vs. 76 ng/L, respectively). In addition, a total of 17 samples had detectable AFM_1 at a level greater than the legal limit of 50 ng/L set by the EC (2001) for liquid milk (Table 2). The percentage of samples exceeding the EU limit was highest during the winter (72.2%) and lowest during spring (5.6%) and autumn (5.6%), with no sample above the legal limit of 500 ng/L set by either the US FDA (1996, p. 219) or China (MoH, 2011).

The above results indicate that AFM_1 contamination in raw milk is season-dependent, which may be because of seasonal variation in the type and quality of feed given to dairy cows in the YRD region. Because of a shortage of fresh green feed during the winter, the dairy cows were offered a large amount of conserved or stored feeds, such as corn, cotton seed and silage, which are easily contaminated by aflatoxins under inadequate storage conditions. Wang and Liu (2006) reported that 70.3% of corn was contaminated with aflatoxins at an average level of 36.5 mg/kg. An average level of 39.6 mg AFB_1/kg was detected in 74.6% of corn from a Chinese market (Gao et al., 2011). Silage was also reported to be a vector for AFB_1 contamination in some studies (Garon et al., 2006; Pereyra

Table 3
Review of the incidence and range of AFM_1 in raw milk during different seasons reported in previous studies.

Season	Total sample	Positive (%)[a]	Sample over 50 ng/L (%)	Range (ng/L)	Mean[b] (ng/L)	Country	Ref[c]
Spring	23	23 (100)	–[d]	8.7–81.9	52.9 ± 4.4	Iran	1
	12	0 (0)	0 (0)	–	–	Morocco	2
Spring-summer	38	28 (73.6)	17 (44.7)	2.6–12.6	60.4	Lebanon	3
Summer	100	48 (48)	1 (1.0)	2–80	26.0	Korea	4
	22	22 (100)	–	2.9–55.9	17.4 ± 3.1	Iran	1
	80	80 (100)	38 (47.5)	14–102	50.0 ± 21.0	Thailand	5
	12	2 (16.7)	0 (0)	10–30	20.0	Morocco	2
	27	–	9 (33.3)	14–95	22.0 ± 6.0	Pakistan	6
Summer-autumn	26	–	0 (0)	0.6–14.9	4.2 ± 4.4	Croatia	7
Autumn	22	22 (100)	–	7.8–28.9	22.3 ± 0.9	Iran	1
	12	7 (58.3)	3 (25.0)	10–100	52.9	Morocco	2
Winter	12	12 (100)	12 (100)	160–500	–	China	8
	23	23 (100)	–	5.8–85.0	56.3 ± 6.6	Iran	1
	80	80 (100)	64 (80.0)	28–197	89.0 ± 34.0	Thailand	5
	50	43 (86)	0 (0)	1–30	8.7 ± 6.8	Turkey	9
	12	4 (33.3)	1 (8.3)	10–90	37.5	Morocco	2
	27	–	15 (55.6)	65–150	89.0 ± 2.0	Pakistan	6
Winter-spring	35	–	1 (2.9)	3.6–58.7	18.4 ± 11.7	Croatia	7

[a] The values in parentheses indicate the percentage of the positive samples in the total.
[b] Means ± SD.
[c] 1: Nemati et al. (2010); 2: Marnissi et al.(2012); 3: Assem et al. (2011); 4: Lee, Kwak, Ahn, and Jeon (2009); 5: Ruangwises & Ruangwises. (2010); 6: Asi et al. (2012); 7: Bilandžić, Varenina, and Solomun (2010); 8: Pei et al. (2009); 9: Ertas, Gonulalan, Yildirim, and Karadal (2011).
[d] Not mentioned in the reference.

et al., 2011). A large amount of fresh animal feed, such as pasture and green fodder, are available during the other three seasons compared to the winter, resulting in a low contamination of milk by AFB_1 (Asi et al., 2012).

The seasonal differences in the AFM_1 levels in raw milk are summarized in Table 3. Consistent with the results of the current study, the milk produced during the hot seasons has less AFM_1 contamination than cold seasons. This may be because of the similarities in climate changes and feed management practices in the districts studied. However, Marnissi, Belkhou, Morgavi, Bennani, and Boudra (2012) found that the AFM_1 concentration in raw milk during autumn was higher than other seasons, which is different from our findings. This difference may be explained by the wide variations in geography, different study design, analytic methods and dairy farm management practices. Additionally, the AFM_1 levels detected in the raw milk in the current study are lower than a previous study by Pei, Zhang, Eremin, and Lee (2009). One possible reason is that the Chinese government, dairy farms and dairy plants have recently focused attention on AFM_1 levels in milk and milk products. Supervisory organizations regularly inspect for AFM_1 concentrations in milk and milk products, and dairy farms have improved the feed quality and hygiene by many quality control measures, such as importing high quality feed and improving storage conditions. In addition, Zheng et al. (2013) reported that AFM_1 was detected in 54.9% of UHT milk with levels of 6–160 ng/L, indicating that the safety of Chinese milk has improved. Moreover, the majority of dairy farms in the current study are modern, large and have enough funds to organize skilled technicians and advanced instruments for feed and milk quality analysis.

The present study is only an initial survey of AFM_1 contamination in raw milk in the YRD using LC-MS/MS. A large-scale investigation is necessary to complete the risk assessment.

4. Conclusions

The incidence and concentration of AFM_1 in raw milk from the YRD during the winter was higher than in other seasons and seasonal factors should be considered for the management of aflatoxins in both the feed and the milk. It was recommended that raw milk from the YRD during the winter season should be strictly supervised to produce safer milk and dairy products.

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